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Isolation and molecular characterization of toxinogenic and antimicrobial resistant Clostridium perfringens from broilers.

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ABSTRACT

Clostridium perfringens is linked to necrotic enteritis, necrotic dermatitis, and cholangiohepatitis in birds, resulting in significant financial losses for the poultry industry. This research aimed to isolate and characterize *C. perfringens* from seemingly healthy and clinically affected poultry between February 2023 and August 2024. The specimens were gathered from 30 broiler flocks in El-Menoufia, El-Gharbia, and El-Beheira. A total of 150 specimens, which included intestine (n= 130) and liver (n=20), were analyzed. A total of twenty-one (14%) *C. perfringens* isolates were collected, with 19 (14.6%) coming from sick broilers and 2 (10%) from those that appeared healthy.

All the isolates were verified using biochemical identification and polymerase chain reaction (PCR) by detecting the *alph*A, *net*B, and *tpe*L genes. All analyzed isolates (n= 21) possessed the alphA toxin at 401 bp, with 9 isolates containing *net*B and 6 isolates having *tpe*L genes at 316 bp and 466 bp, respectively. The in-vitro antimicrobial sensitivity testing was conducted on all isolates and showed a high sensitivity to amoxicillin 100%, gentamycin 76.1%, norfloxacin 66.6%, and levofloxacin 61.9%, while it demonstrated significant resistance to metronidazole 61.9% and penicillin 52.3%. Additionally, PCR analysis of the antibiotic-resistant genes *bla* and *tet*K showed a high occurrence of *bla* resistance genes with no presence of *tet*K.

It can be inferred that certain isolates of *C. perfringens* harboring the *alph*A, *net*B, and *tpe*L genes are capable of causing necrotic enteritis (NE) independently. Moreover, the significant prevalence of *bla* resistance genes reduces the likelihood of NE management.

1. INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming, anaerobic bacillus that typically resides in the intestines of humans and animals. This bacterium is the main reason for NE in chickens and can be readily located in soil, dust, droppings, feed, poultry bedding, and digestive

contents **[1,2]**. Broilers between 2 to 5 weeks old are primarily impacted by NE, which is marked by severe diarrhea and increased mortality linked to necrotic inflammation in the intestinal tract, as well as necrotic lesions in the liver, kidneys, and cecum**[3]**. Crucially, C. perfringens causes subclinical infections linked to long-term intestinal mucosa injury, which leads to decreased weight gain and poor performance, ultimately resulting in substantial economic losses even in the absence of mortality**[4]**.

Clostridium perfringens is classified into seven groups (toxinotypes A–G) based on the combination of toxin types it produces (α-toxin, β-toxin, ε-toxin, ι-toxin, enterotoxin (CPE), and netB) **[5]**. Every toxinotype of *C. perfringens* generates alpha toxin, exhibiting lecithinase activity, leading to pathological changes and necrosis in tissues. Alpha toxin is mainly accountable for necrotic enteritis in birds **[6]**.

Pathogenic strains of C. perfringens are known to generate and release over 20 distinct extracellular toxins and/or hydrolytic enzymes **[7]**. *Clostridium perfringens* bacteria can penetrate the fragile intestinal tissues of broilers due to their recognized endo- and exotoxins. When enterotoxins from *C. perfringens* attach to a protein named claudin, they adversely influence weight gain, feed conversion ratio (FCR), and crude protein digestibility, compromising the tight junctions of the intestinal epithelium and impairing barrier function **[8]**. The primary toxin that causes necrotic enteritis in chickens is the α-toxin **[9]**. The disease is marked by a reduced growth rate and poor feed conversion rate due to cholangiohepatitis and intestinal mucosal necrosis **[10]**.

Different techniques exist for detecting toxins; however, many are laborious, require considerable time, and are costly, exhibiting low sensitivity and specificity. In contrast, PCR provides a more specific and quicker detection method for C. perfringens toxins and antibiotic resistance **[1,11,12]**. The detection of alpha toxin using polymerase chain reaction is crucial for the accurate identification of alpha toxigenic *C. perfringens* strains **[13]**. During the initial phases of the illness, proper antibiotic therapy might effectively manage the infection **[14]**.

