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Toxin Genotyping of *C. perfringens* Isolated from Broiler Cases of Necrotic Enteritis

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Abstract: *Clostridium perfringens* organisms have an economic concern in poultry production. The purpose of this study was to investigate *Net B* and β_2 *C. perfringens* positive isolates in broiler farms and their clinic-pathological effects in broiler chicks. A bacteriological examination of *C. perfringens* was carried upon 92 Necrotic enteritis (NE) diseased cases and 55 apparently healthy broilers of different ages in Egypt. *C. perfringens* type A was only recovered (49.7%). *cpa* gene was detected in 100% of samples with PCR technique. NE diseased cases exhibited both *Net B* (87.5%) and *cpb*$_2$ (75%) toxin genes. Experimentally, an intra-gut induction of *Net B* and β_2 *C. perfringens* toxins were evaluated in chicken models. The hematological studies revealed hemolytic anemia 5 days post infection (p.i) in *Net B* and β_2 inoculated groups (G1&G2). Leucogram revealed neutrophilia and lymphopenia 5 days p.i. A significant increase in ALT, AST, uric acid and creatinine serum levels were recorded in the infected groups at 5th and 12th day p.i. DNA Sequencing for *net B* gene revealed an amino acid replacement from glutamate into arginine at codon 379 with silent mutation was also detected at nucleotide 1134. Sequencing of both toxin genes were recorded in the gene bank for the first time in Egypt. This study pointed out that *C. perfringens Net B* toxin, is a new key virulent factor for the development of NE. Further studies of *Net B* toxiod for vaccine production could minimize the clostridial problems in broiler farms.

Keywords: *C. perfringens*, PCR, *NET B*, Cpb$_2$, Broiler, Virulance, Enteritis, Sequencing

1. Introduction

Necrotic Enteritis (NE) is one of the most important diseases in poultry which destroys the intestinal lining cells of the digestive tract occurring outbreaks in broilers from 2-5 weeks of age. It is caused by *C. perfringens*, which is an important pathogen of a wide spectrum of veterinary diseases [1]. The Clinical signs include depression, decreased appetite, reduced growth rates, diarrhoea, and severe necrosis of the intestinal tract. Indeed, the bacteria live commensally in the gut under normal conditions, but when the gut microecology is drastically altered, these bacteria can proliferate. In acute form, NE causes sudden death of many birds within a few hours, without showing any clinical signs of the disease [2], however, Sub-clinical form may be the most important manifestation of enteritis as it is likely to go undetected and hence untreated [3]. In the global poultry industry, NE is considered an emerging billion-dollar disease [4, 5].

Molecular characterization and toxinotyping are the rapid tools for the detection of *C. perfringens* from suspected necrotic enteritis cases [6]. *C. perfringens* had been classified into five toxigenic types (A, B, C, D and E) according to its ability to produce the major lethal toxins [7]. Alpha toxin of *C. perfringens* is the major virulence factor responsible for producing lesions in NE disease through inducing mucosal

damage in the intestinal tract of chickens [8].

Net B toxin and its encoding gene, *net B* is a pore forming toxin of *C. perfringens* that was firstly discovered in chicken *C. perfringens* isolates of type A. It was thought to be critical to the development of NE in chickens. It is thought to be a critical for the pathogenesis of NE in broilers through causing damage to host cell [9]. Moreover, it was found be associated with *net B* positive *C. perfringens* type A strains [10]. $Beta_2$ toxin (β_2) and its encoding gene cpb_2 had been demonstrated in avian *C. perfringens* type A strains [11] but its exact role in pathogenesis was needed to be further elucidated. The amino acid sequence of cpb_2 showed no significant homologies with cpb_1 from the beta toxin (15%) or other known proteins. Although its biological activity was similar to that of beta toxin, it may possess weaker cytotoxic activity [12]. A possible pore formation or other mechanisms leading to cell membrane disruption appear to be its most plausible function [13].

The genomic variation between *C. perfringens* isolates from poultry is considered an important tool to enhance our understanding of the genetic basis of strain pathogenicity and the epidemiology of virulent and avirulent strains within the context of necrotic enteritis (NE) [14]. Here we report an investigation of *C. perfringens* toxins and particularly, *net B* and $beta_2$ toxins occurrence with respect to NE disease in broilers farms and also DNA sequencing study for both genes.

2. Material and Methods

2.1. Sampling

Intestinal and liver specimens of one hundred and forty seven cases (92 from NE diseased and 55 from apparently healthy broiler) were collected in different ages from different broiler farms in Egypt. The samples were collected aseptically in sterile separate labeled bags in an ice box then were transferred to the bacteriological laboratory to be examined.

2.2. Isolation and Identification of C. perfringens

The samples were inoculated into tubes of freshly prepared boiled then rapidly cooled cooked meat medium (CMM) (Oxoid) and incubated anaerobically for 24 hours at 37°C in a Gaspak anaerobic jar [15]. A loopful of inoculated fluid medium was streaked onto neomycin sulphate (200ug/ml) sheep blood agar plates then re-incubated anaerobically for 24 h at 37°C [16]. The lecithinase activity of suspected *C. perfringens* colonies were tested on egg yolk agar medium. Typical colonies (lecithinase producer and showed double zone of haemolysis on blood agar medium) were picked up, sub-cultured and purified for further biochemical identification tests [17].

2.3. PCR Amplification of C. perfringens Toxin Genes

2.3.1. DNA Extraction

Fifteen *C. perfringens* isolates were screened for the presence of alpha (*cpa*), beta (*cpb*), epsilon (*cpe*), iota (*cpi*) *Net B* (*net B*) and β_2 (cpb_2) toxins. To extract bacterial DNA from the recovered isolates, few *C. perfringens* colonies of each isolate grown overnight on blood agar plate at 37°C then they were suspended in 100 µl distilled water in a clean 1.5 ml microtube, boiled for ten minutes in a heat block for cell lysis then cooled on refrigerator for 15 minutes and centrifuged for ten minutes at 10,000 x g. The supernatants were carefully removed and used as template DNA [18]. Oligonucleotides primer sets (Fermentas) were selected from previously published papers and the amplification cycling conditions were listed in tables (1 & 2).

