Isolation and Genotyping Study of Clostridium Perfringens From Broiler Farms Infected with Necrotic Enteritis in Sulaimania Province

Sazan Qadr Amin¹, Nawzad Rasheed Abdulrahman¹* and Sadat Abdulla Aziz²

¹Department of Anatomy and Histopathology, College of Veterinary Medicine, University of Sulaimani, Kurdistan Region, Iraq.
²Department of Microbiology, College of Veterinary Medicine, University of Sulaimani, Kurdistan Region, Iraq.

*Corresponding author: nawz_ra@yahoo.com

Doi: https://doi.org/10.37940/AJVS.2019.12.2.7
This article is licensed under a CC BY (Creative Commons Attribution 4.0) http://creativecommons.org/licenses/by/4.0/.

Abstract

The study was conducted to isolate and toxintype the suspected cases of Clostridium perfringens infections of broiler farms in Sulaimania province. A total of 108 samples were collected from intestinal contents, mucosal scraping, and hemorrhagic lymphoid nodules from suspected cases of necrotic enteritis in broilers. The result of isolated and identified bacteria were revealed that 63 (58%) out of 108 samples were positive for C. perfringens. The results revealed that the isolates were only positive for alpha and beta2 toxin genes. Phylogenetic and DNA sequence analysis of cpa and cpb2 gene showed that cpa genes were highly identical to isolates from broiler in Iran, poultry stool and broiler in Brazil, and blue calves in Belgium. While cpb2 gene is closely related to the isolates of broiler in Iran, India and isolates of goat in Pakistan. The results indicated that the causative agent of necrotic enteritis in broiler farms in the region was mainly due to C. perfringens type A infection.

Keywords: Clostridium perfringens; Characterization; Necrotic enteritis

Introduction

Necrotic enteritis is the most common clostridial enteric disease in poultry, which typically occurs in broiler aged between two to six weeks (1) that caused by Clostridium perfringens, which is a Gram-positive, rod-shaped, spore forming, anaerobic bacteria. They are widely distributed in the environment and as a part of the normal gastrointestinal tract flora of pathogen (3). The organism has been classified traditionally into five toxintypes (A- E) basis on the production of toxins including; alpha (α), beta (β), epsilon (ε) and iota (ι) (4). But this typing system was recently expanded to include two additional types, such as type F strains produce enterotoxin and alpha-toxin; type G strain produces alpha and NetB (5). C. perfringens is responsible for many histotoxic and enterotoxic
In poultry, *C. perfringens*, especially type A and type C, can cause both clinical and subclinical form of necrotic enteritis (7). The coccidial pathogen is the most important known predisposing factor that enhances the induction of necrotic enteritis by damaging the intestinal epithelium, allowing *C. perfringens* to penetrate and replicate rapidly to produce sufficient amount of the toxins that causes the disease (8,9). In addition, some dietary components such as fish meal, high level of indigestible-non-starch polysaccharides, dysbacteriosis although have been widely accepted as predisposing factors (10).

There was not any data about the type of the *C. perfringens* that caused necrotic enteritis in broiler farms in Kurdistan region. Accordingly, the aims of the study were to isolate and toxinotype the suspected cases of *Clostridium perfringens* in broiler farms, basis on the presence or absence of the major and the minor toxin genes, including alpha (*cpa*), beta (*cpb*), epsilon (*etx*), and iota (*itx*) toxins, beta2, necrotic enteritis toxin B (*netB*), using selective medium, multiplex and uniplex PCR.

**Materials and methods**

**Samples collection:**

The samples from intestinal content, mucosal scraping, and hemorrhagic lymphoid tissue were aseptically collected from 108 diseased broiler aged between two to six weeks from 18 farms in Sulaimania province from August 2018 to May 2019. The samples were collected from farms where the birds had not been taken any antibiotic supplementation for about 72 hrs before sampling. Diseased broiler were characterized by having suspected clinical signs of necrotic enteritis, including depression, ruffled feathers, and diarrhea. The samples were taken from most affected parts of the intestines and collected in sterile plastic bags and processed immediately for isolation and identification of *C. perfringens*.