In addition to virulence factors in *C. perfringens*, antibiotic resistance is another characteristic that threatens affected poultry, primarily because it allows severe and life-threatening infections to resist antibiotic treatment **[5]**. Consequently, numerous research investigations have been carried out on antibiotic resistance patterns in *C. perfringens* employing phenotypic approaches (e.g., minimal inhibitory concentration (MIC) testing **[15,16,17,18]**. Due to the economic significance of this disease, this study aimed to isolate, identify, and examine the antimicrobial sensitivity of *C. perfringens* obtained from private broiler farms, as well as to screen for toxigenic properties of the prevalent strains using PCR.

2. MATERIALS AND METHODS

2.1. Sampling:

A total of 150 samples were gathered from both diseased and seemingly healthy broiler flocks (n=30). Intestinal samples totaled n=130 (with 115 from diseased chickens and 15 from apparently healthy ones), while liver samples amounted to n=20 (including 15 from diseased chickens and 5 from apparently healthy ones). These samples were collected from various farms in El-Beheira (n=15), El-Menoufia (n=8), and El-Gharbia (n=7) from February 2023 to August 2024. The ages of the surveyed birds varied between 2 to 5 weeks, and the affected chickens exhibited signs of diarrhea, lethargy, decreased growth performance, and higher mortality rates. Samples were transferred aseptically into ice boxes for the isolation and identification of C. perfringens at the Laboratory of Microbiology, Faculty of Veterinary Medicine, Menoufia University.

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2.2. Bacterial isolation and identification:

Specimens were introduced into tubes with cooked meat medium (Oxoid, UK) and incubated in an anaerobic jar for 24 hours at 37oC **[19]**. Aliquots of 0.1 ml were spread over Clostridium perfringens agar (supplemented with Tryptose Sulphite Cycloserine TSC, Oxoid) and 10% sheep blood agar, containing 200 ug/ml of neomycin sulfate, then incubated anaerobically at 37°C for 24 hours **[20]**. The biochemical identification of *C. perfringens* isolates revealed the presence of catalase and indole, nitrate reduction, and lecithinase (phospholipase C; α-toxin) activity on egg yolk Agar **[21,22]**.

2.3. Antimicrobial sensitivity test:

All confirmed isolates were subjected to in-vitro antimicrobial sensitivity test against 14 different types of antibiotics using Kirby–Bauer disk diffusion method and the results were calibrated and interpreted according to the Clinical and Laboratory Standards Institute (2017) guidelines. The list of used antibiotics included amoxicillin (25 µg), levofloxacin (5 mcg), doxycycline (30 mcg), ceftriaxone (30 mcg), ampicillin (10 mcg), apramycin (15 µg), norfloxacin (10 µg), metronidazole (5 mcg), amikacin (30 µg), gentamycin (10 µg), streptomycin (10mcg), penicillin (10 µg), lincomycin (10 µg) and colistin (10 mcg).

2.4. Molecular characterization of *C.perfringens*

All *C. perfringens* isolates were tested for toxin and multidrug resistance genes using PCR. DNA extraction was performed using the QIAamp DNA mini kit (Qiagen, Germany, GmbH) **[23]**. Oligonucleotide primers were obtained from Metabion (Germany), with the sequences of the primers and their PCR thermal profile displayed in table 1.

