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Original Article

Toxinotyping of *Clostridium perfringens* strains isolated from broiler flocks with necrotic enteritis and evaluation of the effect of toxins on Leghorn Male Hepatoma cells

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Abstract

Background: Necrotic enteritis (NE) is an economically important disease, caused by *Clostridium perfringens* type G strains, and is one of the major targets of antibiotics used in poultry feed. **Aims:** This study aimed to genotypically characterize virulent strains of *C. perfringens* isolated from healthy and diseased birds in Iran. **Methods:** Eleven isolates were derived from necrotic enteritis cases, and 27 were from healthy chickens. Isolations were performed using blood agar. To assess whether zmp is generally associated with avian NE, 38 *C. perfringens* isolates were screened using PCR and western blotting. The involvement of these toxins as virulence factors was investigated using cytotoxicity assays. **Results:** All isolates carried the phospholipase c (plc) gene regardless of their origin and virulence. The zinc metallopeptidase (zmp) gene was found in the isolates collected from birds affected by necrotic enteritis. Furthermore, Necrotic enteritis like B (NetB) was only found in 36.36% of the isolates derived from necrotic enteritis-infected birds. Western blot analysis further confirmed the expression of Alpha toxin, NetB, and Zmp in different isolates. Incubation of Leghorn Male Hepatoma (LMH) cells with crude *C. perfringens* toxins indicated that the supernatants of all bacterial strains were toxic toward LMH cells at different dilutions. In addition, crude toxins of the Cp28 strain expressing Alpha toxin, Zmp, and NetB showed an approximately 50% cytotoxic dose (CD50) at a 1:34 dilution. Strain Cp119.2, which produces both ZMP and the Alpha toxin, and strain Cp48, which only produces the Alpha toxin, showed CD50 at 1:23 and 1:4 dilutions, respectively. **Conclusion:** It seems that both NetB and Zmp play major roles in the cytotoxicity and pathogenicity of this organism.

Key words: *Clostridium perfringens*, LMH cell line, Necrotic enteritis, NetB, Zinc metallopeptidase

Introduction

Clostridium perfringens is a Gram-positive, spore-forming anaerobe considered one of the fast-growing bacteria in nature (Shimizu *et al*., 2002). Since the recent ban on using in-feed antimicrobials, necrotic enteritis (NE) disease caused by *C. perfringens* has become one of the most devastating poultry diseases worldwide. The economic damage caused by this disease to the poultry industry is estimated to be approximately $ 6 billion annually (Wade and Keyburn, 2015).

Several predisposing factors are involved in NE, including high protein content in the diet, wet litter, coccidiosis infection, stress, and other diseases such as infectious bursal disease and Marek in domestic fowls (Caly *et al*., 2015). In addition, the pathogenesis of *C. perfringens* is attributed to the production and secretion of one or more of the 23 toxins and enzymes in virulent strains (Kiu and Hall, 2018). Furthermore, various transporter systems, degradative enzymes, and toxins can play crucial roles in the rapid degradation of the intestinal mucosa in birds (Prescott *et al*., 2016).

Characterization the infectious strains of C. *perfringens* and their toxins is essential to control NE through vaccination. Different *C. perfringens* toxinotypes secrete various combinations of toxins that cause disease in different hosts (Kiu and Hall, 2018).

For a long time, the Alpha toxin encoded by the chromosomal *plc* gene has been proposed as a critical virulence factor in NE (Kulkarni *et al*., 2006). However, the main role of the Alpha toxin in *C. perfringens* pathogenesis was unclear when Keyburn *et al.* (2008) demonstrated that an Alpha toxin null mutant was pathogenic in birds. It was also found that the severity of NE depends on the necrotic enteritis B-like toxin (NetB), which is a membrane Beta pore-forming toxin whose gene is located on the pathogenicity locus (NELoc-1) of a virulent plasmid (Keyburn *et al*., 2010; Keyburn *et al*., 2013; Lepp *et al*., 2013). The discovery of NetB toxin and its impact on disease control are two important factors that change the toxinotyping of *C. perfringens* (Rood *et al*., 2018). Previously, *C. perfringens* strains that cause NE and gas gangrene were classified as toxinotype A. Recently, with the identification of the role of NetB in NE, *C. perfringens* toxinotyping was expanded to seven toxinotypes (A-G). Based on this classification, toxinotype G has been reported to be responsible for NE in birds (Rood *et al*., 2018). Thus, toxinotype G refers to virulent isolates that produce NetB in addition to the Alpha toxin.

Culture supernatants of *C. perfringens* have previously been shown to be specifically cytotoxic to Leghorn Male Hepatoma cells (LMH) and have been widely used to assess NetB activity. It has been shown that the culture supernatant of *netB* null mutant strains does not induce cytotoxicity in LMH cells (Keyburn *et al*., 2008). In some cases, it has been reported that proteins other than NetB may be involved in causing cytopathic effects on LMH cells (Cheung *et al*., 2010; Lanckriet *et al*., 2010).

Although these toxins are involved in the pathogenesis of *C. perfringens*, the presence of other degradative enzymes and glycoside hydrolases is essential for the penetration of bacteria into intestinal epithelial cells or the mucosal layer (Nakjang *et al*., 2012; Noach *et al*., 2017).