**Isolation and identification *C. perfringens*:**

The samples directly inoculated into tubes that contained freshly prepared cooked meat broth and incubated at 37°C for 24 hrs in an anaerobic jar (BD company, U.S.A) with gaspacks (Thermo scientific, U.S.A) were used to create the anaerobic condition. A loopful of inoculated fluid medium was streaked on sheep blood agar (Himedia, India) supplied with cycloserine (400 mg/1L), at 37°C for 24 hrs under anaerobic conditions. For the purpose of bacterial purification, the single bacterial colonies were picked up and inoculated into tryptose sulfite cycloserine agar (Quelab, Canada) with and without egg yolk, which was incubated at 37°C for 24 hrs under strict anaerobic condition. The isolated colonies were further characterized by looking at the morphology of the bacteria using Gram stain.

**DNA extraction:**

The boiling technique was used to extract DNA from all the isolates (11). Briefly, one well-isolated colony was selected, and suspended in 100µl of (Milli Q) water in an Eppendorf tube, incubated at 100°C for 15 min. using thermo-shaker (Biotech, Spain), and exposed to a pulsatile vortex (5-6 times/15 min.). Then cooled down and centrifuged (Maan lab, Sweden) at 14,000 × g for 10 min. Finally, the supernatant was kept, and the precipitate was discarded.

**Multiplex and Uniplex PCR:**

The multiplex PCR reaction was performed in a thermo-cycler (Techne ® Prime/ U.K) by adding 1µl of the primers mix (Table 1), 5 µl DNA into 10 µl PCR master mix cocktail (Genetbio Inc.). Then the volume was completed to the total reaction volume of 20 µl with ultrapure water. Uniplex PCR for detecting Beta2, NetB, and Alpha toxin gene was set in a
total reaction volume of 20 μl by mixing 1μl of the forward and reverse primers of each gene separately (Table 2), 5 μl DNA and the volume was completed with ultrapure water. The PCR amplification was as follow: initial denaturation was 95 °C for 5 min, denaturation at 95°C/30 sec, annealing at 55°C/30 sec, and extension at 72°C/1 min. The final extension was at 72°C/10 min. DNA bands were visualized on 1.5% agarose gel (Ingenius/ U.S.A).

DNA sequence analysis and Phylogenic Tree:

Both sense and antisense strands of the amplified DNA sequences were sequenced using Sanger DNA sequencer (Macrogen Co., Korea). The obtained DNA sequences subjected to DNA analysis using Clustal Omega (Multiple Sequence Alignment) and NCBI nucleotide blast. Phylogram was created using MEGA-X software. The amino acid sequences and their corresponding codons were predicted using the ExPASy Server (https://web.expasy.org/translate/). The obtained DNA sequences of both alpha and beta-2 toxin genes recorded in the National Center for Biotechnology Information (NCBI) under different accession numbers, including MN224676, MN224677, MN224678, and MN224679 for alpha-toxin, and MN239885-MN239886 and MN239887 for beta-2 toxin genes.

Results and Discussion

Isolation and identification C. perfringens:

In this study, 63 (58 %) of the isolates out of 108 samples suspected cases were positive for C. perfringens. The isolation and characterization of bacterial colonies were revealed basis on the cultural properties of the isolated bacteria on cooked meat broth, blood agar, and TSC agar with and without egg yolk.

Culturing of isolates on sheep blood agar produced small to medium-sized smooth-colonies, which had a gray and glistening appearance, surrounded by an inner zone of complete hemolysis due to theta toxin and an outer zone of partial hemolysis due to alpha toxin (Fig. 1). The bacteria produced a typical round and flat colonies, which had smooth and black color on tryptose sulfite cycloserine agar (Fig. 1). While the growth of the organism on TSC agar with egg yolk produced opalescence around the black colonies as a result of the breaking down of lecithin indicates to lecithinase activity (Fig. 1). The isolates were Gram-positive bacilli appearance (Fig. 1).