Table 1. *PCR primer sets for detection of C. perfringens toxins.*

Toxin gene	Primer sequence (5`-3`)	References
Alpha (cpa)	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	18
Beta (cpb)	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	
Epsilon (cpe)	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	
Iota (cpi)	GCGATGAAAAGCCTACACCACTAC GGTATATCCTCCACGCATATAGTC	
net B	GCTGGTGCTGGAATAAATGC TCGCCATTGAGTAGTTTCCC	
cpb2	AAATATGATCCTAACCAACAA CCAAATACTCTAATCGATGC	11

Table 2. *Cycling conditions and predicted sizes of PCR products for C. perfringens toxins.*

Target gene	Initial denaturation °C/min	Actual cycles (30-35) °C/min			Final extention °C/min	Amplified product Size (bp)
		Denaturation	Annealing	Extension		
Alpha (cpa)	94/5	94/60	55/60	72/60	72/10	400
Beta (cpb)	94/5	94/60	55/60	72/60	72/10	236
Epsilon (cpe)	94/5	94/60	55/60	72/60	72/10	541
Iota (*cpi*)	94/5	94/60	55/60	72/60	72/10	317
net b	94/5	94/30	55/30	72/60	72/10	383
cpb_2	94/5	94/30	53/90	72/90	72/10	548

2.3.2. PCR Amplification

DNA samples were amplified in a total of 50 µl of the following reaction mixture: 5µl 10X buffer, 1.5µl $MgCl_2$, 4µl dNTPs, 1µl *Taq* polymerase, 0.5µl of each primers, 5µl template DNA and completed to 50 µl by DNase-RNase-free deionized water for multiplex PCR detection for typing of *C. perfringens* toxin genes (alpha, beta, epsilon and iota) while the primers of *NET B* and β_2 *C. perfringens* toxins 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 concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

2.3.3. 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 was loaded in each gel slot. A Gelpilot100 bp Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. DNA bands were visualized and the gel was photographed by a gel documentation system.

2.4. Experimental Design [19]

Ninety (one-day-old) broiler chicks were divided into 3 groups (30 of each). The chicks were kept in cleaned, fumigated and well-ventilated separated units. The birds were fed on high protein diet during the period of the experiment. The chicks in 1st and 2nd groups were intra-gut inoculated with 2 ml inoculum of approximately 1.5×10^8 CFU/ml of CMM culture of PCR positive *Net B* and β_2 of *C. perfringens.* The culture was prepared in sterile CMM in two flasks for each toxin separately under anaerobic conditions 24 hours prior to inoculation. The culture was inoculated per OS via sterile soft tubes to be easily inoculated. The 1st group (G1) was inoculated with positive *Net B C. perfringens* culture, 2nd group inoculated with positive β_2 *C. perfringens* culture (G2) while the 3rd one (G3) acts as control negative (non-inoculated). At the end of each week p.i., the blood samples were collected aseptically from the wing vein from ten chicks for each group. The dead birds were examined macroscopically for any lesions. Intestinal and liver specimens were also, collected from the dead chicks for re-isolation and identification of *C. perfringens* and the experiment continued for 2 weeks.

2.5. Heamogram and Serum Biochemical Parameters

Blood samples were collected aseptically from wing vein of 10 chicks from each group on 5th and 12th days post infection. Erythrocytic and total leucocytic count was performed using improved Neuober hemocytometer and Natt and Herrick solution as diluting fluid [20]. Hemoglobin and packed cell volume (PCV) were measured as described by [21, 22], respectively. Blood films stained with Giemsa stain were prepared for the determination of differential leucocytic count [23]. For biochemical tests, Serum samples were collected from infected (G1 and G2) and control (G3) groups (10 /group). Aspartate and alanine aminotransferase (AST and ALT) activities were determined calorimetrically according to, [24] Total proteins and Albumin were determined according to, [25] serum creatinine was determined according to [26] and uric acid [27]. Protein electrophoresis using SDS- Polyacrylamide gel electrophoresis [28], calcium [29] and Inorganic phosphorus [30] were also, done. In addition, Sodium, potassium and chloride were determined using flame photometer [31].

2.6. Statistical Analysis

After obtaining the data, they were analyzed by variance method (ANOVA) considering $P < 0.05$ using SPSS 18.0 software. The significant differences were taken to Duncan multiple range tests to compare the means.

3. Results

3.1. The Prevalence Ratio of C. perfringens

In this study, *C. perfringens* was isolated in both NE diseased and healthy broiler 49.7% (73/147). It was recorded from liver and intestine of diseased broilers in 47.8% (44/92) and in 29 apparently healthy broilers in a ratio of (52.7%). In relation age, the highest incidence rate of *C. perfringens* was recorded in 2-3 weeks of age (52.8%) as shown in (Table 3).

Table 3. *Distribution of C. perfringens isolates at different ages in broilers.*

Age	No. of positive isolates from		Total positive
	Diseased	Healthy	
2-3 weeks (53)	17/38	11/15	28/53 (52.8%)
4 weeks (45)	13/26	9/19	22/45 (48.9%)
Over 4 weeks(49)	14/28	9/21	23/49 (46.9%)
Total (147)	44/92	29/55	73/147 (49.7%)

3.2. Bacteriological Isolation and Identification of C. perfringens

With bacteriological cultivation, *C. perfringens* colonies appear on neomycin sulphate sheep blood agar medium as rounded, raised colonies showing double zones of haemolysis (β-heamolysis). They are Gram-positive short plumb bacilli, which rarely had central oval non bulging endospores. Biochemically, they were catalase and indole negative; glucose fermenters and positive for litmus milk (stormy fermentation). They characterized by an opalescence areas on egg yolk agar medium (on the side without antitoxin) while this was inhibited on the other side of the plate with antitoxin [32]. Typing of *C. perfringens* isolates with dermonecrotic test in mice confirmed that type A was the most predominant in all isolates (which appeared as an irregular area of yellowish necrosis tended to spread downward) as shown in table (4).