Table (1): Oligonucleotide primers sequences and cycling condition for PCR:

Toxin	Gene sequence	Cycling conditions of the different primers during conventional PCR	Amplified product	Reference
Alpha	GTTGATAGCGCAGGACATGTTAAG	1 cycle (95 °C, 8 min) 30 cycles (95 °C, 30 s/58 °C, 30 s/72 °C, 30 s) 1 cycle (72 °C, 7 min	402 bp	Yoo *et al*., 1997
	CATGTAGTCATCTGTTCCAGCATC			
*tpe*L	ATATAGAGTCAAGCAGTGGAG	1 cycle (95 °C, 5 min) 30 cycle (94 °C, 30 s/62 °C, 90 s/ 72 °C, 1 min) 1 cycle (72 °C, 10 min	466 bp	Bailey *et al*., 2013
	GGAATACCACTTGATATACCTG			
*net*B	CGCTTCACATAAAGGTTGGAAGGC	1 cycle (94 °C, 5 min) 30 cycle (94 °C, 30 s/55 °C, 30 s/72 °C, 30 s) 1 cycle (72 °C, 7 min	316 bp	
	TCCAGCACCAGCAGTTTTTCCT			
*tet*K	TTATGGTGGTTGTAGCTAGAAA	1 cycle (94 °C, 5 min) 30 cycle (94 °C, 30 s/55 °C, 30 s/72 °C, 30 s) 1 cycle (72 °C, 7 min)	382 bp	Gholamiandehkordi *et al*., 2009
	AAAGGGTTAGAAACTCTTGAAA			
bla	ATGAAAGAAGTTCAAAAATATTTA	1 cycle (94 °C, 5 min) 30	780 bp	Catalán

	GAG	cycle (94 °C, 30 s/55 °C, 30 s/72 °C, 30 s) 1 cycle (72 °C, 7 min		*et al*., 2010
	TTAGTGCCAATTGTTCATGATGG			

3. RESULTS

Identification of *C. perfringens* isolates

Following 24 hours of incubating the samples in cooked meat medium, colonies emerged with a characteristic black pigmentation on C. perfringens agar, indicative of *C. perfringens*. Suspected colonies of *C. perfringens* were additionally sub-cultured on *C. perfringens* agar plates to achieve pure culture. On sheep blood agar, the isolates exhibited a double zone of hemolysis surrounding the colony, which is characteristic of *C. perfringens*. Notably, the central area of total hemolysis results from theta toxin, whereas the external area of partial hemolysis is a result of the phospholipase action of alpha toxin.

During Gram's staining, the black colonies obtained from C. perfringens agar revealed Gram-positive rods indicative of *C. perfringens*. When inoculated onto egg yolk agar plates, C. perfringens generated opalescence surrounding the colonies, indicating lecithinase activity, which is a hallmark of the organism.

A total of 21 out of 150 (14%) isolates of *C. perfringens* were retrieved through bacteriological testing, with 19 out of 130 (14.6%) from unhealthy and 2 out of 20 (10%) from seemingly healthy broilers (table 2). The occurrence of *C. perfringens* isolated based on the samples included 115 intestinal samples from sick chickens 17 (14.7%), 15 liver samples from sick chickens 1(6.6%), 15 intestinal samples from seemingly healthy chickens 2 (13.3%), and 5 liver samples from seemingly healthy chickens 1 (20%).

Antimicrobial sensitivity testing:

The in-vitro antimicrobial susceptibility testing for the isolated *C. perfringens* strains (n=21) showed that all isolates were 100% highly sensitive to amoxicillin, 76.2% to gentamycin, 66.6% to norfloxacin, and 62% to levofloxacin, while they demonstrated high resistance of 62% to metronidazole and 52.5% to penicillin. Six strains exhibiting multi-drug resistance included no. 10, 16, 25, 35, 93, and 116, as illustrated in Fig. 1.