Multiplex and Uniplex PCR:

The multiplex PCR reaction showed that almost all isolates were positive for C. perfringens alpha-toxin gene and negative for other toxin-forming genes (Fig. 2). This result indicated that the isolated type was C. perfringens type A, because of the presence of alpha-toxin gene alone, which is only present in C. perfringens type A.

The uniplex PCR results reconfirmed the presence of the alpha-toxin gene; besides, the cpb2 gene (548 bp) was detected in 20 from 63 isolates of the C. perfringens type A (Fig. 3). However, all CPA gene-positive samples were negative for the NetB toxin gene.

DNA sequence analysis and Phylogenic Tree:

The DNA sequence analysis of the partially sequenced C. perfringens, which had been isolated from different broiler farms, using conventional software, including Clustal Omega (Multiple Sequence Alignment), NCBI nucleotide blast, MEGA-X software, and ExPASy Server showed that C. perfringens type A alpha gene (Fig. 5) (MN224677 and MN224678, and MN224676 and MN224679), had 100% to 99.22% homology respectively. The phylogenetic tree analysis showed that the first two sequences were 100% similar to that of taxon
L43548.1 (isolated from blue calves in Belgium), and 99.87% identical to that of taxon JQ071544.1 (isolated from poultry stool in Brazil). Still, it has a low rate identity of 84.18% with AF204209.1, which has been isolated from a diseased swan in the UK (Fig. 4). The other two sequences MN224676 and MN224679 were 100% similar to taxon X13608.1, L43547.1, and KT020614.1, which isolated from veterinary isolate, blue calves and broiler chicken in the UK, Belgium and Brazil respectively. However, the sequences were 99.68% identical to taxon GU581194 (isolated from the intestinal tract of diseased broiler chicken in Iran), and 84.31% homology with taxon AF204209.1 which was isolated from a diseased swan in the UK (Fig. 4).

The DNA sequence of the isolates showed that there were several nucleotides substitutions within the sequence of the partially sequenced alpha-toxin genes (MN224677 and MN224678, MN224676 and MN224679) at the site 164 (C with T), 266 (T with C), 263 (A with C), 605 (C with A), 613 (A with G) and 783 (G with A) (Fig. 5). Codons at the site 164, 266, 263, and 783 were replaced with alternative codons for the same amino acids. While, nucleotides substitutions at the site 605 and 613 altered the corresponding codons that lead to the replacement of the amino acid alanine (MN224676 and MN224679) with aspartate (MN224677 and MN224678), and threonine (MN224676 and MN224679) with alanine (MN224677 and MN224678) (Fig. 6).

Similar to the alpha gene, C. perfringens type A beta2 toxin gene was amplified from 20 of alpha-toxin gene positive isolates. Beta2 toxin gene was found to be highly conserved. The sequence analysis showed that there was only one nucleotide substitution at site 428 of the Beta2 toxin gene (MN239887) (Fig. 8). The substitution of the nucleotide Guanine (MN239886 and MN239885) with adenine that leads to the alteration of the corresponding codon (UGU to UAU) and replacement of the amino acid cysteine with tyrosine (Fig. 9). The phylogenetic analysis of cpb2 partially sequenced gene revealed that MN239886 and MN239885 were 100% identical with the sequence MF471365.1 (isolated from broiler chicken in India), AY884037.1 (from an unknown source), GU581183.1 (isolated from diseased broiler chicken in Iran) and MF191716.1 (isolated from goat in Pakistan). The sequence homology with the other taxons was ranged between 93.75% to 99.75%, respectively (Fig. 7). The MN239887 sequence of the isolate was 100% identical to that of the taxon KX924463.1 isolated from gout in Pakistan and taxon GU581182.1 and GU581185.1, which had been isolated from broiler chicken in Iran. Also, the sequence homology with other sequences were ranged between 93.50% to 99.50%, respectively (Fig. 7).

**Discussion**

**Isolation and identification C. perfringens:**

*Clostridium perfringens* infections are of economic concern in poultry production, resulting in gastrointestinal dysbacteriosis and necrotic enteritis (15). Proper characterization and identification of the causative agent’s disease are very crucial in minimizing economic losses due to the clostridial infections in the poultry.