Among these pathogenic factors, zinc metallopeptidase contains a carbohydrate-binding domain that destroys the mucosal layer to access the underlying epithelial cells of the host intestine (Kulkarni *et al*., 2008; Noach *et al*., 2017; Wade *et al*., 2020). Zinc metallopeptidase is a member of the peptidase_M60 family that contains a gluzincin motif (HEXXHX(8,28)E), which is a key element for zinc binding and acts as the catalytic center of M60 peptidases (Pluvinage *et al*., 2021). A noteworthy characteristic of peptidase-M60 proteins is the presence of Carbohydrate Binding Modules (CBMs) at the N and C termini. ZMPs are found in the genome and on the pathogenic locus of the virulence plasmid NELoc-1 (Wade *et al*., 2020).

Recently, a study using an avian model system showed that the deletion of the genes encoding ZmpC and ZmpB profoundly contributes to necrotic enteritis; therefore, both ZmpC and ZmpB are required for full virulence in avian disease models (Wade *et al*., 2020).

This study characterized different toxinotypes of *C. perfringens* isolated from healthy and diseased flocks. The presence of the *plc*, *netB*, and *zmp* genes in their genomes and the expression of their related proteins were investigated. In addition, to quantitatively determine the cytotoxicity of the culture supernatants, the isolates were tested using an LMH cell cytotoxicity assay.

Materials and Methods

Bacteria

The *C. perfringens* strains used in this study were isolated from 11 NE-positive broiler flocks (necrotic enteritis-infected birds) with typical symptoms of NE disease and 27 healthy farms (NE-negative birds) from 2014 to 2017. Samples were obtained from Tehran and Mashhad, Iran. Several birds were selected from each flock and sampled to isolate the bacteria. The isolation and genotyping procedures have been previously detailed in the previous researches (Razmyar *et al*., 2014; Afshari *et al*., 2015; Razmyar *et al*., 2017).

Bioinformatics analysis

The nucleotide sequences of *alpha-toxin*, *netB*, and *zmps* were extracted from the NCBI website (https://www.ncbi.nlm.nih.gov) (Accession No. D63911 and NZ_CP075979.1 and CP000246.1). The molecular weight (MW) of the Alpha toxin, NetB, and Zmp proteins were analyzed using ProtParam (https://web.expasy.org/protparam/).

The protein sequences of ZmpA, ZmpB, and ZmpC were obtained from UniProtKB (Accession No. A0A0H2YN38, A0A0H2YW34, and F8UNJ8).

The sequence similarity between Zmp proteins was calculated using the SMS website (https://www.bioinformatics.org/sms2/ident_sim.html). MEME Suite (https://meme-suite.org/meme/tools/meme) was used to identify and analyze motifs in Zmp protein sequences, and multiple sequence alignments were performed using CLC Genomics Workbench (version 22.0.2, QIAGEN, Venlo. NL). Primers for *zmp* gene were designed based on regions with high similarity to the M60 domains in *zmpB*.

Preparation of anti-NAM antibody

The procedures for expression, purification, and raising antibodies in rabbits have been described in our previous studies (Katalani *et al*., 2020a, b). In brief, the chimeric construct with GenBank accession number MN266289 consisting of the selected immunogenic fragments of $\text{NetB}_{146\text{-}322}$, $\text{Alpha-toxin}_{284\text{-}398}$, and $\text{Zmp}_{698\text{-}1022}$ (NAM) was designed using bioinformatic tools and synthesized as a chimeric construct. The bacterial prokaryotic vector pET-28a-NAM was constructed and expressed in the *E. coli* strain BL21 (DE3). Recombinant NAM (rNAM) was purified and used as an antigen to raise the antiserum. rNAM was emulsified in Complete Freund's adjuvant to prime the immune response and two boosters with Incomplete Freund's adjuvant at 400 and 250 µg on days 1, 14, and 28, respectively. Finally, Blood samples were collected on day 42, serum antibody levels were monitored by indirect ELISA using the purified NAM protein, and serum was used as an anti-NAM antibody in subsequent experiments. The serum control was collected before injection.

Detection of *plc*, *netB* and *zmp* genes

The presence of *plc*, *netB*, and *zmp* in the *C. perfringens* isolates was determined by PCR. Genomic DNA was isolated using a boiling procedure as described previously (Razmyar *et al*., 2017). A single colony of each strain was suspended in 100 µL of distilled water, boiled for 10 min, and then centrifuged at 10000 ×g for 10 min. The supernatants were collected and 5 µL was used as the template for PCR. Taq DNA polymerase (0.2 unit, Sinaclon, Iran) and 0.4 µM concentration of each primer were used to amplify the target regions. Denaturation (94°C for 1 min), annealing (55°C (for *netB*) and 58°C (for *plc* and *zmp*) for 1 min), and extension (72°C for 1 min) steps were performed for 30 cycles. Primer pairs including Atf (5´-GAA CTG GTC GCG TAC ATC-3´), Atr (5´-TTT GAT ATT GTA GGT AGA GTT AC-3´), mptf (5´-ATG TTT TGG GGA TTT GAT AAT TCA AAA GAT G-3´) and mptr (5´-TTA CTC TTC ACC CAA AGC AAG TG-3´) were used to screen for the presence of *plc* and metallopeptidase, respectively. Primers Ntf (5´-ATT GGT TAT TCT ATT GG-3´) and Ntr (5´-CAG GTA ATA TTC GAT TTT GTG-3´) were used to amplify *netB*. The NAM construct was used as a control. PCR products were visualized by electrophoresis on a1.5% agarose gel.