Molecular characterization of 3 different toxins, and 2 resistance genes in all the 21 isolates of *C. perfringens*

Standard PCR testing outcomes indicated that all 21 analyzed isolates contained alpha toxin at 401 bp. Nine isolates (numbers 10, 12, 15, 16, 25, 27, 84, 89, and 93) possessed *netB*, while six isolates (numbers 7, 10, 12, 16, 28, and 30) had tpeL genes at 316 bp and 466 bp, respectively. Out of 21 isolates, two (no. 10 and 16) tested positive for 3 toxin genes (*alph*A, *net*B, and *tpe*L) along with the bla gene, whereas one (isolate no. 12) was positive for only the 3 toxin genes (*alph*A, *net*B, and *tpe*L). Out of 21 isolates, six (No. 15, 27, 44, 84, 89, and 93) tested positive for 2 toxin genes (*alph*A and *net*B), with three isolates (No. 27, 44, 93) also being positive for the bla gene. Two C. perfringens strains (No. 28 and 30) tested positive for 2 toxigenic genes (*alph*A and *tpe*L), but only isolate No. 28 also possessed the *bla* gene. Nine *C. perfringens* isolates (Nos. 25, 26, 35, 40, 82, 92, 94, 110, and 116) tested positive solely for the alphA toxin gene, of which 3 isolates (Nos. 35, 110, and 116) also tested positive for the *bla* gene and exhibited resistance to β-lactam. The overall isolates carrying the *bla* gene and resistant to β-lactam numbered 11 out of 21 (specifically no. 7, 10, 16, 27, 28, 35, 44, 82, 93, 110, and 116). All isolates of *C. perfringens* tested negative for the tetracycline resistance gene (*tet*K), as demonstrated in Table (3) and Fig.(2)

Table (2): Incidence of *C. perfringens* according to sample types:

Samples	Positive intestinal samples	Incidence %	Positive liver samples	Incidence %	Total
Diseased broilers	17/115	14.7 %	1/15	6.6%	18/130 (13.8%)
Apparently healthy broilers	2/15	13.3%	1/5	20%	3/20 (15%)
Total	19/130	14.6%	2/20	10%	21/150 (14%)

Table (3): Conventional PCR assay to identify the presence of *Alpha*, *tpe*L and *net*B toxin genes in the *C. perfringens* isolates.

Sample No.	**Locality**	**Total No.**	**Age (days)**	**Mortality***	**Conventional PCR results**					
					***alpha* toxin**	***net*B**	***tpe*L**	***bla***	***tet*K**	**Total**
7	El-Beheira	4000	23	3.1%	+ve	-ve	+ve	+ve	-ve	3/5
10	El-Beheira	6000	22	0.5%	+ve	+ve	+ve	+ve	-ve	4/5
12	El-Beheira	2000	20	1.5%	+ve	+ve	+ve	-ve	-ve	3/5
15	El-Beheira	5000	12	0.94%	+ve	+ve	-ve	-ve	-ve	2/5
16	El-Menoufia	20000	21	1%	+ve	+ve	+ve	+ve	-ve	4/5
25	El-Menoufia	15000	18	0.33%	+ve	-ve	-ve	-ve	-ve	1/5
26	El-Menoufia	1500	18	0.33%	+ve	-ve	-ve	-ve	-ve	1/5
27	El-Gharbia	35000	16	1.5%	+ve	+ve	-ve	+ve	-ve	3/5
28	El-Beheira	5000	21	75 %	+ve	-ve	+ve	+ve	-ve	3/5
30	El-Gharbia	10000	32	0.01%	+ve	-ve	+ve	-ve	-ve	2/5
35	El-Menoufia	15000	22	0.7%	+ve	-ve	-ve	+ve	-ve	2/5
40	El-Gharbia	2000	25	0.4%	+ve	-ve	-ve	-ve	-ve	1/5
44	El-Gharbia	2000	25	0.4%	+ve	+ve	-ve	+ve	-ve	3/5
82	El-Menoufia	20000	22	0.8%	+ve	-ve	-ve	+ve	-ve	2/5
84	El-Menoufia	1000	21	0.92%	+ve	+ve	-ve	-ve	-ve	2/5