The characteristic of the colonial morphology, which included, appearance of inner zone of complete hemolysis due to theta toxin and an outer zone of incomplete hemolysis due to alpha toxin on sheep blood agar and blackish colonies on TSC agar which is due to reduction of sulfate to sulfide by *C. perfringens* which in turn react with iron and form a black iron sulfide precipitate, is concurred with observation by other authors (16, 17). The growth of the isolates on medium contained egg yolk produced opalescence around the colony due to the breaking down of lecithin by alpha toxin (14). Microscopically the bacteria characterized by having gram-positive rod-shaped with blunt ends
after being stained with gram stain.

**Multiplex and Uniplex PCR:**

Alpha and beta2 toxin genes, which have been detected in the present study, appear to be associated with the virulence of the bacteria. Alpha toxin is one of the most important lethal, hemolytic, and dermonecrotic toxins produced by *C. perfringens*, is considered to be the major virulence factor and lethal toxin in the pathogenesis of necrotic enteritis (15, 18). The beta2 toxin was found to have in-vitro cytotoxicity and be lethal in mice (19). Although it seems to be associated with enteric diseases in piglet (20), horses (21). Although Beta2 toxin gene was isolated from sheep dysentery and an African elephant ulcerative enteritis (22, 23).

NetB toxin gene is plasmid coded, pore-forming toxin plays a crucial role in the pathogenesis of necrotic enteritis in broiler (24). The isolates in the present study were found to be negative for netB gene similar to that which have been reported by Nakano et al. (25) and Merati et al. (26).

**DNA sequence analysis and Phylogenetic Tree:**

The *cpa* sequence (100% to 99.22%) similarity with the recorded sequences, including isolated *C. perfringens cpa* gene from blue calves in Belgium (L43548.1 and L43547.1), veterinary isolate in UK (X13608.1), broiler in Iran (GU581194), poultry stool (JQ071544.1) and broiler (KT020614.1) in brazil, and *cpb2* gene of *C. perfringens* homology with *C. perfringens* isolates from broiler in India (MF471365.1), Iran (GU581183.1, GU581182.1, and GU581185.1), goat in Pakistan (MF191716.1 and KX924463.1), with a minor point mutations which were observed in the present study might be related to the fact that all isolates were obtained from broiler of the same age group at a limited geographical region, they appears to be epidemiologically related (27). Although those homologies with other various isolates from different countries might be associated with a substantial border and a large trade relation with other countries, especially, regarding the sources of chicks, and litter in the farms. Eggshell fragments, chick fluff, and paper pads in commercial hatcheries were reported to be contaminated with *C. perfringens* (28). The wild migratory birds might have a contribution in introducing the *C. perfringens* strains across countries. Wild birds have been reported as a reservoir of *C. perfringens* and might have a role in the transmission of the bacteria (29). The ration of animal origin contained high protein, particularly in fish meal followed by meat, bone meal and dry fish, was detected to be contaminated with *C. perfringens* (30). Also, a high level of *C. perfringens* contamination was found in processed animal proteins (31). It has been revealed that *C. perfringens* strains of mammalian species can cause necrotic enteritis in chickens (32).

Nucleotides substitutions that altered the corresponding codons were expected to be followed by replacement of the amino acid alanine with aspartate, and threonine with alanine in the polypeptide chain of alpha toxin protein, and replacement of the amino acid cysteine with tyrosine in the structure of beta2 toxin protein that might be associated with the alteration of the structural and functional properties of alpha and beta2 toxin in *C. perfringens* (33). Meanwhile, it was found that induced substitution of amino acid with another amino acid by point mutation alters the activity and function of the alpha-toxin protein (34) and epsilon toxin (35). However, further study is needed to investigate the role of the predicted amino acid alterations on *C. perfringens* virulence.
### Table 1: Primers used for multiplex PCR to detection of the types of *C. perfringens* toxin genes

