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### Antibiotic resistance pattern of *Clostridium perfringens* isolated from necrotic enteritis cases of broiler chickens in Luxor city

Shimaa El-Nagar<sup>\*</sup>, Azhar G. Shalaby<sup>\*\*</sup>, Heba Badr<sup>\*\*</sup>

\* Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute (Luxor branch), (ARC)

\*\*Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute (AHRI), Agricultural Research Centre, (ARC), Dokki, Giza, Egypt

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#### ABSTRACT:

**C***lostridium perfringens* is one of the highest dominant spore-forming foodborne pathogens, which is widely distributed and causes severe diseases in humans and animals. Poultry is the main host of this pathogen. In this study, we investigated the prevalence, antibiotic resistance pattern, and toxin-encoding genes of *C. perfringens* isolated from chicken with necrotic enteritis. Twelve of *C. perfringens* strains were isolated from 100 intestinal samples (Native and Hubbard chickens) with a percentage of 12% and subsequently confirmed by PCR; all strains carried alpha-toxin gene and were negative for other major toxin genes (Beta, Epsilon and Iota). The *C. perfringens* isolates showed highly resistance (100%) to most of the antibiotics tested; Ampicillin, Amoxicillin, Amoxicillin-clavulanic acid, Erythromycin, Lincomycin, Metronidazole, Neomycin, Oxytetracycline, Penicillin, Spectinomycin, Streptomycin, but showed sensitivity with a low percentage (25% or low) for Colistin sulphate, Doxycyclin, Enrofloxacin, Norfloxacin and Sulfamethoxazole-trimethoprim. However, virulence genes were detected with a different percentage of 100%, 75% and 16.7 with *Cpe*, *netB* and *Tpel* respectively. In the examination of antimicrobial-resistant genes the examined isolates showed 100%, 75% and 25% with (tetK) tetracyclines, (Bla) B. lactamase resistant gene and macrolide (ermB) respectively. Our findings confirmed that the diseased cases with different sings of multidrug-resistant *C. perfringens* strains could be regarded as one of the concerning pathogens in broiler chickens and its products. Widespread monitoring of *C. perfringens* isolates is strongly suggested.

#### INTRODUCTION

*Clostridium perfringens* (*C. perfringens*) is a Gram-positive anaerobic bacterium that is commonly found in nature also it is one of the constituents of normal flora in the gastrointes-

tinal tract of animals and humans (Miller et al. 2010). *C. perfringens* has emerged necrotic enteritis (NE) as an important disease of digestive tract infections and a major concern in the poultry industry and leading to severe econom-

Corresponding Author: Shimaa El Nagaar, Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute (Luxor branch), (ARC)

E-mail address:

DOI:

ic losses (Mwangi et al. 2019, Salem and Attia 2021). However, *C. perfringens* is normal intestinal content but it developed to NE under presence of some risk factor; high temperature and or high protein constituent based diet, that lead to increase the *C. perfringens* rate up ten times than normal cases (Cooper and Songer 2009). Necrotic enteritis (NE) present in two forms either clinical or subclinical which consider the main enteric poultry disease that affect productivity in the broiler so badly affecting the poultry industry (Bansal et al. 2021 Salem et al. 2021). There are five toxin types of *C. perfringens* strains (A to E) this typing is according to the major toxin production abilities such as Alpha toxin, beta, epsilon, and iota toxins (Schlegel et al. 2012). The main toxin type associated with NE in poultry is toxin type A (Wilder et al. 2001 Uzal et al. 2014). Also, it has been suggested that Alpha toxin is the key virulence element for NE in broilers (Wages 2003). However, (NetB), which is a spore-forming toxin, shows a major role in the pathogenesis of NE produced by some strains of *C. perfringens*, (Keyburn et al. 2008 Keyburn et al. 2010). The disease is distributed worldwide in two forms clinical and subclinical forms, causing a reduction in body weight or body weight gain (Abd El-Hack et al. 2022). As the extensive use of antibiotics as growth promoters and to control diseases in poultry such as NE leads to re-emergence of antibiotic resistance, especially in Healthy poultry (Collier et al. 2008 Lanckriet et al. 2010 and Lyhs et al. 2013). Moreover, recent studies showed that most *C. perfringens* strains isolated from NE are resistant to commonly used antibiotics such as streptomycin and gentamicin (Park et al. 2015).