89	El-Menoufia	1000	21	0.92%	+ve	+ve	-ve	-ve	-ve	2/5
92	El-Menoufia	15000	19	0.32%	+ve	-ve	-ve	-ve	-ve	1/5
93	El-Menoufia	15000	19	0.32%	+ve	+ve	-ve	+ve	-ve	3/5
94	El-Gharbia	5000	23	0.72%	+ve	-ve	-ve	-ve	-ve	1/5
110	El-Menoufia	20000	21	0.80%	+ve	-ve	-ve	+ve	-ve	2/5
116	El-Menoufia	20000	30	0.01%	+ve	-ve	-ve	+ve	-ve	2/5
Total					21/21	9/21	6/21	11/21	0/21	

Fig. 1. Antibiotic sensitivity testing of *C. perfringens* isolates (*n* = 21)

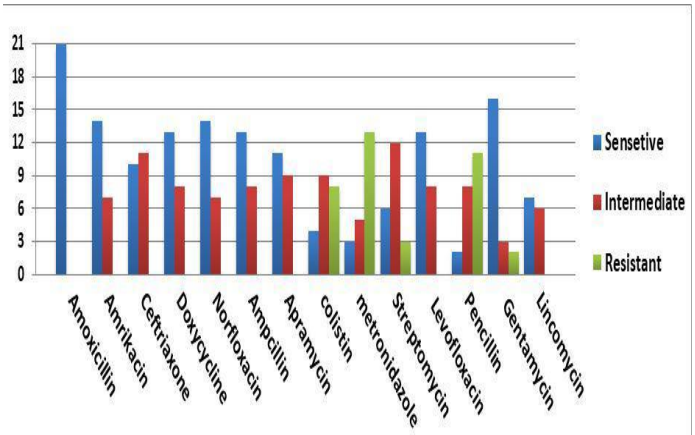
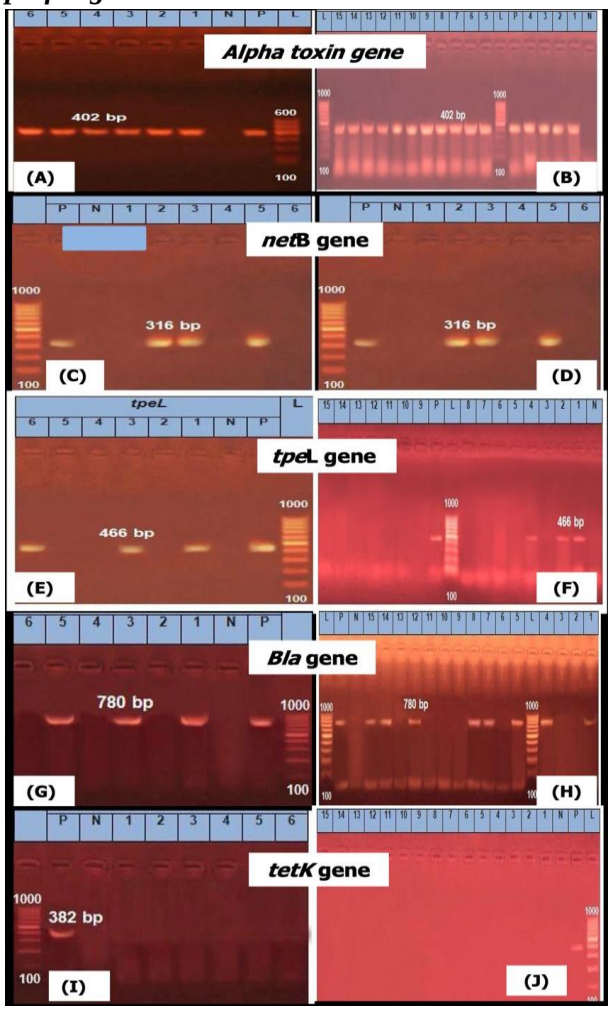