| Primer | Sequence | Gene | Size of product | Annealing temp. | Reference |
|--------|----------|------|-----------------|-----------------|-----------|
| Alpha  | 5`-GTGATAGCGCAGGACATGTAAG-3`<br>5`-CATGTAAGTCATCTGTCCAGCAATC-3` | cpa  | 400             | 55 °C           | (1)       |
| Beta   | 5`-ACTATACAGACAGATCATCAACC-3`<br>5`-TTAGGAGCAGTTAGAACTACAGAC-3` | cpb  | 236             | 55 °C           | (11)      |
| Epsilon| 5`-ACTGCAACTAACTCATACTGTG-3`<br>5`-CTGGTGCTTAATAGAAAGACTCC-3` | etx  | 541             | 55 °C           |          |
| Iota   | 5`-GCAGAAAGCTACACCACCTAC-3`<br>5`-GGTATATCCCTCCAGCATATAGTC-3` | iap  | 317             | 55 °C           |          |

### Table 2: Uniplex PCR primer sets for detection of *C. perfringens* toxins.

| Toxin | Primer sequence (5`-3`) | Gene | Size | Annealing temp. | Reference |
|-------|--------------------------|------|------|-----------------|-----------|
| Alpha | 5`-AGTCTACGCTGGGATG AA-3`<br>5`-TTTCTGGGTGTTCCATTTC-3` | cpa  | 900  | 55 °C           | (12)      |
| Net-B | 5`-GCTGGTGCTGGAATAATGC-3`<br>5`-TCGCCATTGAGTAGTTTCCC-3` | netB | 383  | 55 °C           | (13)      |
| Beta2 | 5`-AAATATGATCTAACCACAA-3`<br>5`-CCAAATACTCTAATCGATG-3` | cpb2 | 548  | 55 °C           | (143)     |
Figure 1: A; *C. perfringens* culture on blood agar. B; TSC agar culture. C; TSC agar with egg yolk. D; photograph of *C. perfringens* stained with gram stain under oil immersion (100X).

Figure 2: Multiplex PCR of *C. perfringens* isolates. Lane 1: hyper DNA ladder 100. Lane 2-9: *C. perfringens* types, which is only positive for type A.

Figure 3: Panel A: Lane 1: Hyper ladder 100. Lane 3, 4 and 5: amplified alpha-toxin gene (900 bp). Panel B: Lane 1: Hyper ladder 100. Lane 2 and 3: amplified beta2 toxin gene (548 bp).
Figure 4: Phylogenetic tree of partially sequenced *Clostridium perfringens* type A alpha toxin genes. The phylogram were created using MEGA-X software.

GU581194.1  ----------------------------------------  0
L43547.1    TGCTATGATTGTAACTCAAGGGTTTCAATCTTAGAAAATGATCTG  TCC  AAAATGAACC  176
MN224676    ----------------------------------------  AAATGATCTG  TCC  AAAATGAACC  24
MN224679    ----------------------------------------  AAATGATCTG  TCC  AAAATGAACC  24
X13608.1    TGCTATGATTGTAACTCAAGGGTTTCAATCTTAGAAAATGATCTG  TCC  AAAATGAACC  960
KT020614.1  ----------------------------------------  0
MN224677    ----------------------------------------  AAATGATCTG  TCC  AAAATGAACC  24
MN224678    ----------------------------------------  AAATGATCTG  TCC  AAAATGAACC  24
JQ071544.1  ----------------------------------------  ATGATCTG  TCC  AAAATGAACC  2
GU581194.1  ----------------------------------------  0
L43547.1    AGAAAGTGTAAGAAAAAATCTTAGATTTAAAAAGAGAACATGCATGAGCTTCAATTAGG  236
MN224676    AGAAAGTGTAAGAAAAAATCTTAGATTTAAAAAGAGAACATGCATGAGCTTCAATTAGG  84
MN224679    AGAAAGTGTAAGAAAAAATCTTAGATTTAAAAAGAGAACATGCATGAGCTTCAATTAGG  84
X13608.1    AGAAAGTGTAAGAAAAAATCTTAGATTTAAAAAGAGAACATGCATGAGCTTCAATTAGG  1020
KT020614.1  ----------------------------------------  0
MN224677    AGAAAGTGTAAGAAAAAATCTTAGATTTAAAAAGAGAACATGCATGAGCTTCAATTAGG  84
MN224678    AGAAAGTGTAAGAAAAAATCTTAGATTTAAAAAGAGAACATGCATGAGCTTCAATTAGG  84
JQ071544.1  AGAAAGTGTAAGAAAAAATCTTAGATTTAAAAAGAGAACATGCATGAGCTTCAATTAGG  84
GU581194.1  ----------------------------------------  0
L43547.1    TTCTACTTATCAGATTATGATAAG  PATGATCTATATCAAGATCTATTCTGGGA  296
MN224678 ATATGCAAGAGGTTTTTGCTAAAAACAGGAAAAATCAATATACATAGGCTACATGAGGAG 564
JQ071544.1 ATATGCAAGAGGTTTTTGCTAAAAACAGGAAAAATCAATATACATAGGCTACATGAGGAG 562