The current study was designed to distinguish the NE status, In Luxor city to isolate *C. perfringens* from suspected cases in broiler birds (Native and Hubbard), identification and typing of the isolates by PCR technique in addition to detection of their antimicrobial resistance pattern and identified different virulent and antibiotic relevant genes using specific primers for accurate identification and consciousness of the infection and antibiotic resistant pattern in the study area.

## Materials and Methods

### Sampling

One hundred of chicken intestinal samples (50 Native breed broilers and 50 Hubbard broilers) were collected from Native breed chickens of 6–8 weeks old and from Hubbard of 40–45 days old. Samples were collected randomly from 20 farms (5 individual samples from each farm) with a flock size of 1000–3000 birds in Luxor city, Egypt. Samples were collected from diseased chicken showing clinical signs of necrotic enteritis like severe depression, decreased appetite, and reluctance to move, ruffled feathers, diarrhea (frothy and foamy pings with clear zone of fluidity) dehydration, and emaciation. Intestinal scrapings from freshly dead or diseased birds were collected for screening of *C. perfringens*.

### Isolation and identification of *Clostridium perfringens* according to (Dar et al. 2017)

For isolation of *C. perfringens*, samples were inoculated in cooked meat medium (Himedia®, India) and sterile liquid paraffin was poured to make a layer over the medium (anaerobic condition). Inoculated medium was incubated anaerobically at 37°C for 24 hrs. Enriched samples were streaked onto 5% sheep blood agar plates (Oxoid®, UK) supplied with 200 µg/ml neomycin sulphate incubated anaerobically at 37°C for 24 hrs using Gaspak anaerobic jar. The colonies producing characteristic double zone of hemolysis on blood agar were tentatively identified as *C. perfringens*. The pure cultures of *C. perfringens* were re-inoculated in cooked meat medium for further identification and typing of the isolates by PCR.

### Antimicrobial Sensitivity Test (AST)

All *C. perfringens* isolates were subjected to AST using the disc diffusion test on Mueller-Hinton agar as previously described (WHO-CDC 2003) against 19 antibiotics (Oxoid UK), and inhibition zones were interpreted following the guidelines of Clinical and Laboratory Standards Institute (CLSI 2021). The following antibiotic discs were used; Ampicillin (AMP) 10 µg, Amoxicillin (AMX) 10 µg, Amoxicillin - clavulanic acid (AMC) 30 µg, Ciprofloxacin

(CIP) 5 µg, Ceftriaxone (CRO) 30µg, Colistin sulphate (CT) 10 µg, Doxycyclin (DO) 30µg, Erythromycin (E) 15 µg, Enrofloxacin (ENR) 5µg, Lincomycin (L) 2 µg, Metronidazole (MT) 5 µg, Neomycin (N) 10 µg, Norfloxacin (NOR) 10 µg, Oxytetracycline (OT) 30 µg, Penicillin (P) 10µg, Spectinomycin (SPT) 100 µg, Streptomycin (S) 10µg, Sulfamethoxazole-trimethoprim (SXT) 23.75+ 1.25=25µg and Tetracycline (TE) 30 µg.

### **Molecular identification:**

#### **DNA extraction.**

DNA extraction from pure characterized isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from 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.

#### **Oligonucleotide Primer.**

Primers used were supplied from **Metabion (Germany)** and are listed in Table (1).

#### **PCR amplification.**

##### **Multiplex PCR for detection of Alfa- Beta-Epsilon and Iota toxin genes (*cpa*, *cpb*, *ctx* and *iap*):**

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, 12 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

##### **Uniplex PCR for other genes (*netB*, *tpel*, *ermB*, *Bla*, *tetK* and *cpe*):**

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp 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. The reaction was performed in an Applied biosystem 2720 thermal cycler.

#### **Analysis of the PCR Products.**

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 analysis, 20 µl of the products were loaded in each gel slot. Generuler 100 bp ladder (fermentas, Thermo) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