Table 4. *Typing of C. perfringens isolates in diseased and healthy broilers.*

C. perfringens	Toxigenic (Type A)	Non toxigenic
Diseased chickens (44)	38 (86.4%)	6 (13.6%)
Healthy chickens (29)	19 (65.5%)	10 (34.5%)
Total (73)	57 (78.1%)	16 (21.9%)

3.3. Genotypic Detection of C. perfringens Toxins

Multiplex PCR showed that characteristic clear bands at 400 bp (Figure 1) for α toxin (*cpa*) in the examined fifteen *C. perfringens* isolates were shown; however no bands were shown for *cpb* or *cpe* toxin genes. Hence, all isolates were of type A due to the presence of alpha toxin only. Uniplex PCR

detected the presence of *NET B* toxin gene in the examined isolates, and it was found in 46.7% (7/15) of the isolates at 383 bp (Figure 2). Also, Beta (β_2) toxin was examined using uniplex PCR at 548 bp where *cpb_2* gene was detected in (73.3%) of fifteen *C. perfringens* isolates (Figure 3). Interestingly, A positive correlation of *net B* gene with NE diseased status was studied. This paper reported that *C. perfringens net B* toxin gene was recorded only in NE diseased broilers (87.5%) while β_2 toxin was detected in both diseased and healthy cases in percentages of 75% and 71.4% respectively (Table 5).

Table 5. *Detection of NET B and β_2 toxins in C. perfringens recovered iso.*

***C. perfringens* isolates from**	**+ve *NET B***	**+ve B_2**
Diseased chickens	7/8 (87.5%)	6/8 (75%)
Healthy chickens	--	5/7 (71.4%)

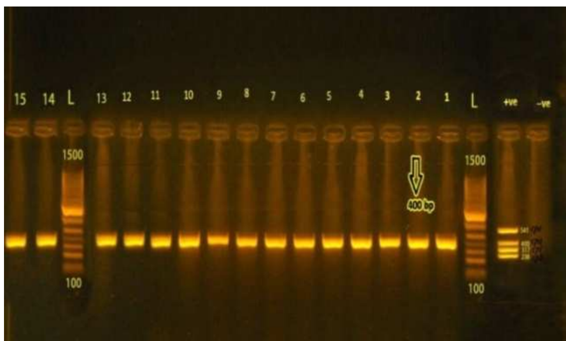

Figure 1. *Multiplex PCR for toxin typing of C. perfringens isolates. L lane: 100 bp DNA ladder "Ladder," all lanes from lane 1- lane 15: Positive C. perfringens isolates for alpha toxin gene (cpa) at 400 base pair (bp), +ve= positive control and –ve= negative control.*

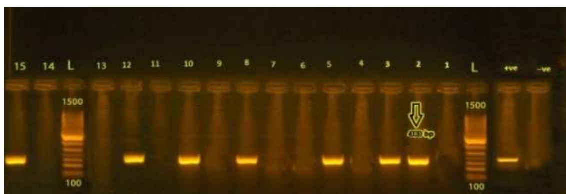

Figure 2. *Agarose gel electrophoresis of C. perfringens DNA product for (net B toxin gene). L:100 bp DNA ladder "Marker", lanes 2,3,5,8,10,12 and 15 only +ve for net B toxin gene at 383 base pair (bp) +ve= positive control and –ve= negative control.*

Figure 3. *Agarose gel electrophoresis of C. perfringens DNA product (cpb $_2$ toxin gene). L: 100 bp DNA ladder "Marker", lanes 1, 2, 3,4,6,7,8,10,13, 14 and 15: +ve for cpb_2 toxin gene at 548 base pair (bp) +ve= positive control and –ve= negative control.*

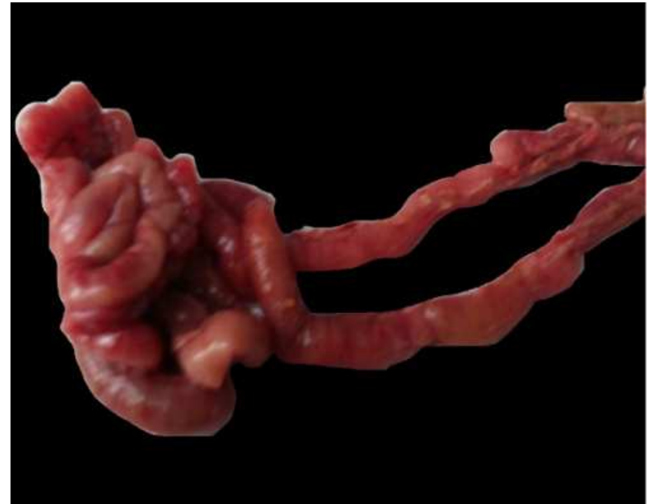

Figure 4. *Sever haemorrhgaic diarrhoea with severe haemorrhage in the intestine of a dead bird 12 days post inoculation, that was inoculated with +ve NET B C. perfringens toxin.*

Figure 5. *Yellowish diarrhoea with some haemorrhage in intestine of a dead bird 12 days post inoculation that was inoculated with +ve β_2 C. perfringens toxin.*

3.4. Experimental Challenge in Chicken Models

Depression, anorexia, ruffled feathers, bloody diarrhea and weight loss were the most predominant signs in infected groups (G1, G2) which were inoculated with *NET B* and β_2 toxins, respectively. Post mortem examination of *NET B* inoculated group (G1) showed sever haemorrhagic enteritis, congested liver, spleen and soft friable intestine with accumulation of gases (Figure 4). Lesser haemorrhage and lesser gases in intestine with congestion in liver and spleen were shown in β_2 inoculated (G2) (Figure 5). On the other hand, the control group (G3) didn't show any signs. Mortalities were observed also, in relation to each group. At 1st week post inoculation, five chicks were died in *NET B* group (G1) then all chicks were died due to *Net B* toxin at the end of 2nd week (p.i) however, 4 chicks only were died due to β_2 toxin in G2 at 1st week (p.i) followed by 8 chicks were died at 2nd week (p.i) as shown in (Table 6).