GU581194.1 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 200
L43547.1 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 776
MN224676 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 624
MN224679 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 624
X13608.1 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 1560
KT020614.1 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 196

MN224677 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 622
MN224678 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 684
MN224679 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 684
X13608.1 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 1620
KT020614.1 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 256

MN224677 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 744
MN224678 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 744
JQ071544.1 TCAATAGTTGGGATGATTGGGAATTATGCAGCAAAGGTAACTTTAGCTAATCTCTCAAAGG 742

GU581194.1 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 244
L43547.1 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 836
MN224676 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 684
MN224679 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 684
X13608.1 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 1680
KT020614.1 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 257

MN224677 TGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGG 744
MN224678 TGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGG 744
JQ071544.1 TGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGG 742

GU581194.1 TGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGG 1680
L43547.1 TGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGG 257

MN224677 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 744
MN224678 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 744
JQ071544.1 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 742

GU581194.1 TGGAAAGAATGTAAAAGAACTAGTAGCTTACATATCAACTAGTGGTGAGAAAGATGCTGG 244
L43547.1 AACA GCG GGATATATTTATAGATTCTTACACGATGTATCAGAGGGTAATGATCCATCAGTCTCAAGG 896
Figure 5: Alignment of partially sequenced alpha toxin gene using Cluster omega multiple sequence alignment software.
Figure 6: Amino acid sequence of alpha toxin gene, created using Expasy bioinformatics software.

Figure 7: Phylogenic tree of partially sequenced *Clostridium perfringens* type A beta-2 toxin genes.
MF191716.1  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 203
GU581183.1  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 291
AY884037.1  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 299
MN239885  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 299
MN239886  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 224
MF471365.1  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 255
GU581185.1  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 300
GU581182.1  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 293
MN239887  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 224
KX924463.1  TGACGAATTAAGTCAATATTGAGACGCTGTTAGTTTTACACGTTCTAGTAAATTTCA 288
************************************************************
MF191716.1  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 263
GU581183.1  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 351
AY884037.1  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 359
MN239885  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 284
MN239886  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 284
MF471365.1  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 315
GU581185.1  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 360
GU581182.1  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 353
MN239887  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 284
KX924463.1  ATATAGTTCTAATACGATTACATTAAACTTTAGACAATATGCAACTTCTGGATCAAGATC 348
************************************************************
MF191716.1  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 323
GU581183.1  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 411
AY884037.1  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 419
MN239885  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 344
MN239886  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 344
MF471365.1  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 375
GU581185.1  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 420
GU581182.1  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 413
MN239887  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 344
KX924463.1  CTTAAAGGTAAAATACAGTGTAGTAGACCATTGGATGTGGGGGGATGACATTAGAGCTTC 408
MF191716.1  TCAATGGGTATATGGTGAAAATCCGGATTATGCTAGACAGATAAAATTATATCTAGTTC 383
GU581183.1  TCAATGGGTATATGGTGAAAATCCGGATTATGCTAGACAGATAAAATTATATCTAGTTC 471
AY884037.1  TCAATGGGTATATGGTGAAAATCCGGATTATGCTAGACAGATAAAATTATATCTAGTTC 479
Figure 8: Alignment of partially sequenced beta-2 toxin gene using Cluster omega multiple sequence alignment software.