Preparation of crude toxin extract from supernatant

A single colony of *C. perfringens* on blood agar was incubated overnight in 10 ml Fluid Thioglycolate broth (FTG, Difco) and grown anaerobically at 37°C. An overnight culture with a 1:100 (v/v) ratio was used to inoculate fresh medium and grown to a turbidity of 0.6 at 600 nm to maximize the expression of lethal toxins in the mid-log phase (Yu *et al*., 2017; Zhou *et al*., 2017).

Subsequently, the culture was centrifuged at 10000 ×g for 20 min at 4°C and the culture supernatant was filter sterilized through a 0.22 µm filter (Millipore) to prepare the crude toxin mixture, which was then subjected to SDS-PAGE analysis. The protein concentration was quantified using the Bradford assay.

Western blot analysis of supernatant of *C. perfringens* culture

One hundred microliters of the crude toxin mixture was boiled in lysis buffer (5% SDS, 20% glycerol, 250 mm Tris-HCl, pH 6.8, 500 mM DTT) and resolved by SDS-PAGE in a 5% stacking gel and 10% separating gel (Laemmli, 1970). The separated proteins were transferred onto a nitrocellulose membrane (Sigma) using a Mini Protein blotting system (Bio-Rad). The membrane was blocked in 5% skimmed milk overnight at 4°C and then incubated with primary rabbit anti-NAM antibody at a 1:10000 dilution for 1 h at room temperature. Subsequently, goat anti-rabbit IgG (Bethyl, USA) was used as the secondary antibody at a 1:10000 dilution. The blots were developed by diaminobenzidine (1 mg/ml) and H_2O_2 (o.3 µL/ml) (DAB Substrate System, Sigma-Aldrich, USA).

Cytotoxicity assay

The LMH cell line (ATCC CRL-2117 Pasteur Institute (IRAN)) was grown in Waymouth's complete medium (90% Waymouth's MB 752/1 (Invitrogen) supplemented with 10% fetal bovine serum, L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin). LMH cells were incubated in a humidified environment with 5% CO_2 at 37°C. For the cytotoxicity assay, 96 well plates (SPL, Korea) were seeded at a density of 5 × 10^3 cells/well and incubated until they reached almost 100% confluence. Sterile supernatants from different isolates were prepared in a two-fold dilution series, from 1:2 to 1:128 (v/v), added to LMH medium in duplicate, and incubated for up to 8 h at 37°C with 5% CO_2. Cells treated with 1% Triton X-100 and medium without LMH were used as positive and negative controls, respectively. The conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell viability. After incubation, MTT solution at a final concentration of 1 mg/ml was added to the LMH cells and incubated for 4 h. The precipitated dark blue formazan crystals in viable cells were solubilized with acidified isopropanol for 10 min, and then absorbance at 570 nm was measured using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The 50% cytotoxic dose (CD50) was determined as the reciprocal dilution of crude toxin that caused 50% LMH cell death compared with untreated control cells. The results of the MTT cytotoxicity assay with two replicate experiments were calculated as follows:

1 - [(absorbance of sample - absorbance of negative control) / (absorbance of negative control - absorbance of positive control)] × 100

Ethics statement

Research on experimental animals was approved by the Poultry Disease Section, Faculty of Veterinary Medicine, University of Tehran, and according to the guidelines of the NIGEB Animal Care and Use Committee (Ethic code No. IR. NIGEB.EC.1397.11.30 F).

Statistical analysis

Significant differences between the various dilutions and strains were determined using One-way ANOVA, followed by Tukey's honestly significant difference (HSD) test. The values are expressed as mean±SD, and differences were considered significant at a P-value of less than 0.05. All statistical analyses were performed using Prism v.5.02 (GraphPad Software, San Diego, CA).

Results

Motif analysis

According to the sequence similarity results, the M60 catalytic domains were highly conserved among ZmpA, ZmpB, and ZmpC, even though ZmpA and ZmpB are located on the chromosome, and ZmpC was found on the virulence plasmid of *C. perfringens*. Thus, there was a 67.16% similarity between the M60 domains in ZmpA and ZmpB, 85.39% similarity between ZmpC and ZmpB, and 65.68% between ZmpC and ZmpA. Motif analysis showed that Zmp genes in the M60 region had similar motifs, regardless of their origin, and the gluzincin motif was highly conserved (Fig. 1).

Toxinotyping on gene and protein level

The bacterial strains were isolated from 27 healthy and 11 infected birds that showed important clinical signs of necrotic enteritis, including severe depression, ruffled feathers, diarrhea followed by wet litter, and in some acute cases sudden mortality.

The PCR results using specific primers related to the three toxins revealed that all 38 isolates were *plc* (*alpha toxin*)-positive, whereas *zmp* was found among 11 strains isolated from birds suffering from NE. However, 36.36% of the strains isolated from the infected birds were netB-positive (Table 1). In order to confirm the presence of these proteins, western blots were performed on different virulent *C. perfringens* isolates, and protein bands were detected for NetB at 36.46 kD, Alpha toxin at 45.52 kD, and Zmp at 189.89 kD, similar to those detected by ProtParam. Immunoblotting of crude toxins revealed cross-reactivity of the sera prepared against NetB-Alpha toxin-Zmp (NAM) recombinant protein (Fig. 2).