**Table (1)** Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Second denaturation	Annealing	Extension		
Alpha ( <i>cpa</i> )	GTTGATAGCGCAG-GACATGTTAAG CATGTAG-TCATCTGTTCCAGCA TC	402	94°C 5 min.	94°C 45 sec.	50°C 45 sec.	72°C 45 sec.	72°C 10 min.	Yoo et al., 1997
Beta ( <i>cpb</i> )	ACTATACAGA-CAGATCATTCAACC TTAGGAGCAGTTA-GAACTACAGAC	236						
Epsilon ( <i>etx</i> )	ACTGCAAC-TACTACTCATACTGT G CTGGTGCCTTAA-TAGAAAAGACTCC	541						
Iota ( <i>iap</i> )	GCGATGAAAAGCC-TACACCACTAC GGTATATCCTCCAC-GCATATAGTC	317						
<i>netB</i>	CGCTTCACATAAAAGG TTGGAAGGC TCCAGCACCAGCAG-TTTTTCT	316	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 40 sec.	72°C 10 min.	Bailey et al., 2013
<i>tpcL</i>	ATATAGAGTCAA-GCAGTGGAG GGAATACCACTTGA-TATACCTG	466	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	
<i>ermB</i>	GAA AAG GTA CTC AAC CAA ATA AGT AAC GGT ACT TAA ATT GTT TAC	638	94°C 5 min.	94°C 30 sec.	57°C 40 sec.	72°C 45 sec.	72°C 10 min.	Soge et al., 2009
<i>Bla</i>	ATGAAA-GAAGTTCAAAAATAT TTAGAG TTAGTGCCAATT-GTTCATGATGG	780	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 45 sec.	72°C 10 min.	Catalán et al., 2010
<i>tetK</i>	TTATGGTGGTT-GTAGCTAGAAA AAAGGGTTA-GAAACTCTTGAAA	382	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 40 sec.	72°C 10 min.	Gholamiandeh kordi et al., 2009
Enterotoxin ( <i>cpe</i> )	ACATCTGCAGA-TAGCTTAGGAAAT CCAGTAGCTGTAATT-GTTAAGTGT	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

**RESULTS:**

Twelve *C. perfringens* strains were isolated from 100 intestinal samples with a percentage of 12% with characteristic colonies which was

subsequently confirmed by PCR; there are 16% (8/50) from Native breed and 8% (4/50) from Hubbard broiler. Table (2).

**Table (2)** Incidence of isolation for *C. perfringens* strains.

Intestinal samples/ breed	No of positive/50	Percentage of positivity (%)
Native	8	16
Hubbard	4	8
<b>Total</b>	<b>12</b>	<b>12</b>

**Antimicrobial Sensitivity Test (AST)**

The *C. perfringens* isolates showed highly resistance to most of antibiotic tested; Ampicillin, Amoxicillin, Amoxicillin-clavulanic acid, Erythromycin, Lincomycin, Metronidazole, Neomycin, Oxytetracycline, Penicillin, Spec-

tinomycin, Streptomycin with 100% resistance, but the sensitive result were present with low percentage (25% or low) for Colistin sulphate, Doxycyclin, Enrofloxacin, Norfloxacin and Sulfamethoxazole-trimethoprim as shown in Table (3).

**Table (3)** Antimicrobial susceptibility pattern of the isolated *C. perfringens* strains (n=12)

Antimicrobial agent	Resistant No. (%) *	Intermediate No. (%) *	Sensitive No. (%) *
Ampicillin (AMP <sup>10</sup> )	12/12 (100%)	-	-
Amoxicillin (AMX <sup>10</sup> )	12/12 (100%)	-	-
Amoxicillin-clavulanic acid (AMC <sup>30</sup> )	12/12 (100%)	-	-
Ciprofloxacin (CIP <sup>5</sup> )	8/12 (66.7%)	4/12 (33.3%)	-
Ceftriaxone (CRO <sup>30</sup> )	9/12 (75%)	3/12 (25%)	-
Colistin sulphate (CT <sup>10</sup> )	9/12 (75%)	-	3/12 (25%)
Doxycyclin (DO <sup>30</sup> )	3/12 (25%)	6/12 (50%)	3/12 (25%)
Erythromycin (E <sup>15</sup> )	12/12 (100%)	-	-
Enrofloxacin (ENR <sup>5</sup> )	7/12 (58.3%)	3/12 (25%)	2/12 (16.7%)
Lincomycin (L <sup>2</sup> )	12/12 (100%)	-	-
Metronidazole (MT <sup>5</sup> )	12/12 (100%)	-	-
Neomycin (N <sup>10</sup> )	12/12 (100%)	-	-
Norfloxacin (NOR <sup>10</sup> )	8/12 (66.7%)	2/12 (16.7%)	2/12 (16.7%)
Oxytetracycline (OT <sup>30</sup> )	12/12 (100%)	-	-
Penicillin (P <sup>10</sup> )	12/12 (100%)	-	-
Spectinomycin (SPT <sup>100</sup> )	12/12 (100%)	-	-
Streptomycin (S <sup>10</sup> )	12/12 (100%)	-	-
Sulfamethoxazole-trimethoprim (SXT <sup>25</sup> )	9/12 (75%)	-	3/12 (25%)
Tetracycline (TE <sup>30</sup> )	5/12 (41.7%)	7/12 (58.3%)	-