Figure 9: Amino acid sequence of beta2 toxin gene, created using Expasy bioinformatics software.
Conclusion

The results of the present study indicated that the causative agent of necrotic enteritis in broiler farms in Sulaimania province is caused by *C. perfringens* type A, which is characterized by having alpha-toxin gene along with beta2 toxin gene.

Acknowledgments

We want to acknowledge the College of the veterinary medicine research center, the University of Sulaimani, for the laboratory facilities that utilized during the study.

References:

1. Cooper KK, Songer JG, Uzal FA. Diagnosing clostridial enteric disease in poultry. Journal of Veterinary Diagnostic Investigation. 2013 May;25(3):314-27.

2. McClane BA, Uzal FA, Miyakawa MF, Lyerly DA, Wilkins TR. The enterotoxigenic clostridia. The prokaryotes. 2006;4:698-752.

3. Revitt-Mills SA, Rood JI, Adams V. Clostridium perfringens extracellular toxins and enzymes: 20 and counting. Microbiology Australia. 2015 Sep 17;36(3):114-7.

4. Songer JG. Clostridial enteric diseases of domestic animals. Clinical microbiology reviews. 1996 Apr;9(2):216.

5. Rood JI, Adams V, Lacey J, Lyras D, McClane BA, Melville SB, Moore RJ, Popoff MR, Sarker MR, Songer JG, Uzal FA. Expansion of the Clostridium perfringens toxin-based typing scheme. Anaerobe. 2018 Oct 1;53:5-10.

6. Uzal FA, Freedman JC, Shrestha A, Theoret JR, Garcia J, Awad MM, Adams V, Moore RJ, Rood JI, McClane BA. Towards an understanding of the role of Clostridium perfringens toxins in human and animal disease. Future microbiology. 2014 Mar;9(3):361-77.

7. Timbermont L, Haesebrouck F, Ducatelle R, Van Immerseel F. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathology. 2011 Aug 1;40(4):341-7.

8. Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in microbiology. 2009 Jan;17(1):32-6.

9. Abdullah IN. Isolation and identification of some bacterial isolates from table egg. Al-Anbar Journal of Veterinary Sciences. 2010;3(2):59-67.

10. Engberg RM, Hedemann MS, Jensen BB. The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. British poultry science. 2002 Aug 1;43(4):569-79.

11. Ibrahim GA, Mahmoud BS, Ammar AM, Youssef FM. Toxin genotyping of *C. perfringens* isolated from broiler cases of necrotic enteritis. Animal and Veterinary Sciences. 2017 Nov 11;5(6):108.

12. Baums CG, Schotte U, Amtsberg G, Goethe R. Diagnostic multiplex PCR for toxin genotyping of Clostridium perfringens isolates. Veterinary microbiology. 2004 May 20;100(1-2):11-6.
13. Keyburn AL, Yan XX, Bannam TL, Van Immerseel F, Rood JI, Moore RJ. Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin. Veterinary research. 2010 Mar 1;41(2):1-8.

14. Dar PS, Wani SA, Wani AH, Hussain I, Maqbool R, Ganaie MY, Kashoo ZA, Qureshi S. Isolation, identification and molecular characterization of Clostridium perfringens from poultry in Kashmir valley. India.J Entomol Zool Stud. 2017, 5(5): 409-414.

15. McDevitt RM, Brooker JD, Acamovic T, Sparks NH. Necrotic enteritis; a continuing challenge for the poultry industry. World's Poultry Science Journal. 2006 Jun;62(2):221-47.

16. Miah MS, Asaduzzaman M, Sufian MA, Hossain MM. Isolation of Clostridium perfringens, Causal agents of necrotic enteritis in chickens. Journal of the Bangladesh Agricultural University. 2011;9(1):97-102.

17. Skariyachan S, Mahajannakatti AB, Biradar UB, Sharma N, Abhilash M. Isolation, identification and characterization of Clostridium perfringens from cooked meat-poultry samples and in silico biomodeling of its delta enterotoxin. Int J Pharm Sci, 2010, 4: 164-172.