Cytotoxicity on LMH

To investigate if zmp plays a role in toxicity toward the LMH cell line and to determine whether these isolates produced a distinct secreted toxin, three strains were initially subjected to the cytotoxicity assay: strains that were able to produce Alpha toxin (Cp 48), Alpha toxin and Zmp (Cp 119.2), and Alpha toxin, NetB, and Zmp (Cp 28). The results indicated that the cytotoxicity level of the culture supernatant from Cp 28 was significantly higher than that of Cp 48.

The culture supernatants of the Cp28 and Cp119.2 strains showed potent cytotoxic effects on the LMH cell line at a dilution of 1:2, with 93.57% and 90.48% lethality, respectively (Fig. 3), and there was no significant difference (P=0.056) in cytopathic effects between Cp28 and Cp119.2. In contrast, supernatants derived from Cp48 displayed 57.36% lethality at 1:2 dilutions, suggesting that the toxicity of Cp28 and Cp119.2 strains might be caused by common toxins such as Zmps.

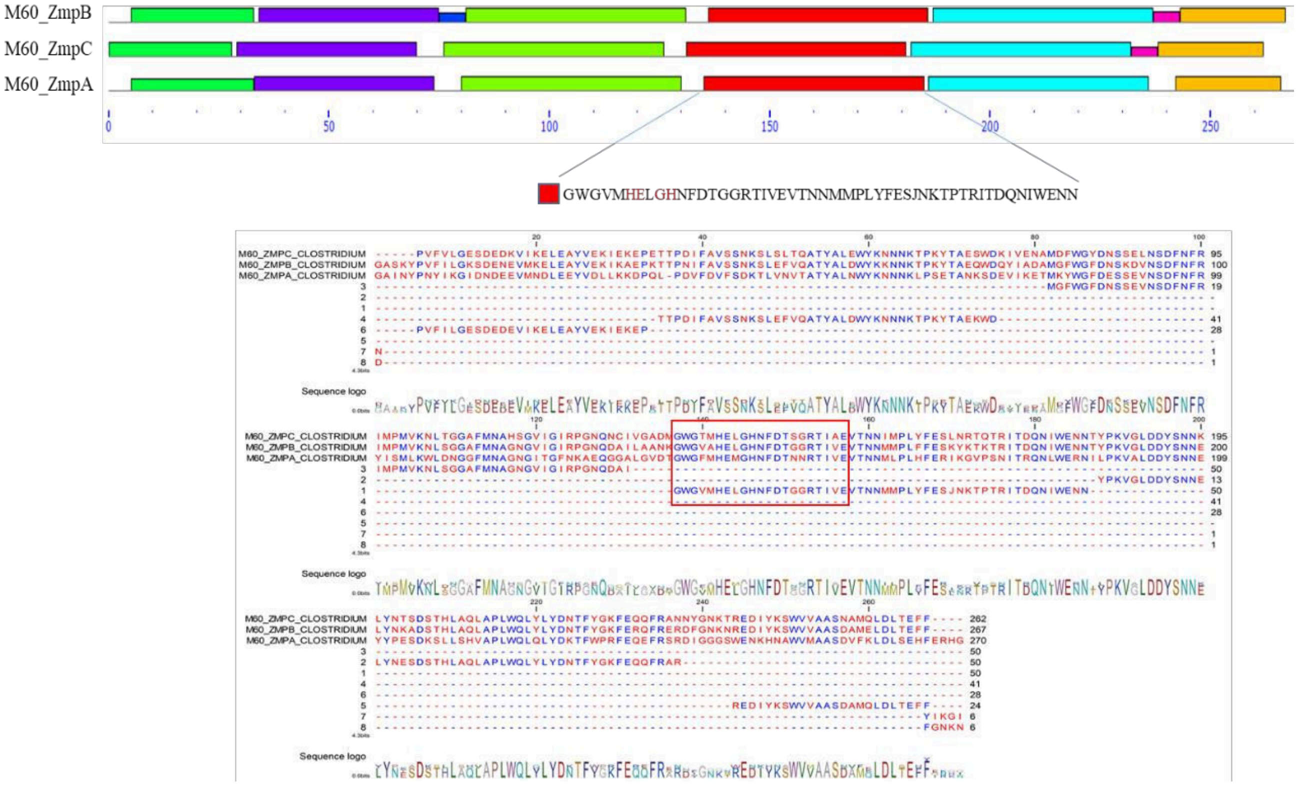
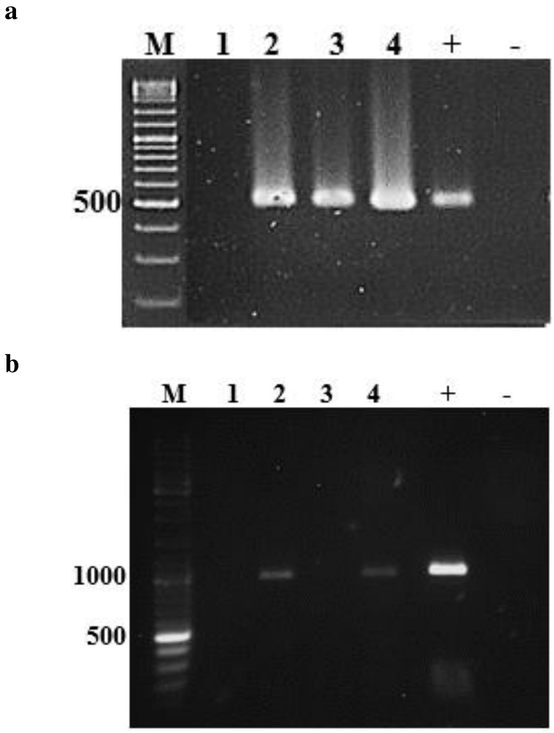