Fig. 2. Conventional PCR results of *C. perfringens* isolates



4. DISCUSSION

Clostridium perfringens is a well-known bacterium located in poultry facilities and their environments, such as water, poultry waste, feed, soil, and air. *C. perfringens* is thought to be responsible for several economically important diseases in livestock. Genetically, *C. perfringens* is a varied organism that has been effectively isolated from the gastrointestinal tract of both animals and humans. Especially in poultry, *C. perfringens* is found along nearly the entire length of the GIT; it has been recovered from the crop, gizzard, and caeca **[24]**. In the current research, the bacteriological analysis of 150 broiler samples from El-Menoufia, El-Gharbia, and El-Beheira governorates showed the isolation of *C. perfringens* strains, yielding an overall incidence of 14%. This finding correlates with **[25]**, who identified 12 isolates as *C. perfringens* among 100 examined samples, resulting in a total percentage of 12% from broilers. Conversely, **[26]** indicated that *C. perfringens* was obtained from 40 samples, showing an occurrence rate of 44.4% in broilers.

C. perfringens toxinotypes contribute to different disease syndromes in poultry and livestock animals. In this research, 15% of samples taken from healthy broilers were positive for C. perfringens. The characteristics of the colonial morphology featured blackish colonies, resulting from the reduction of sulfite to sulfide by *C. perfringens*, which then reacts with iron to form a black iron sulfide precipitate. Additionally, on sheep blood agar media, glistening round colonies were noted, surrounded by a central zone of complete hemolysis and an outer zone exhibiting discoloration and incomplete hemolysis after 48 hours of incubation at 37 ºC in anaerobic conditions. These findings align with **[27,28]**. At the microscopic level, all isolates in this study were Gram-positive spore-forming bacilli, and biochemical analysis revealed that the isolates were positive for both catalase and lecithinase, as previously documented by **[29]**. On egg yolk agar, every isolate degraded lecithin in the medium, leading to opalescence around the streak. These results align with those of **[30]**.

Initial infections with *C. perfringens* within the initial three weeks of life, as shown by isolates no. (12, 15, 25, 26, 27, 28, 84, 89, 92, 93, and 110), could be attributed to contamination in hatcheries with *C. perfringens*. This finding aligns with **[31]**, who established a link between hatchery contamination and a new form of enteritis in young broilers, characterized by necro-ulcerative enteritis in chickens at three weeks of age, which is linked to the isolation of *C. perfringens.*

Numerous predisposed factors contribute to the onset of necrotic enteritis, such as stress, coccidiosis, damage to the intestinal mucosa, inadequate sanitation, nutritional imbalance, mycotoxins, and substandard housing conditions. These elements facilitate the substantial development and significant toxin generation of C. perfringens **[32]**.

All toxigenic strains of *C. perfringens* can produce alpha toxin, which exhibits lecithinase activity and leads to tissue necrosis, particularly in the small intestine. Alpha toxin is primarily accountable for necrotic enteritis in chickens **[6]**. Our results showed that the *C. perfringens* strains analyzed by PCR possess alpha, *net*B, and *tpe*L toxin genes. In this investigation, a PCR assay was utilized to identify the *alph*A toxin gene, revealing that all isolates tested positive at 402 bp, which aligned with the findings reported by **[33]**. Additionally, **[13]** indicated that alpha toxin is the main cause of necrotic enteritis in chickens.

The findings of detected *net*B in 9 out of 21 isolates were consistent with **[34]**, suggesting the presence of the *net*B gene in *C. perfringens* isolates from unhealthy broiler chickens in NE, with a significant rate of 35% but a reduced occurrence in healthy broiler chickens at 22%. These findings align with **[35]**, which indicated that netB toxins are the primary virulence factors linked to the onset of NE, and these toxins are solely generated by type A strains of C. perfringens. Additionally, these findings aligned with **[6]**, which highlighted the

significance of the netB toxin in inducing NE. It indicated that 70% of *C. perfringens* isolates from poultry suffering from NE tested positive for the *net*B gene, suggesting the *net*B toxin plays a crucial role in inducing NE.