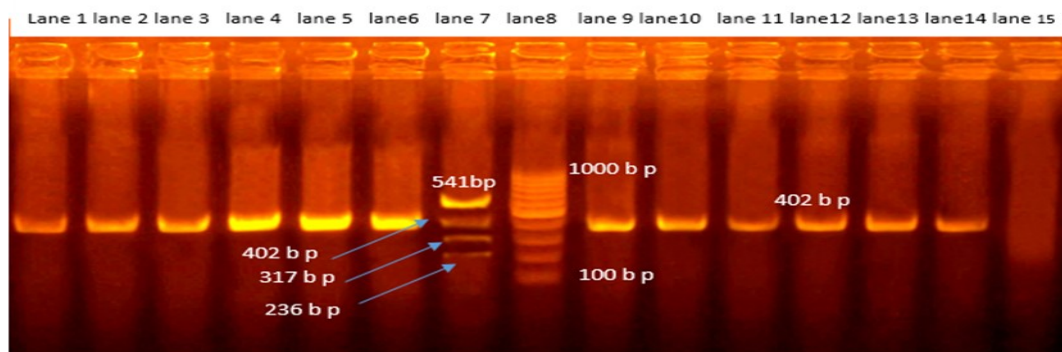
\* Percentage of positive samples.

**PCR Results:**

All the isolated strains were undergoing detection and confirmation of toxin types by multiplex PCR through screening for the common toxin genes *cpa*, *cpb*, *etx* and *iap* (*Alpha*, *Beta*, *Epsilon* and *Iota*) *toxins*

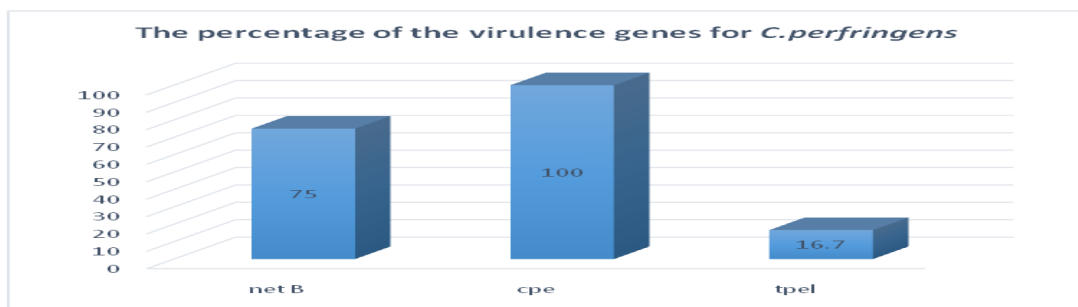
All twelve examined strains were confirmed as alpha producing toxin only and thus typed as *C. perfringens* type A. As shown in Fig. (1). Conducting conventional PCR technique for

strains using dedicated primers by different virulent genes like (*Cpe*, *netB*, and *tpel*). The examined strains showed 100% (12/12) presence of enterotoxin *Cpe* gene, in addition to the 75% (9/12) of strains showed positivity to *netB* gene and the presence of *tpel* gene was the least percentage about 16.7% (2/12). as shown in Fig. (2). All individual genes for virulence were represented in Fig. (3) to Fig.(5).

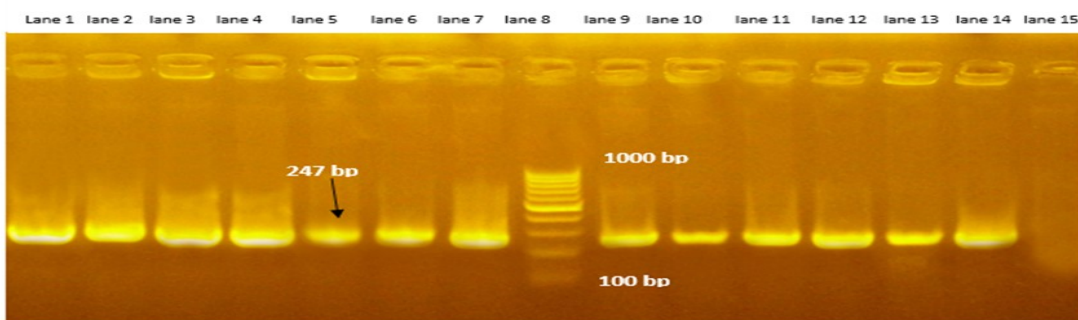


**Fig. (1):** Typing pattern for toxins (alpha, Beta, epsilon and Iota) for *C. perfringens* isolates by multiplex PCR for detection of toxin genes (*cpa*, *cpb*, *etx* and *iap*) respectively:

Lane 1 to lane 6 (first six examined *C. perfringens* strains which sowed Alpha type toxin at 402 bp, lane 7 different toxin positive controls, lane 8 Generuler 100 bp ladder, lane 9-14 (the rest of strains) lane 15 negative control



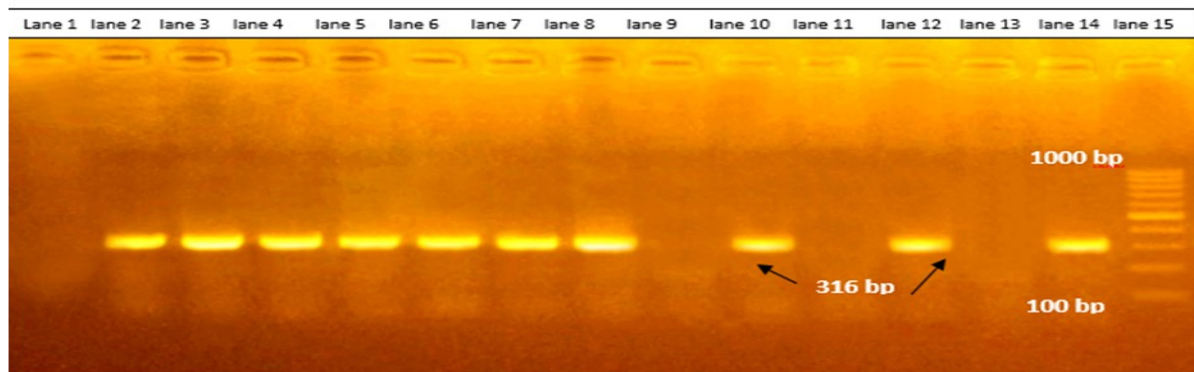
**Fig (2)** The different virulence genes for *clostridium perfringens*.



**Fig (3):** Gel electrophoresis for *cpe* gene for *C. perfringens*.

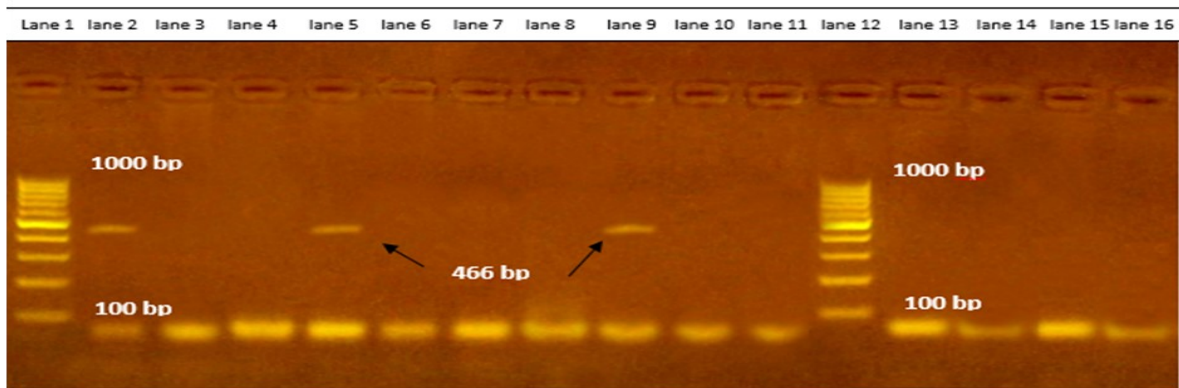
Lane 1 to lane7 represented positive amplification for *cpe* gene at 247 bp for *C. perfringens*, Lane 8 (Generuler 100bp), lane 9 to lane 13 the rest of *C. perfringens* strains, lane 14 and 15 represented positive and negative control.





**Fig (4): Gel electrophoresis for *netB* gene for *C. perfringens*.**

Lane 1 to lane12 represented the results for *NetB* gene for *C.perfringens*, the positive amplification appeared at 316 bp, lane 13 and 14 represented negative and positive control, lane 15 represented the Generuler 100bp.