Fig. 1: Schematic representation of the gluzincin motif on zinc-metallopeptidase (HEXXHX(8,28)E). Motifs were predicted by the MEME suite search program. Different motifs are represented by different colored boxes. The red box depicts the gluzincin motif, and the rectangular box corresponds to its sequence on multiple alignments sequence alignments

Table 1: Prevalence of *alpha toxin*, *netB*, and *zmp* genes among *C. perfringens* isolates

	Strain	Source	*alpha toxin*	*netB*	*zmp*
1	CP 18	Infected farm	+	-	+
2	CP 18.2	Infected farm	+	-	+
3	CP 19	Healthy farm	+	-	-
4	CP 24	Healthy farm	+	-	-
5	CP 24.1	Healthy farm	+	-	-
6	CP 27	Healthy farm	+	-	-
7	CP 28	Infected farm	+	+	+
8	CP 28.2	Infected farm	+	+	+
9	CP 32	Healthy farm	+	-	-
10	CP 33.1	Healthy farm	+	-	-
11	CP 39	Healthy farm	+	-	-
12	CP 40	Healthy farm	+	-	-
13	CP 41	Infected farm	+	-	+
14	CP 42	Infected farm	+	-	+
15	CP 44	Infected farm	+	+	+
16	CP 48	Healthy farm	+	-	-
17	CP 51	Healthy farm	+	-	-
18	CP 56	Infected farm	+	-	+
19	CP 57	Infected farm	+	-	+
20	CP 58	Infected farm	+	+	+
21	CP 60	Healthy farm	+	-	-
22	CP 61	Healthy farm	+	-	-
23	CP 79	Healthy farm	+	-	-
24	Cp 81.2D	Healthy farm	+	-	-
25	CP 84	Healthy farm	+	-	-
26	CP 96	Healthy farm	+	-	-
27	CP 103	Healthy farm	+	-	-
28	CP 119.2	Infected farm	+	-	+
29	CP 125	Healthy farm	+	-	-
30	Cp 125.2	Healthy farm	+	-	-
31	CP 129	Healthy farm	+	-	-
32	CP 129.2	Healthy farm	+	-	-
33	CP 134	Healthy farm	+	-	-
34	CP 135	Healthy farm	+	-	-
35	CP 137d	Healthy farm	+	-	-
36	CP 137.2	Healthy farm	+	-	-
37	CP 142	Healthy farm	+	-	-
38	CP 143.2	Healthy farm	+	-	-



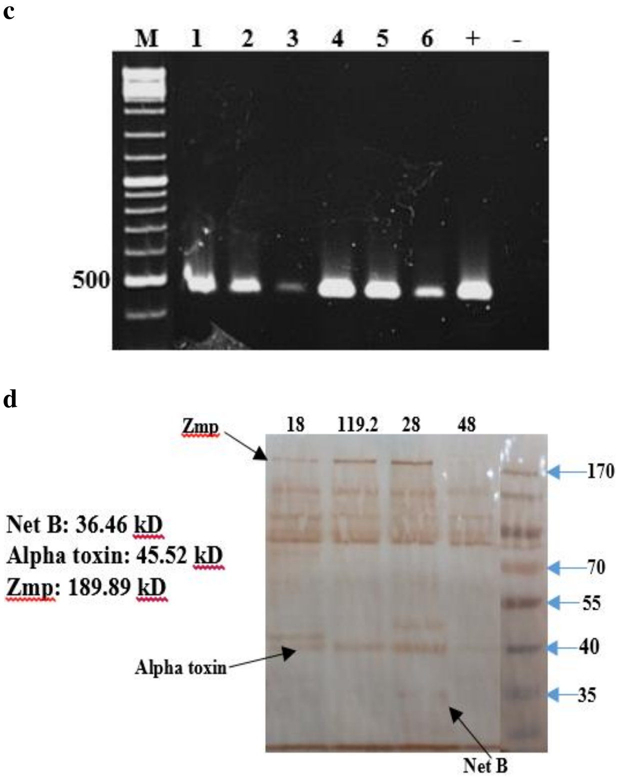


Fig. 2: Presence of three toxins, Net B, Alpha toxin, and Zmp, in different *C. perfringens* isolates at the gene and their corresponding protein levels. (**a**) PCR result of the *netB* gene, showing a band of 531 bp on an agarose gel, (**b**) PCR result of the *zmp* gene showing a band of 1008 bp that is not present in some isolates, (**c**) PCR result for *plc* gene encoding Alpha toxin showing a band of 442 bp on agarose gel for all isolates, and (**d**) Western blot analysis of secreted toxins of *C. perfringens* using a polyclonal anti-NAM antibody against three proteins. Lane M: Gene ruler DNA ladder mix (Fermentas)