18. Titball RW, Naylor CE, Basak AK. The Clostridium perfringens-toxin. Anaerobe. 1999 Apr 1;5(2):51-64.

19. Gibert M, Jolivet-Renaud C, Popoff MR. Beta2 toxin, a novel toxin produced by Clostridium perfringens. Gene. 1997 Dec 5;203(1):65-73.

20. Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW. Occurrence of Clostridium perfringens β2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiology & Infection. 2000 Feb;124(1):61-7.

21. Herholz C, Miserez R, Nicolet J, Frey J, Popoff M, Gibert M, Gerber H, Straub R. Prevalence of β2-toxigenic Clostridium perfringens in horses with intestinal disorders. Journal of clinical microbiology. 1999 Feb 1;37(2):358-61.

22. Gkiourtzidis K, Frey J, Bourtzi-Hatzopoulou E, Iladiis N, Sarris K. PCR detection and prevalence of α-, β-, β2-, ε-, t-and enterotoxin genes in Clostridium perfringens isolated from lambs with clostridial dysentery. Veterinary Microbiology. 2001 Sep 3;82(1):39-43.

23. Bacciarini LN, Boerlin P, Straub R, Frey J, Gröne A. Immunohistochemical localization of Clostridium perfringens β2-toxin in the gastrointestinal tract of horses. Veterinary pathology. 2003 Jul;40(4):376-81.

24. Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Di Rubbo A, Rood JI, Moore RJ. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS pathogens. 2008 Feb;4(2).

25. Nakano V, Ignacio A, Llanco L, Bueris V, Sircili MP, Ávila-Campos MJ. Multilocus sequence typing analyses of Clostridium perfringens type A strains harboring tpeL and netB genes. Anaerobe. 2017 Apr 1;44:99-105.
26. AAAF M. Identification and Characterization of Clostridium perfringens Isolated from necrotic Enteritis in Broiler Chickens in Tiaret, Western Algeria. KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ.;23(4).

27. Johansson A, Aspan A, Bagge E, Båverud V, Engström BE, Johansson KE. Genetic diversity of Clostridium perfringens type A isolates from animals, food poisoning outbreaks and sludge. BMC microbiology. 2006 Dec 1;6(1):47.

28. Craven SE, Cox NA, Stern NJ, Mauldin JM. Prevalence of Clostridium perfringens in commercial broiler hatcheries. Avian Diseases. 2001 Oct 1;1050-3.

29. Ghazi AM, Amer MM. Role of some wild birds in transmission of bacterial pathogens of zoonotic importance and poultry health and production. Vet Med J Giz, 2014, 60(2): 1110-1423.

30. Udhayavel S, Ramasamy GT, Gowthaman V, Malmarugan S, Senthivel K. Occurrence of Clostridium perfringens contamination in poultry feed ingredients: Isolation, identification and its antibiotic sensitivity pattern. Animal Nutrition. 2017 Sep 1;3(3):309-12.

31. Wojdat EL, Kwiatek KR, Kozak MA. Occurrence and characterization of some Clostridium species isolated from animal feedstuffs. BULLETIN-VETERINARY INSTITUTE IN PULAWY. 2006 Jan 1;50(1):63.

32. Smyth JA, Martin TG. Disease producing capability of netB positive isolates of C. perfringens recovered from normal chickens and a cow, and netB positive and negative isolates from chickens with necrotic enteritis. Veterinary microbiology. 2010 Nov 20;146(1-2):76-84.

33. Lugo-Martinez J, Pejaver V, Pagel KA, Jain S, Mort M, Cooper DN, Mooney SD, Radivojac P. The loss and gain of functional amino acid residues is a common mechanism causing human inherited disease. PLoS computational biology. 2016 Aug;12(8).

34. Guillouard I, Garnier T, Cole ST. Use of site-directed mutagenesis to probe structure-function relationships of alpha-toxin from Clostridium perfringens. Infection and immunity. 1996 Jul 1;64(7):2440-4.

35. Oyston PC, Payne DW, Havard HL, Williamson ED, Titball RW. Production of a non-toxic site-directed mutant of Clostridium perfringens ε-toxin which induces protective immunity in mice. Microbiology. 1998 Feb 1;144(2):333-41.