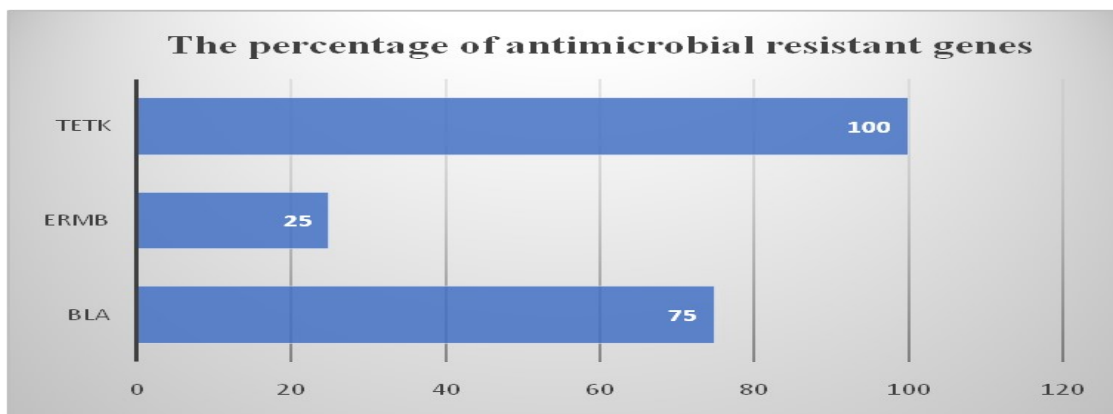


**Fig (5): Gel electrophoresis for *tpeL* gene for *C. perfringens*.**

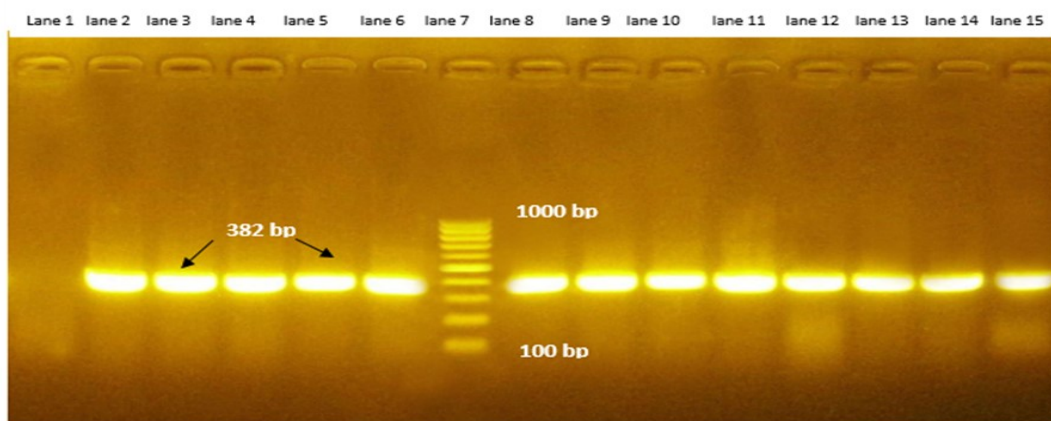
Lane 1 and 12 represented the Generuler (100bp), lane 2 and 3 represent the positive and negative control, lane 4 to lane 16 represented the twelve strains of *C. Perfringens*. The positive amplification for *tpeL* gene was at 466 bp.

On examination of the antibiotic resistance genes, All the examined strains showed 100% (12/12) presence of tetracycline-resistant gene (*tetK*), and a percentage of 75% (9/12) for the B. lactamase resistant gene (*Bla*) and the least

gene was macrolide (*ermB*) with a percentage of 25% (3/12). As shown in Fig. (6). All the individual genes for antibiotic resistance were represented in Fig (7) to Fig (9).