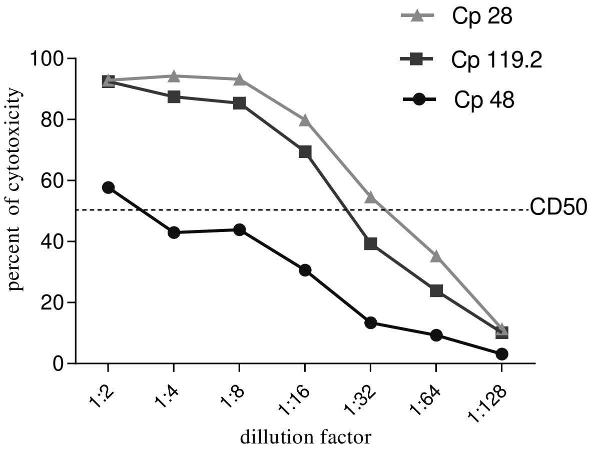


Fig. 3: LMH cytotoxicity assay using culture supernatant in several dilutions prepared from three selected isolates of Cp 28, Cp 48, and Cp 119.2 from the farm. Data are presented as the ratio of the CD50 of the *C. perfringens* supernatant after incubation with LMH for up to 18 h at 37°C with 5% CO_2. The amount of cytotoxicity induced by each dilution of secreted toxin is expressed as the mean±SD of two separate experiments. The standard errors are shown in the graph

The results of the cytotoxicity assay are expressed as 50% of cytotoxicity (CD50) on the regression curve of the cytotoxicity graphs. The CD50 of Cp28 strain (*plc*, *netB*, and *zmp* positive) was assessed at a dilution of 1:34. The culture supernatants of the two strains Cp119.2 strain (*plc* and *zmp*-positive) and the Cp48 (*plc*-positive) strain showed approximately CD50 at a dilution of 1:23 and 1: 4, respectively (Fig. 3). The higher toxicity of Cp28 and Cp119.2 toward LMH cells might be caused by a common secreted component, such as Zmp, which is distinct from other unknown toxins.

Therefore, further investigation is required to determine whether the presence of other immunoreactive proteins had a statistically significant effect on cytotoxicity.

Discussion

The pathogenesis of *C. perfringens* infection is associated with the production and secretion of various toxins and enzymes. Identifying high-potential virulence-associated genes and understanding their roles in pathogenesis could help improve the severity of NE in chickens. Vaccination with multiple immunogens may protect against NE completely (Katalani *et al*., 2020b; Yuan *et al*., 2022). New virulent toxins and enzymes have been shown to play a role in pathogenesis, which calls for developing effective vaccines (Van Immerseel *et al*., 2009).

In the current diagnostic approach for NE, PCR is commonly used to identify virulence genes involved in the disease (Razmyar *et al*., 2017). In the present study, 38 strains of *C. perfringens* isolated from infected and non-infected broiler chicken farms were examined, and 71% of the isolates derived from healthy birds belonged to type A (Alpha toxin, *plc* positive). Alpha toxin in all pathogenic and non-pathogenic strains confirms previous studies showing that Alpha toxin alone cannot cause NE disease in poultry. Therefore, the presence of type A in the intestines of healthy chickens can be explained by the fact that *C. perfringens* is found in low numbers (10^2 to 10^4 CFU per gram of intestinal content) (Shojadoost *et al*., 2012; Razmyar *et al*., 2014).

In addition, since the discovery of other toxins, such as NetB and Zmps, the role of Alpha toxin as a major virulence factor has almost diminished (Cooper and Songer, 2009; Wilde *et al*., 2019). However, the fact that all *C. perfringens* clones are *plc*-positive suggests that they are appropriate controls. The *netB*-producing isolates of *C. perfringens* are categorized as type G. However, the results of toxinotyping showed that in 7 cases of NE positive isolates, *netB* were negative, and disease was still observed in *Clostridium*-infected birds that produced Zmp and Alpha toxin (Table 1). Zmp has also been shown to be involved in the development of NE in poultry (Wade *et al*., 2020).

Multiple sequence alignment analysis has revealed similarities in the *zmp* genes, including a conserved catalytic M60 domain, suggesting that they may have a conserved function. Using in situ analysis, Pluvinage *et al*. (2021) suggested that ZMPA is not catalytic. However, further experiments in chicken necrotic enteritis disease models are required to validate this hypothesis. Our results suggest that Zmp may be a more prevalent virulence factor in the disease compared to NetB.