Table 6. *Clinical signs and mortalities of positive NET B and β_2 C. perfringens experimentally inoculated chicks.*

Groups Weeeks (P.I.)	Group 1 (G1) (*NET B* toxin inoculated Group)			Group 2 (G2) (β_2 toxin inoculated Group)			Group 3 (G3) (Control Group)		
	Signs	**P.M Score**	**Mortality (30)**	**Signs**	**P.M Score**	**Mortality (12)**	**Signs**	**P.M Score**	**Mortality**
1st Week P.I.=	No apparent signs except soft faeces	2	5	No apparent signs	0	4	-	0	0
2nd Week P.I.=	Depression, decrease in body weight, sever diarrhoea and some with bloody faeces	4	25	Soft faeces and some diarrhea	1	8	-	0	0

Score Lesions: 0 = No gross lesions. 1= Thin or friable walls, or diffuse superficial fibin. 2 = Focal necrosis or ulceration.
3 = Variable patches of necrosis 2 to 3 cm long. 4 = Extensive diffuse necrosis typical of field case. P.I. = Post Inoculation.

3.5. Hematological and Serum Biochemical Results

The hematological examination of experimental animals showed a significant reduction in RBCs, Hb conc. and PCV and non-significant changes in blood indices as shown in (Table 7). In a comparsion with the control group, significant increase in total leucocytic count, neutrophil and monocyte values was observed 5 days post inoculation in both *NET B* and β_2 inoculated groups (G1, G2). In addition, the *NET B* inoculated group (G1) showed microcytic hypochromic anemia accompanied with leucocytosis, neutropenia, lymphocytosis and monocytosis at 12 days post infection. On the other hand, β_2 inoculated group (G2) exhibited a normocytic normochtomic anemia, leucocytosis, neutrophilia, lymphopenia and monocytosis.

Concerning to serum biochemical analysis, Table (8) revealed that the experimental chicks showed a significant increase in their liver enzymes (ALT and AST), globulin, uric acid, and creatinine in the infected groups with *C. perfringens NET B* and β2 toxins (G1, G2). The electrophoretic pattern of serum protein of infected broiler chicks (Table 9) showed a decrease in total albumin, an increase in alpha and gamma globulins of all infected groups (G1, G2). Also, serum electrolytes cleared a significant decrease in serum sodium and chloride levels of both inoculated groups with *NET B* and β2 toxins meanwhile; non-significant variance in the serum potassium level was recorded (Table 10). In addition, serum calcium, inorganic phosphorus and magnesium levels were recorded a significant decrease in both experimentally infected groups (G1, G2).

Table 7. *Mean values of Haemogram picture of experimentally broiler infected with NET B and β_2 C. Perfringens toxins (n=10).*

Conditions Parameters	G 1		G2		G3	
	5th day (P.I)	**12th day (P.I)**	**5th day (P.I)**	**12th day (P.I)**	**5th day (P.I)**	**12th day (P.I)**
Hb(gm /dl)	11.4 ± 1.2^b	9.97 ± 0.9^b	11.7 ± 04^c	5.74 ± 0.54^c	14.2 ± 0.61^a	$13.8 \pm 0\ 4.^a$
R.B.Cs (10^6 /μl)	3.8 ± 0.35^b	$3.5\pm0.\ 5^b$	3.47 ± 0.24^b	2.58 ± 0.3^c	4.3 ± 0.4^a	5.1 ± 0.7^a
P.C.V (%)	36.2 ± 1.55^b	29.14 ± 0.7^b	35.60 ± 0.9^c	18.2 ± 0.76^c	42.2 ± 1.6^a	40.2 ± 2.3^a
M.C.V(F1)	95.26 ± 3.4^a	83.26 ± 3.4^a	102.59 ± 311^a	70.59 ± 311^b	$98.14 \pm 1.7\ ^a$	$80.24 \pm 1.7\ ^a$
M.C.H (Pg)	31.49 ± 1.6^a	28.49 ± 1.6^a	$33{,}72 \pm 2.5\ ^a$	$22.24 \pm 2.5\ ^b$	33.00 ± 2.3^a	27.1 ± 2.3^a
M.C.H.C.(gm /dl)	$34.\ 34 \pm 1.8^a$	$34.\ 22 \pm 1.8^a$	32.87 ± 2.01^a	31.5 ± 0.71^b	33.63 ± 2.6^a	34.37 ± 2.6^a
WBCs 103/ μl	12.1 ± 01.3^c	14.4 ± 0.9^a	13.3 ± 0.5^a	14.3 ± 0.65^a	10.9 ± 0.3^b	12.00 ± 0.8^b
Neutrophil 103/μl	6.2 ± 0.3^b	3.90 ± 0.3^c	7.2 ± 1.2^a	7.0 ± 1.2^a	4.20 ± 0.2^c	5.20 ± 0.2^b
Lymphocyte 103/μl	2.0 ± 0.6^b	6.7 ± 0.6^a	2.5 ± 1.1^b	3.3 ± 1.1^a	3.5 ± 0.5^b	4.5 ± 0.5^b
Monocyte 103/ μl	2.3 ± 0.4^b	2.4 ± 0.4^a	2.1± 0.3	3.0 ± 0.3^a	1.5 ± 0.3	$1.5 \pm 0.3\ ^b$
Eosinophil 103/ μl	1.2 ± 0.3^a	1.2 ± 0.3^a	1.5± 0.2	1.0 ± 0.2^a	0.80 ± 0.04	0.80 ± 0.04^a

Table 8. *Mean value of liver and kidney function in experimentally boilers chicks infected with NET B and β_2 C. Perfringens toxins (n=10).*

Conditions Parameters	G 1		G2		G3	
	5th day (P.I)	**12th day (P.I)**	**5th day (P.I)**	**12th day (P.I)**	**5th day (P.I)**	**12th day (P.I)**
ALT (J/ml)	15.9± 2.9	26.0±1.6	20.2±1.6	33.4±2.4	10.4±0.8	12.5±.1.2
AST (J/ml)	46 ± 1.6	9.1±0.7	64.1 ± 2.9	8.6± 0.3	26.0 ± 1.7	37.2±1.3
Creatinine (mg/dl)	2.3 ±0.6	3.5±1.8	3.7± 0.8	4.20± 2.4	1.1 ± 0.3	1.67± 1.7
Uric acid (mg/dl)	7.2 ± 0.4	6.9±1.5	9.4± 0.5	7.8± 1.3	4.6 ± 0.34	3.4± 1.0