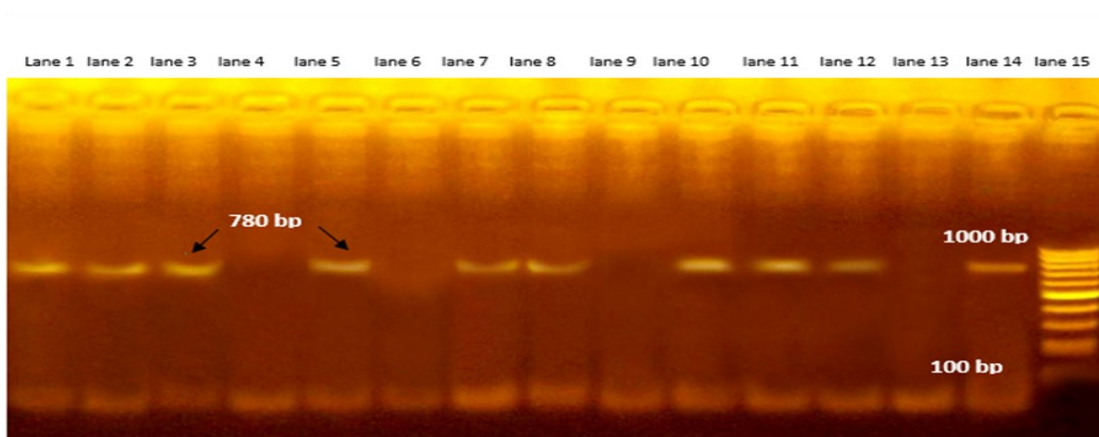


**Fig. (6): The percentage of different antimicrobial resistant genes for *Clostridium perfringens***



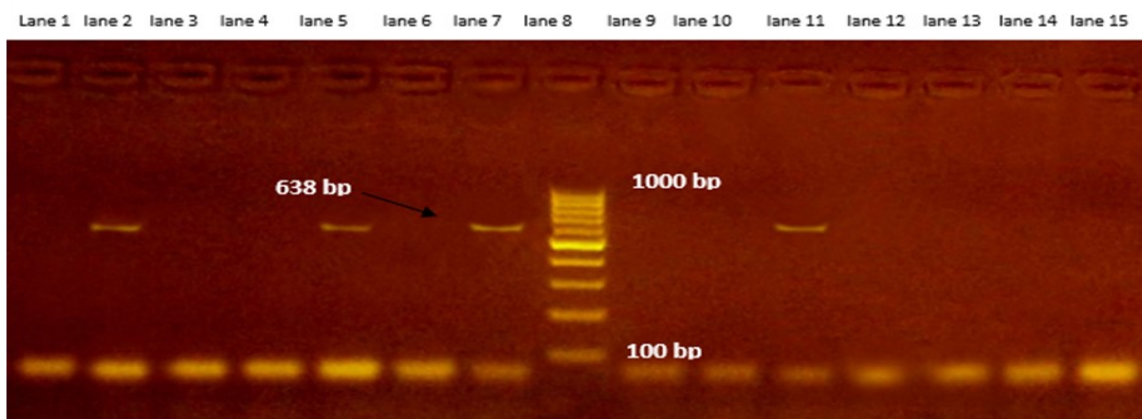
**Fig (7):** Gel electrophoresis for *tetK* gene for *C. perfringens*.

Lane 1 and 2 represented the negative and positive control, lane 3 to lane 5 represented positive amplification for tetK at 382 bp., lane 7 represented the Generuler (100 bp). Lane 9 to lane 15 the rest of *C. perfringens* strains



**Fig (8):** Gel electrophoresis for *Bla* gene for *C. perfringens*.

Lane 1 to lane 12 represented the twelve *C. perfringens*, positive amplification for bla gene appeared at 780 bp. Lane 13 and 14 represented negative and positive control, lane 15 represented the Generuler (100 bp).



**Fig (9):** Gel electrophoresis for *ermB* gene for *C. perfringens*.

Lane 1 and 2 represented negative and positive control, lane 3 to lane 7 represented strains of *C. perfringens*, the positive amplification for *ermB* appeared at 638 bp lane 8 represented Generuler (100bp), lane 9 to lane 15 represented the rest of *C. perfringens* strains