In agreement with our results, Wade *et al*. (2020) demonstrated that Zmp is a major pathogenic factor required for full virulence in experimental model of NE. Another study on turkey flocks infected with *C. perfringens* showed that NetB toxin was not involved in 90% of NE cases and concluded that other toxins or enzymes may also be involved in the NE outbreak (Razmyar *et al*., 2017). Western blot analysis of toxins secreted from different strains confirmed the PCR results and proteins of 36, 45, and 189 kDa were identified for NetB, Alpha toxins, and Zmp, respectively. In this case, non-specific protein bands below the ZmpB protein band might be homologous to Zmp proteins detected by polyclonal antibodies. However, the possibility that the related protein forms are due to proteolytic degradation cannot be ignored. According to the cytotoxicity results, the supernatant of the Cp28 strain, which contained all three toxins, had the most toxic effects against the LMH cell line. Because the Cp48 supernatant showed less cytotoxicity than the Cp119.2 supernatants, metallopeptidases may be responsible for the increased cytotoxicity of Cp119.2. There is evidence of the cytotoxic effects of NetB toxin on LMH cells in culture supernatants from wild-type and mutant strains of *C. perfringens* (Zhou *et al*., 2017). Hence, the major cytotoxic effect of crude *C. perfringens* extracellular toxins is specifically attributed to the presence of NetB (Cheung *et al*., 2010; Keyburn *et al*., 2010). However, other toxins in bacterial culture supernatants have not been investigated *in vitro* (Yu *et al*., 2017). It is worth mentioning that the results of various studies on this subject are contradictory. For example, Lanckriet *et al*. (2010) found that Alpha toxin-containing supernatants in strains lacking NetB and containing Alpha toxin were moderately cytotoxic (61%). The researchers concluded that other proteins might also play a role in the cytotoxicity of LMH cells. This is consistent with our findings that Cp1192 culture supernatant induces high levels of cytotoxicity in the LMH cell line, suggesting that other virulence factors, such as zinc metallopeptidase, may also be required to induce high levels of cytotoxicity.

The findings of the present study, which shed new light on the impact of different bacterial toxins or immunogenic proteins on the severity of NE disease in birds, are of significant importance in the field of veterinary medicine and the development of a vaccine for this disease. Further experiments are crucial to fully comprehend the role of all virulence factors in the pathogenesis of *C. perfringens*.

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Conflict of interest

The authors declare no conflicts of interest.

References

Afshari, A; Jamshidi, A; Razmyar, J and Rad, M (2015). Genotyping of *Clostridium perfringens* isolated from broiler meat in northeastern of Iran. Vet. Res. Forum. 6: 279-284.

Caly, DL; D'Inca, R and Auclair Eand Drider, D (2015). Alternatives to antibiotics to prevent necrotic enteritis in broiler chickens: A microbiologist's perspective. Front. Microbiol., 6: 1336-1348 https://doi.org/10.3389/fmicb.2015.01336.

Cheung, JK; Keyburn, AL; Carter, GP; Lanckriet, AL; Van Immerseel, F; Moore, RJ and Rood, JI (2010). The VirSR two-component signal transduction system regulates NetB toxin production in *Clostridium perfringens*. Infect. Immun., 78: 3064-3072. https://doi.org/10.1128/IAI.00123-10.

Cooper, KK and Songer, JG (2009). Necrotic enteritis in chickens: a paradigm of enteric infection by *Clostridium perfringens* type A. Anaerobe. 15: 55-60. https://doi.org/10.1016/j.anaerobe.2009.01.006.

Katalani, C; Ahmadian, G; Nematzadeh, G; Amani, J; Ehsani, P; Razmyar, J and Kiani, G (2020a). Immunization with oral and parenteral subunit chimeric vaccine candidate confers protection against Necrotic Enteritis in chickens. Vaccine. 38: 7284-7291. https://doi.org/10.1016/j.vaccine.2020.09.047.

Katalani, C; Nematzadeh, G; Ahmadian, G; Amani, J; Kiani, G and Ehsani, P (2020b). *In silico* design and *in vitro* analysis of a recombinant trivalent fusion protein candidate vaccine targeting virulence factor of *Clostridium perfringens*. Int. J. Biol. Macromol., 146: 1015-1023. https://doi.org/10.1016/j.ijbiomac.2019.09.227.

Keyburn, AL; Boyce, JD; Vaz, P; Bannam, TL; Ford, ME; Parker, D; Di Rubbo, A; Rood, JI and Moore, RJ (2008). NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog., 4: e26.

Keyburn AL; Portela RW; Sproat K; Ford ME; Bannam TL; Yan X; Rood JI and Moore RJ (2013). Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis. Vet Res., 44: 1-8. https://doi.org/10.1186/1297-9716-44-54.

Keyburn, AL; Yan, XX; Bannam, TL; Van Immerseel, F; Rood, JI and Moore, RJ (2010). Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. Vet. Res., 41: 1-8.

Kiu, R and Hall, LJ (2018). An update on the human and animal enteric pathogen *Clostridium perfringens*. Emerg. Microbes. Infect., 7: 1-15. https://doi.org/10.1038/s41426-018-0144-8.

Kulkarni, RR; Parreira, VR; Sharif, S and Prescott, JF (2006). *Clostridium perfringens* antigens recognized by broiler chickens immune to necrotic enteritis. Clin. Vaccine Immunol., 13: 1358-1362. https://doi.org/10.1128/CVI.00292-06.

Kulkarni, RR; Parreira, VR; Sharif, S and Prescott, JF (2008). Oral immunization of broiler chickens against necrotic enteritis with an attenuated *Salmonella* vaccine vector expressing *Clostridium perfringens* antigens. Vaccine. 26: 4194-4203. https://doi.org/10.1016/j.vaccine.2008.05.079.