Table 9. *Mean value of protienogram experimentally boilers chicks infected with NET B and β_2 C. Perfringens toxins (n=10).*

Conditions Parameters	G 1		G2		G3	
	5th day (P.I)	**12th day (P.I)**	**5th day (P.I)**	**12th day (P.I)**	**5th day (P.I)**	**12th day (P.I)**
Total protein (gm /dl)	7.07 ± 0.4^b	$6.66 \pm 0.14\ ^b$	6.78 ± 0.04^b	6.54 ± 0.4^b	8.5 ± 0.14^a	$7.\ 57\pm 0.14\ ^a$
Albumin (gm /dl)	$2.\ 5 \pm 0.3^b$	$1.9 \pm 0.64\ ^b$	1.85 ± 0.4^a	1.53 ± 0.64^b	4.53 ± 0.64	3.6 ± 0.43^a
Globulin (gm /dl)	4.57 ± 0.13^a	4.76 ± 0.69^a	5.03 ± 0.13^a	5.01 ± 0.5^a	3.47 ± 0.69^b	3.97 ± 0.56^b
α– globulin (gm /dl)	1.67 ± 0.33^a	1.52 ± 0.4^a	1.82 ± 0.5^a	1.76 ± 0.3^a	1.12 ± 0.4^b	1.02 ± 0.58^b
β – globulin (gm /dl)	0.80 ± 0.4^a	0.84 ± 0.5^a	0.81 ± 0.4^a	0.85 ± 0.6^a	0.85 ± 0.5^a	0.80 ± 0.3^a
γ– globulin (gm /dl)	2.10 ± 0.6^a	2.40± 0.5	2.40 ± 0.6^a	2.50 ± 0.4^a	1.50 ± 0.5^b	1.3 ± 0.5^b

Table 10. *Mean value of serum electrolyte experimentally boiler chicks infected with NET B and β_2 C. Perfringens toxins (n=10).*

Conditions Parameters	G 1		G2		G3	
	5th day P.I)	12th day (P.I)	5th day (P.I)	12th day (P.I)	5th day (P.I)	12th day (P.I)
Potassium mEq/l	7.5 ± 0.3	6.0±0.6	6.5± 0.9*	5.4±0.4	9.2 ± 0.13*	8.5±.22
Phosphorous mg/dl	8.4 ± 0.42	9.1±0.7	7.9 ± 0.60	8.6±0.3	6.49 ± 0.5	7.2±0.6
Sodium mEq/l	140 ± 0.9	155±2.5	112±0.4	120± 2.4	160± 0.82	167± 1.7
Chloride (mmol/L)	89.4 ± 0.7	80.9±1.5	81.46 ± 0.6	78.5± 1.3	100.9 ± 0.98	95.4± 1.0
Calcium mg/dl	6.3 ± 0.13	7.0±0.3	5.8±0.12**	6.1± 0.6	8.8 ± 0.1	9.3± 0.4

3.6. Sequencing of Net B and Cpb$_2$ Toxin Genes of C. perfringens

Sequencing of *net B* toxin gene in this study revealed that it was highly conserved in both nucleotide and amino acid sequence. Only one difference in this gene was identified where a replacement of an amino acid was occurred at codon 379 (glutamate GAA → arginine AGG), while a silent mutation was detected at nucleotide 1134 (GAG→GAA, both are glutamate) (Figure 6, 7). In addition to very few changes in nucleotides of eight strains in which alanine changed into threonine at position 168 attributing that into the use of different strains that obtained from different countries. A more systematic nucleotide variation of *net B* gene (A replaced by G) was recorded in 6 isolates in CDS position 502 leading to a shift from threonine (ACT) to alanine (GCT) in amino acid position 168 of *NET B* protein. The nucleotide and amino acid sequences of *C. perfringens net B* toxin gene were deposited into GenBank under accession number (KJ724530). Additionally, phylogenetic tree of nucleotides and amino acids based on *net B* toxin gene sequences of the *C. perfringens* isolate is shown (Figure 8). The difference in nucleotide sequence and amino acids replacement of (*NET B*) toxin in this study opens significant opportunities for further studies in Egypt for the development of novel vaccines against NE. On the other hand, no mutations were recorded in *cpb$_2$* gene when compared with its identical mutant sequence (accession number FJ493474.1). The nucleotide and amino acid sequences of *C. perfringens cpb$_2$* toxin gene were deposited into GenBank under accession number (KJ874348) (Figure 9). Additionally, distance and standard error between *net* B and *cpb$_2$* toxin genes of *C. perfringens* strains under study indicated that identity percentage of both toxin genes was 86.7% (Table 11). According to nucleotide sequencing of the consensus *cpb$_2$* gene in this study, frame shift mutations were recorded as 21 bp deletions and 4 bp additions when it was compared with the complete wild genome (accession number AY609161.1) however, no mutations were recorded when it was compared with its identical mutant sequence (accession number FJ493474.1).

Table 11. *Distance and standard error between net B and beta$_2$ toxin genes of C. perfringens strains under study.*