In the current research, the *tpe*L gene was identified in 6 out of 21 isolates. This finding is consistent with **[25]**, which indicated that the *tpe*L gene is among the toxin-producing genes in C. perfringens, identified in 28.5% of the tested isolates. These findings also align with **[36]**, which found the *tpe*L gene in 9% and 2% of isolates from deceased and seemingly healthy broilers, respectively.

C. perfringens isolates carrying the *bla* gene were 11/21, whereas the *tetK* gene was not found in any of the isolates. This finding aligns with **[25]**, which identified a 75% prevalence of the B. lactamase resistant gene (bla).

The PCR findings showed that 3\21 isolates (10, 12, and 16) tested positive for 3 genes (*alph*A, *net*B, and *tpe*L toxin genes), whereas 3\21 isolates (15, 84, and 89) were positive for two genes (*alph*A and *net*B toxin genes). This indicates a significant level of virulence and pathogenicity in these isolates, allowing them to cause NE on their own without requiring predisposing factors, which results in varying levels of mortality.

These findings align with **[37]**, which indicated that the presence of virulent *alph*A *net*B and *tpeL* carrying strains is crucial for NE development. The elevated occurrence of the bla resistance gene (β-lactam) in the isolates studied limits the options for control and requires the exploration of alternative solutions. These findings were consistent with **[38]**, which stated that antibiotics like lincomycin and bacitracin are utilized in the treatment of necrotic enteritis. Earlier, **[39]** stated that amoxicillin is among the most potent β-lactam antibiotics. It is commonly employed in veterinary medicine due to its broad-spectrum antimicrobial properties, excellent and rapid absorption, and effective tissue penetration, making it an optimal choice for treating necrotic enteritis.

In our research, isolate no. (30) that tested positive for two toxigenic genes (*alph*A and *tpe*L) exhibited resistance to β-lactam and had a high mortality rate (75%), potentially due to mixed infections with other viral, bacterial, and/or coccidial infections.

The antimicrobial sensitivity testing conducted in vitro on the isolated C. perfringens strains (n=21) showed that all isolates exhibited high sensitivity to amoxicillin, gentamycin 76.2%, Norfloxacin 66.6%, and levofloxacin 62%, while demonstrating significant resistance to metronidazole 62% and penicillin 52.5%. These results are consistent with **[38]**, indicating that the mix of amoxicillin and clavulanic acid proved effective against *C. perfringens* strains. In a similar manner, **[25]** discovered that the resistance of *C. perfringens* isolates to metronidazole was 100%.

Typically, NE outbreaks in broilers can be easily managed through the use of specific antibiotics in water, including lincomycin, bacitracin, oxytetracycline, penicillin, and tylosin tartrate. To avoid the illness, antibiotics like bacitracin, lincomycin, penicillin, or nitrovin have been mixed into the feed. Nonetheless, there are indications of resistance in some isolates to specific antimicrobials like bacitracin and lincomycin **[38]**. In this study, all strains tested demonstrated a complete absence of the tetracycline-resistant gene (*tet*K), possibly because tetracycline is rarely used in the treatment of necrotic enteritis on poultry farms. These findings aligned with **[25]**, which indicated that all tested strains exhibited 100% absence of the tetracycline-resistant gene (*tet*K); however, these results contradicted those of **[40]**, who stated that the proportion of tetracycline-resistant isolates differed based on the source of the isolates and the antibiotic utilized in treatment.

5. CONCLUSIONS

Clostridium perfringens is common in the broiler farms studied in Egypt. The existence of C. perfringens in healthy poultry draws focus on them as carriers of Clostridial infection,

potentially resulting in disease under immunocompromised conditions that could cause considerable economic losses for the poultry sector. Certain isolates of *C. perfringens* harboring *alpha*, *netB*, and *tpeL* genes that allow them to cause NE independently of predisposing factors. Additionally, the frequent occurrence of *bla* resistance genes reduces the likelihood of effective NE control and requires the exploration of alternative solutions.

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Authors' declarations

Publication consent

Each author has demonstrated their consent for the publication of the current manuscript

Data and material availability:

All data of this study is provided.

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