## DISCUSSION

*C. perfringens* has emerged as necrotic enteritis (NE) which is a significant disease of digestive tract infections causing severe economic losses (Salem and Attia 2021). In this research, we only confirm 12 isolates as *C. perfringens* out of 100 examined samples with a total percentage of 12%, also it was 16% (8/50) from Native breed and 8% (4/50) from Hubbard broiler. our results for isolation were consistence with that obtained by Fancher et al . 2021 that only confirm 9 *C.perfringens* isolates from 734 isolates and all of type A toxin, also our results were compatible with that recorded by (Mwangi, et al. 2019) who found that 81% of isolated *C.perfringens* were from diseased dead birds. Antimicrobial susceptibility testing using a disc diffusion method indicated that 100% of the isolates had a multidrug-resistant profile that comprised of Ampicillin, Amoxicillin, Amoxicillin-clavulanic acid, Erythromycin, Lincomycin, Metronidazole, Neomycin, Oxy-tetracycline, Penicillin, Spectinomycin, Streptomycin, but the sensitivity was (25% or low) for Colistin sulfate, Doxycyclin, Enrofloxacin, Norfloxacin and Sulfamethoxazole-trimethoprim, the current research results were in agreement with (Osman and El-hariri, 2013), that detected 100% (n = 125) of *C. perfringens* isolates from broiler chickens were resistant to gentamicin, erythromycin, streptomycin, lincomycin, and spiramycin. Moreover, the authors reported a major multidrug-resistant profile that covered of gentamicin, streptomycin, lincomycin, and erythromycin. in another study Park et al. (2015) reported that *C. perfringens* strains isolated from NE cases were resistant to many antimicrobial agents like gentamicin, neomycin, streptomycin, colistin and intermediate resistance to bacitracin, tetracycline, clindamycin erythromycin, trimethoprim-sulamethoxazole, and sulfisoxazole. In this research all the examined strains showed 100% presence of tetracycline-resistant gene (*tetK*), and a percentage of 75% of the  $\beta$ . lactamase resistant gene (*Bla*) and the least resistant gene was macrolide (*ermB*) with a percentage of (25%). these results reflected and confirmed the obtained results of disc diffusion results as the Multidurg resistant to tetracyclines and b lactam and also resistant to some macrolides. Another study, which was

performed in Korea by Jang et al. 2020) conveyed *C.perfringens* strains isolated from chicken samples were resistant to tetracycline (with a percentage of 100%). Anju et al. (2020) reported that 44, 40, 40 and 26.6% of *C. perfringens* recovered from poultry were resistant to gentamicin, bacitracin, erythromycin, and tetracycline antibiotics, respectively.

The virulence of *C. perfringens* was dependent on the production of at least 20 different toxins and enzymes (Revitt- mills et al. 2015). Toxin production, which differed significantly among *C. perfringens* strains depending on the presence of 6 different toxin types which encoding for *CPA*, *CPB*, *ETX*, and *ITX* toxins, as well as the recently included enterotoxin *Cpe* and necrotic enteritis B-liketoxin (*netB*) (Rood et al. 2018). *NetB* toxin was identified as a virulence element for necrotic enteritis in chickens (Keyburn et al. 2010) and this gene *netB* was found in 75% of *C. perfringens* isolates in the current study. In *netB* studies, the prevalence for *netB* was typically higher in diseased birds than in healthy birds especially broilers (Keyburn et al. 2010 Park et al. 2015 and Mwangi et al. 2019). However, Healthy birds have a more varied percentage of *C. perfringens*, which may clarify the lower prevalence of *netB* (Engstrom et al. 2003 Abildgaard et al. 2010). Recently, Zhou et al. (2017) determined that while *netB* is essential in the virulence of NE, it could be detected in healthy birds. *Cpe* is a typing toxin which has been confirmed to be responsible for enterotoxigenic infections in humans and animals (Dolan et al. 2016 Ghoneim et al. 2017) also the production of the *CPA* toxin and the *NetB* toxin were responsible for necrotic enteritis in poultry (Uzal et al. 2018). *CPE* gene was present in 100% of the examined isolates in the current study which indicated the highly virulence strains. And this assumption was agreed with that discussed by (freedman et al. 2016). who mentioned that enterotoxin *CPE* produced and associated with type A, C D and E strains also induced food-poisoning and non-foodborne diarrhea. However, Forti et al. 2020 reported that The *CPE* gene can be sited on either the chromosome or on plasmids, and the expression of the toxin only occurs during sporulation. *Tpel* gene was one of the genes

producing toxin in *C.perferingnes* which was present with a percentage of 25% from examined isolates in this study, those results were higher than that recorded by **Mwangi et al. 2019** who detected the *tpel* gene in 9% and 2% of the isolates obtained from dead and apparently healthy birds, respectively.

**I**n conclusion, this study was carried out to characterize *C. perfringens* isolated from broiler chicken affected by NE. multidrug-resistant profile was detected in the isolated strains of *C. perfringens* and However, significant prevalence of *CPE* and *netB* gene in *C. perfringens* isolates from broiler chickens affected by NE suggested that they played a serious role in the pathogenesis of NE. So we recommend good vaccination program for NE and to avoid the up-use of antibiotics in the infection. In addition to *C. perfringens* requires molecular monitoring to update the data about the virulence attitudes and factors to trace its virulence factors and toxins produced.

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