C. Perfringens toxin gene	Distance	Standard error
Net B		0.098
Beta$_2$	0.867	

*Net*B gene complete cds, FJ189484.1	841	C A A A T T T A G C A T C A T G G G	858
EGY2014 *C. perfringens Net*B gene		. . . .	
JRMTK01, KC460332.1		. . . .	
HH09, HQ585982.1		. . . .	
		* * * * * * * * * * * * * * * * * *	
*Net*B gene complete cds, FJ189484.1	859	A T A T A A A A T T T G T T G A G A	876
EGY2014 *C. perfringens Net*B gene		. . . .	
JRMTK01, KC460332.1		. . . .	
HH09, HQ585982.1		. . . .	
		* * * * * * * * * * * * * * * * * *	
*Net*B gene complete cds, FJ189484.1	877	C T A A G G A C G G T T A T A A T A	894
EGY2014 *C. perfringens Net*B gene		. . . .	
JRMTK01, KC460332.1		. . . .	
HH09, HQ585982.1		. . . .	
		* * * * * * * * * * * * * * * * * *	
*Net*B gene complete cds, FJ189484.1	895	T A G A T T C T T A T C A T G C T A	912
EGY2014 *C. perfringens Net*B gene		. . . .	
JRMTK01, KC460332.1		. . . .	
HH09, HQ585982.1		. . . .	
		* * * * * * * * * * * * * * * * * *	
*Net*B gene complete cds, FJ189484.1	913	T T T A T G G A A A T C A A T T A T	930
EGY2014 *C. perfringens Net*B gene		. . . .	
JRMTK01, KC460332.1		. . . .	
HH09, HQ585982.1		. . . .	
		* * * * * * * * * * * * * * * * * *	
*Net*B gene complete cds, FJ189484.1	931	T C A T G A A A T C A A G A T T G T	948
EGY2014 *C. perfringens Net*B gene		. . . .	
JRMTK01, KC460332.1		. . . .	
HH09, HQ585982.1		. . . .	
		* * * * * * * * * * * * * * * * * *	
*Net*B gene complete cds, FJ189484.1	949	A T A A T A A T G G T G A T A A A A	966
EGY2014 *C. perfringens Net*B gene		. . . .	
JRMTK01, KC460332.1		. . . .	
HH09, HQ585982.1		. . . .	
		* * * * * * * * * * * * * * * * * *	
*Net*B gene complete cds, FJ189484.1	967	A T T T C A C A G A T G A T A G A G	984
EGY2014 *C. perfringens Net*B gene		. . . .	
JRMTK01, KC460332.1		. . . .	
HH09, HQ585982.1		. . . .	
		* * * * * * * * * * * * * * * * * *	

*Net*B gene complete cds, FJ189484.1	**985**	A	T	T	T	A	T	C	A	A	C	A	T	T	A	A	T	T	T	**1002**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**1003**	C	T	G	G	T	G	G	A	T	T	T	T	C	A	C	C	C	A	**1020**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**1021**	A	T	A	T	G	G	C	T	T	T	A	G	C	A	T	T	A	A	**1038**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**1039**	C	A	G	C	A	C	C	T	A	A	A	A	A	T	G	C	T	A	**1056**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**1057**	A	A	G	A	A	T	C	T	G	T	A	A	T	A	A	T	A	G	**1074**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**1075**	T	T	G	A	A	T	A	T	C	A	A	A	G	A	T	T	T	G	**1092**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**1093**	A	T	A	A	T	G	A	C	T	A	T	A	T	T	T	T	A	A	**1110**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**1111**	A	T	T	G	G	G	A	A	A	C	T	A	C	T	C	A	A	T	**1128**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**1129**	G	G	C	G	A	**G**	**G**	**A**	**A**										**1137**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	**A**	**A**	**G**	**G**										
JRMTK01, KC460332.1		.	.	.	.	.	A	A	G	G										
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.										
		*	*	*	*	*														

Figure 6. *Nucleotides sequence similarities for C. perfringens net B toxin gene under study and the reference C. perfringens strains. Dots indicate nucleotides positions identical to the corresponding C. perfringens net B sequences. Numbers refer to the nucleotide positions in the C. perfringens net B sequences. Mutations are indicated by the solid bars.*

*Net*B gene complete cds, FJ189484.1	**281**	Q	I	*	H	H	G	I	*	N	L	L	R	L	R	T	V	I	I	*	I	**300**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**301**	L	I	M	L	F	M	E	I	N	Y	S	*	N	Q	D	C	I	I	M	V	**320**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**321**	I	K	I	S	Q	M	I	E	I	Y	Q	H	*	F	L	V	D	F	H	P	**340**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**341**	I	W	L	*	H	*	Q	H	L	K	M	L	K	N	L	*	*	*	L	N	**360**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	
*Net*B gene complete cds, FJ189484.1	**361**	I	K	D	L	I	M	T	I	F	*	I	G	K	L	L	N	G	E	**E**		**379**
EGY2014 *C. perfringens NetB* gene		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**R**		
JRMTK01, KC460332.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R		
HH09, HQ585982.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*			

Figure 7. *Amino acids sequence similarities for C. perfringens net B toxin gene under study and the reference C. perfringens strains. Dots indicate amino acid positions identical to the corresponding C. perfringens net B sequence. Numbers refer to the amino acid positions in the C. perfringens net B sequence. Amino acid substituation of glutamate (E) by arginine (R) at codon 379 is indicated by the solid bar.*

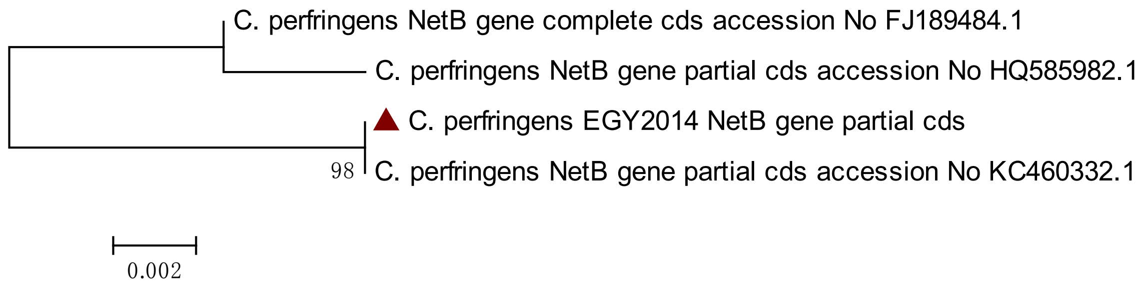

Figure 8. *Phylogenetic tree of net B toxin gene sequence of a C. perfringens strain.*