Laemmli, UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685. https://doi.org/10.1038/227680a0.

Lanckriet, A; Timbermont, L; Eeckhaut, V; Haesebrouck, F; Ducatelle, R and Van Immerseel, F (2010). Variable protection after vaccination of broiler chickens against necrotic enteritis using supernatants of different *Clostridium perfringens* strains. Vaccine. 28: 5920-5923. https://doi.org/10.1016/j.vaccine.2010.06.035.

Lepp, D; Gong, J; Songer, JG; Boerlin, P; Parreira, VR and Prescott, JF (2013). Identification of accessory genome regions in poultry *Clostridium perfringens* isolates carrying the netB plasmid. J. Bacteriol., 195: 1152-1166. https://doi.org/10.1128/JB.01032-12.

Nakjang, S; Ndeh, DA; Wipat, A; Bolam, DN and Hirt, RP (2012). A novel extracellular metallopeptidase domain shared by animal host-associated mutualistic and pathogenic microbes. PLoS One. 7: e30287. https://doi.org/10.1371/journal.pone.0030287.

Noach, I; Ficko-Blean, E; Pluvinage, B; Stuart, C; Jenkins, ML; Brochu, D; Buenbrazo, N; Wakarchuk, W; Burke, JE and Gilbert, M (2017). Recognition of protein-linked glycans as a determinant of peptidase activity. PNAS. 114: E679-E688.

Pluvinage, B; Ficko-Blean, E; Noach, I; Stuart, C; Thompson, N; McClure, H; Buenbrazo, N; Wakarchuk, W and Boraston, AB (2021). Architecturally complex O-glycopeptidases are customized for mucin recognition and hydrolysis. PNAS. 118: e2019220118. https://doi.org/10.1073/pnas.2019220118.

Prescott, JF; Parreira, VR; Mehdizadeh Gohari, I; Lepp, D and Gong, J (2016). The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review. Avian Pathol., 45: 288-294. https://doi.org/10.1080/03079457.2016.1139688.

Razmyar, J; Kalidari, GA; Tolooe, A; Rad, M and Movassaghi, AR (2014). Genotyping of *Clostridium perfringens* isolated from healthy and diseased ostriches (*Struthio camelus*). Iran. J. Microbiol., 6: 31-36.

Razmyar, J; Peighambari, SM and Zamani, AH (2017). Detection of a newly described bacteriocin, perfrin, among C lostridium perfringens isolates from healthy and diseased ostriches and broiler chickens in Iran. Avian Dis., 61: 387-390.

Rood, JI; Adams, V; Lacey, J; Lyras, D; McClane, BA; Melville, SB; Moore, RJ; Popoff, MR; Sarker, MR; Songer, JG; Uzal, FA and Van Immerseel, F (2018). Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe. 53: 5-10. https://doi.org/10.1016/j.anaerobe.2018.04.011.

Shimizu, T; Ohtani, K; Hirakawa, H; Ohshima, K; Yamashita, A; Shiba, T; Ogasawara, N; Hattori, M; Kuhara, S and Hayashi, H (2002). Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci., 99: 996-1001. https://doi.org/

10.1073/pnas.022493799.

Shojadoost, B; Vince, AR and Prescott, JF (2012). The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Vet. Res., 43: 1-12. https://doi.org/10.1186/1297-9716-43-74.

Van Immerseel, F; Rood, JI; Moore, RJ and Titball, RW (2009). Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Microbiol., 17: 32-36.

Wade, B and Keyburn, A (2015). The true cost of necrotic enteritis. World Poult., 31: 16-17.

Wade, B; Keyburn, AL; Haring, V; Ford, M; Rood, JI and Moore, RJ (2020). Two putative zinc metalloproteases contribute to the virulence of *Clostridium perfringens* strains that cause avian necrotic enteritis. J. Vet. Diagn. Invest., 32: 259-267.

Wilde, S; Jiang, Y; Tafoya, AM; Horsman, J; Yousif, M; Vazquez, LA and Roland, KL (2019). *Salmonella*-vectored vaccine delivering three *Clostridium perfringens* antigens protects poultry against necrotic enteritis. PLoS One. 14: e0197721. https://doi.org/10.1371/journal.pone.0197721.

Yu, Q; Lepp, D; Gohari, IM; Wu, T; Zhou, H; Yin, X; Yu, H; Prescott, JF; Nie, SP and Xie, MY (2017). The Agr-like quorum sensing system is required for pathogenesis of necrotic enteritis caused by *Clostridium perfringens* in poultry. Infect. Immun., 85: e00975-16.

Yuan, B; Sun, Z; Lu, M; Lillehoj, H; Lee, Y; Liu, L; Yan, X; Yang, DA and Li, C (2022). Immunization with pooled antigens for *Clostridium perfringens* conferred partial protection against experimental necrotic enteritis in broiler chickens. Vaccines (Basel). 10: 979-990. https://doi.org/10.3390/vaccines10060979.

Zhou, H; Lepp, D; Pei, Y; Liu, M; Yin, X; Ma, R; Prescott, JF and Gong, J (2017). Influence of pCP1NetB ancillary genes on the virulence of *Clostridium perfringens* poultry necrotic enteritis strain CP1. Gut Pathog., 9: 1-7. https://doi.org/10.1186/s13099-016-0152-y.