Sequence	Start																			End
C. perfringens EGY2014 *cpb2* gene	2	A	A	G	T	T	T	C	T	C	C	T	G	A	A	C	C	T	A	19
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	20	G	A	T	A	T	A	A	T	T	T	T	A	T	C	T	G	T	C	37
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	38	T	A	G	C	A	T	A	A	T	C	C	G	G	A	T	T	T	T	55
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	56	C	A	C	C	A	T	A	T	A	C	C	C	A	T	T	G	A	G	73
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	74	A	A	G	C	T	C	T	A	A	T	G	T	C	A	T	C	C	C	91
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	92	C	C	C	A	C	A	T	C	C	A	A	T	G	G	T	C	T	A	109
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	110	C	T	A	C	A	C	T	G	T	A	T	T	T	T	A	C	C	T	127
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	128	T	T	A	A	G	G	A	T	C	T	T	G	A	T	C	C	A	G	145
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	146	A	A	G	T	T	G	C	A	T	A	T	T	G	T	C	T	A	A	163
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	164	A	G	T	T	T	A	A	T	G	T	A	A	T	C	G	T	A	T	181
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	182	T	A	G	A	A	C	T	A	T	A	T	T	G	A	A	A	T	T	199
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	200	T	A	C	T	A	G	A	A	C	G	T	G	T	A	A	A	A	C	217
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	218	T	A	A	C	A	G	C	G	T	C	T	C	C	A	A	T	A	T	235
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	236	A	T	T	G	A	C	T	T	A	A	T	T	C	G	T	C	A	T	253
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	254	C	C	C	A	T	G	T	A	C	A	A	A	C	T	T	G	A	G	271
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	272	T	T	C	T	G	A	A	A	C	T	T	G	G	T	G	T	A	G	289
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	290	G	A	A	T	A	C	A	T	T	T	A	C	C	T	T	C	A	T	307
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	308	T	T	A	C	T	A	T	T	G	A	T	A	T	T	C	T	A	T	325
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	326	T	C	A	T	A	T	T	A	C	T	T	A	C	C	A	T	T	A	343
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens EGY2014 *cpb2* gene	344	A	T	T	T	A	T	T	A	T	C	A	A	T	A	T	A			359
Clostridium sp. accession No. FJ493474.1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			

Figure 9. *Nucleotides sequence similarities for C. perfringens cpb$_2$ toxin gene under study and the reference C. perfringens strain. Dots indicate nucleotides positions identical to the corresponding C. perfringens cpb$_2$ sequences. Numbers refer to the nucleotide positions in the C. perfringens cpb$_2$ sequences. No mutations were recorded.=*

4. Discussion

Clostridium perfringens organisms are of an economic concern in poultry production. They constitute a risk for transmission to humans through the food chain. Colonization of poultry by clostridia is a very early event in the animals' life and can be transmitted within the broiler chicken operation.

The percentage of *C. perfringens* positive isolates in NE diseased broilers was 47.8% while it was isolated in a higher percentage (52.7%) from the healthy broilers. This attributed to a large number of *C. perfringens* could be found in healthy broilers but the proliferation of *C. perfringens* or increase of its number in the gut depends on many factors like contaminated soil, dust, feed, litter and also induced by nutrition, pH and coccidial infection. All these factors might cause hindering of the digestion and decreased feed consumption that lead to low absorption, growth retardation and so appearance of the disease [33]. In the similar trend, higher percentages (41.6%, 58.4%, 75% and 40%) of *C. perfringens* isolation in chickens were recorded with many authors [34, 35, 36, 37]. Meanwhile in previous studies [38, 39] a lower prevalence rate (8 and 5%) of NE diseased cases from the intestinal broiler chickens, respectively were recorded. This variation might be due to the different methodologies used for isolation, classifying the microorganism or using of growth promoting in poultry farms [7].

An acute form of NE disease could be seen from about two weeks of age however, the subclinical form was observed at varying ages of birds, but it was first detected most commonly in birds at 21 to 23 days of age [18]. In current study, the incidence of *C. perfringens* according to the age of the chickens was higher (52.8%) in 2-3 weeks of age as shown in (table 3). These results were in line with many authors [33, 40, 41] who stated that NE disease is most common in broiler chickens causing high mortality rate at 2–3 or 4 weeks of age.

The pathogencity of *C. perfringens* is associated with their ability to secrete major and minor toxins which play important role in pathogenesis and induction of the disease. Multiplex PCR technique showed that all ten isolates in this study harboured *cpa* gene which give characteristic bands at 400 bp confirming that all of *C. perfringens* type. This result goes hand in hand with several anthers [42, 43, 44].

For long time, α-toxin or phospholipase C enzyme of *C. perfringens* was considered the main virulence factor in NE disease. A new discovered virulence determinant (*net B*) toxin recently was discovered and studied [9, 45, 46]. In this paper, *net B* toxin of *C. perfringens* was studied and detected in NE diseased broilers in a percentage of (46.7%) but didn't found in the isolates from healthy birds. These results were in accordance with a study [47] in which they stated that *net B* gene was only detected in Candian isolates that were associated with NE outbreaks but it wasn't found in isolates from healthy birds. In addition, *net B* gene was found in 77.8%, 74.4% and 70% in chickens derived NE *C. perfringens* strains [9, 18, 48]. However the latter study showed also, that 2/15 isolates carried *net B* toxin gene from healthy chickens and they explained the cause for the negative *NET B* strains from the diseased birds (didn't not carry *net B* gene) were that alternative virulence factors may constitute complex associations with other microflora that were required for disease production.

Throughout the last decade, several epidemiological studies showed wide distribution of beta$_2$ (β_2) toxigenic *C. perfringens* strains among human and other animal species [49] but its exact role in pathogenesis would still to be further elucidated [50]. In this study, it was discovered in both diseased and healthy birds in percentages of 75% and 71.4%. Similarl studies [51, 47, 36] detected *cpb$_2$* toxin gene in 75%, 74.2% and 62.6% of *C. perfringens* type A isolates in NE affected chickens. *C. perfringens* isolates were not capable of causing disease without *net B* gene especially it is linked with the health condition of the bird while a weak or no relationship between β_2 toxin and NE disease in birds [8, 46].

The experimental study of the pathogencity of both toxins in chicks revealed post mortem enlargement of the small intestine in NE affected chicks due to gas accumulation that could lead to thinning of the wall of the intestine. Similar macroscopic lesions were also detected by [52, 53, 40]. Eleven *net B* positive strains were able to induce lesions typical of NE in induction chickens models [8]. Importantly in vitro, all of *C. perfringens* isolates that carried *net B* gene expressed also *NET B* protein but only 54.5% of positive strains of *cbp*2 gene, produced β2 toxin [51]. Alpha toxin of *C. perfringens* from healthy birds was confirmed to be failed to induce the disease while 33% of broilers that were inoculated with *NET B* diseased isolates, developed NE specific intestinal lesions [54].

DNA sequencing has been used to investigate the genetic variation in individual genes, such as those encoding alpha and NetB toxins. NE affected birds fall into three distinct sequence based clades while non-pathogenic isolates from healthy birds tend to be more genomically diverse [14].

Nucleotide sequencing of *net B* in this study identified that glutamate amino acid was replaced with arginine at codon 379 in addition a silent mutation was detected at nucleotide 1134. In a similar way, a single nucleotide variation was observed in *net B* gene of four isolates at CDS position 10 (T replaced by with no AA shift) and in 2 isolates in CDS position 497 (C replaced by T with shift from Ala to Val in AA position 166) [55].

The gene sequencing of *cpb$_2$* didn't show mutations in this paper. Differently, the difference of nucleotide sequences at positions 6, 10, 12, 20 and 198 of two Iranian *C. perfringens* isolates was recorded [49] with 99% similarity to each other and 73 % identity with the *cpb$_2$* sequences of *C. perfringens* strains. An absence of β2 toxin expression where almost half of the non-porcine consensus *cpb$_2$* genes (44.4%) carried a frameshift mutation was also, reported [56]. However, 88.5% of 78 non-porcine isolates carried atypical *cpb$_2$*, but β2 toxin

was not expressed. Atypical β2 toxin displayed 62.3% identity and 80.4% similarity to consensus β2 toxin.

The hematological examination of experimentally infected broilers with *NET B* and B_2 toxins of *C. perfringens* revealed a decrease in erythrocytic count, Hb concentration and PCV values. While blood indices didn't show any changes after 5 days of infection. These results could be observed in the hemolytic type of anemia and could be attributed to action of α toxin which causes the breakdown of phospholipids of erythrocytes membrane and cause hemolysis by damaging circulating erythrocytes. Hemolytic anemia which was associated with excessive destruction of erythrocyte might be caused by variety of diseases like bacterial infection like *Clostridium* [22]. Also, *C. perfringens* bacteremia is commonly associated with intravascular hemolysis [57].

A significant reduction in RBCs, Hb, and PCV values were recorded in infected broiler chicks than normal ones. Such results might be attributed to the sequestration of iron in the bone marrow macrophages and hepatocytes during the infection, thus become unavailable to be utilized in hemoglobin synthesis, resulting in inhibition of erythropoiesis [23]. Group (G1) which was infected by *NET B* toxin showed a significant decrease in RBCs count, Hb concentration and PCV in the affected birds. This result indicated microcytic hypochromic anemia as showed by the erythrocytic indices that were proportionally correlated with the severity of infection. These results are in accordance with some researches [58].

Concerning to leucogram revealed neutrophilia and lymphopenia after 5 days post infection in both G1 and G2 groups. In addition, neutrophilia and lymphocytosis were shown after 12 days of infection by β_2 infected group (G3), but lymphocytosis and neutropenia were observed in G1 (*NET B* infected group). These results were common in acute inflammatory response because the inflammatory mediators stimulated the movement of neutrophil during acute inflammation, also stimulated the movement of lymphocytes from the blood to the inflamed tissue and lymphoid tissues. The severity of lymphopenia reflects the severity of systemic inflammatory response [59, 60, 61]. There was an increased TLC (Lymphocytosis) which might be due to the antigenic stimulation of *C. perfringens* that could lead to an increase in the thymus dependent lymphocytes (T lymphocytes) production as reported [22].The results of biochemical tests indicated that a significant increase in ALT and AST transaminase enzymes, uric acid and creatinine were noticed in both infected groups (G1, G2) at 5th and 10th days post infection. This increased in serum AST level had been associated with hepatocellular damage in chickens, turkeys and ducks as well as the worse effect of microorganism or its toxin in the liver and kidney as described by [62]. These results agreed with a study [63] which reported that, a significant elevation in the activities of AST and ALT due to invasion of the liver by pathogenic bacteria which causes liver cell damage. Similar results were obtained by [60, 64]. Also, some authors [61, 65] reported a significant increase in liver and kidney enzymes in broiler chickens post *C. perfringens* infection. Hypoprotienemia and hypoalbuminemia in the infected broiler chicken might be due to cease feeding and diarrhea. Similarly, similar studies [22, 66] mentioned that bacterial toxins, increase the capillary permeability and permitted the escape of plasma proteins into tissue resulting in hypoprotienemia. A Significant increase in gamma and alpha globulins could be associated with bacterial septicemia [22]. The increase in uric acid and creatinine could be due to the effect of the microorganisms and their toxins on the kidneys. Our results were completely agreed with many studies [67, 68, 69] in which the increased levels of creatinine and uric acid in case of renal disease were reported. Hypocalcemia and hyperphosphatemia could be due to decrease calcium resorption by damaged renal tubules and associated with hypoalbuminemia as reported [62, 70]. Decreased calcium level lead to hypoalbuminemia where decreased albumin concentration lowers the total calcium level, while both ionized and complex calcium levels remain normal. Also the metabolism of calcium and phosphorus were closely linked in the body [62, 70]. These results agreed with [61] who reported that the significant decrease in calcium and chloride as well as a significant increase in phosphorus in Guinea pig experimentally infected with *C. perfringens* type A. Additionally, the serum electrolytes showed significant decrease in serum sodium and chloride levels of infected groups while there is no significant variance in the serum potassium level. Similar results reported that sodium and chloride are particularly exposed to loss in diarrhea stools as they are components of the gastrointestinal secretions [61, 70].

5. Conclusion

In summary*, C. perfringens NET B* toxin harbouring isolates exhibited more lethal, pathogenic and virulent effects than β_2 toxin harbouring isolates in broilers. Vaccine preparations that include *NET B* toxoid can protect chickens against disease. A series of single amino acid substitution derivatives of *NET B* have potential value for vaccine formulations. It is likely that *NET B* will be an important antigen to include in an effective, commercially viable, necrotic enteritis vaccine.

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