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NEW KNOWLEDGE ON PATHOGENESIS OF TOXIN PERFRINGENS LARGE (TPEL) FOR PRODUCTION OF NEW GENERATION VACCINE AND MOLECULAR WORKING, A REVIEW

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ABSTRACT

Clostridium perfringens (*C. perfringens*) is an anaerobic and gram-positive, spore-forming bacterium. Clostridial infections are cause of the most important diseases including lamb dysentery, pulpy kidney, black disease, malignant oedema, and blackleg, with heavy losses over many years in Iran. Major and minor toxins have an important role in pathogenesis. The species is classified into five types (types A-E) based on the major toxin. A novel toxin (toxin perfringens large) (TpeL) has recently been identified from *C. perfringens* types A, B and C. This toxin belongs to large glycosylating toxins family which is cause of cytotoxic effects in Vero cells (including enlargement and the rounding of Vero cells). TpeL toxin is involved in the pathogenesis of avian enteric necrosis (including ostrich and chicken flocks) which can lead to the morbidity and mortality rate; therefore TpeL is considered as a virulence factor. This paper reviews the studies on molecular working (PCR) for detection of disease, determination of the biotypes of *C. perfringens*, analyze for genetic diversity using PFGE, immunoinformatics analysis and production of the new generation vaccine was performed on TpeL toxin. Finally, the new generation vaccine was evaluated the amount of antibody using serum neutralization assay in laboratory animals. The results of this study showed that the chimeric vaccine could be used as an effective vaccine against domestic animals disease. It is suggested that more research should be done on isolation, characterization, and purification. Furthermore, the evaluation of immunity could be performed in target animals.

KEYWORDS: *Clostridium perfringens*, new generation vaccine, TpeL toxin.

1. INTRODUCTION

Clostridium perfringens is an anaerobic and Gram-positive bacilli worldwide, commonly are found in the soil and intestinal tract of mammals and birds (Songer, 1996). The first case of *C. perfringens* was identified in the 1994_s from food poisoning -associated enteritis (McClung, 1945). After World War II in Germany, *C. perfringens* infection was detected in necrotic enteritis cases. *C. perfringens* is responsible of infection in humans and animals including enterotoxaemia, lamb dysentery, avian necrotic enteritis, neonatal hemorrhage or necrotic enteritis in calves, foals and piglets and ovine.

C. perfringens produces major and minor toxins that are directly cause of intestinal diseases. It is categorized into five types (A-E), respectively. *C. perfringens* type A causes gas gangrene, gastrointestinal disease and food poisoning in humans, hemorrhagic gastroenteritis in dogs (Songer, 1996) necrotic enteritis in chickens, yellow lamb disease in sheep, enteritis and enterotoxaemia in cattle, goats, pigs and horses (Pilehchian Langroudi, 2015).

It produces four major toxins including iota, alpha, beta and epsilon. Some of them such as *C. perfringens* toxin type B and C are removed during secretion, resulting in an active mature toxin. *C. perfringens* toxin type B causes hemorrhagic dysentery in sheep, whereas type C strains cause enterotoxaemia and necrotic enteritis in several species, and pigbel in humans.

However *C. perfringens* ε toxin type D and B is secreted as prototoxin of ~33 kDa, which is activated by removing of the 13 N-terminal and 29 C-terminal residues using trypsin, α-chymotrypsin, carboxypeptidases, and λ-protease in the gastrointestinal tract (Freedman et al., 2016). Epsilon toxin induces rapidly fatal enterotoxaemia (Silva and Lobato, 2015).

Recently has been reported *C. perfringens* produces up to thirty major and minor toxin. (Lebrun et al., 2010). Toxin

perfringens large (TpeL) recently detected important virulence factor, in *C. perfringens* type A, B and C, which modify an intracellular target. This toxin is involved in the pathogenesis of avian enteric necrosis (Songer, 1996). *C. perfringens* strains which are carried of *TpeL* gene can lead to the mortality rate.

2. TpeL toxin

TpeL toxin is produced by *C. perfringens* of types A, B, and C that belongs to large glycosylating toxins family (Pauillac et al., 2013). The name of the toxin is derived from toxin *C. perfringens* large cytotoxin. This toxin was identified and isolated from *C. perfringens* type C strain MC18 supernatant in 2007 (Amimoto et al., 2007). In 2012, this toxin was isolated from *C. perfringens* of type A and purified using HPLC (Coursodon et al., 2012). TpeL is expressed during the sporulation phase and is regulated by sporulation-specific sigma factor, SigE (Paredes-Sabja et al., 2011). The molecular mass of this toxin was determined about 180 kDa by SDS-Page (Amimoto et al., 2007). TpeL is the largest toxin among *C. perfringens* toxins. The sequences of this toxin was showed homology with TcsL, TcsH, TcnA, TcdA and TcdB, called "LCTS" (Amimoto et al., 2007).

2.1. Toxin Structural organization

The LCTs are large single-chain proteins (Pruitt and Lacy, 2012) which are known AB-toxins. "B domain" is responsible of the binding to target cell receptor and "A domain" is responsible of delivery enzymatic domain into the cytosol of the host cell. A-domain (enzymatically active domain) is located in the N-terminal region, which has glycosyltransferase activity. A-domain characterized by the DXD motif surrounded by a hydrophobic region (Hofmann et al., 1997).

A-domain is consist of 543aa (Nagahama et al., 2012), followed Autocatalytic processing domain (between residues 543-769) is cysteine protease which has catalytic-cleavage activity (Egerer et al., 2007) Delivery domain contains large hydrophobic region (between residues 955 and 1852) that is important for the entry of toxin into the endosome membranes. Delivery domain is known as translocating domain that is likely participated in the delivery of glycosylating domain into the target cell cytosol Fig. 1a, 1b.

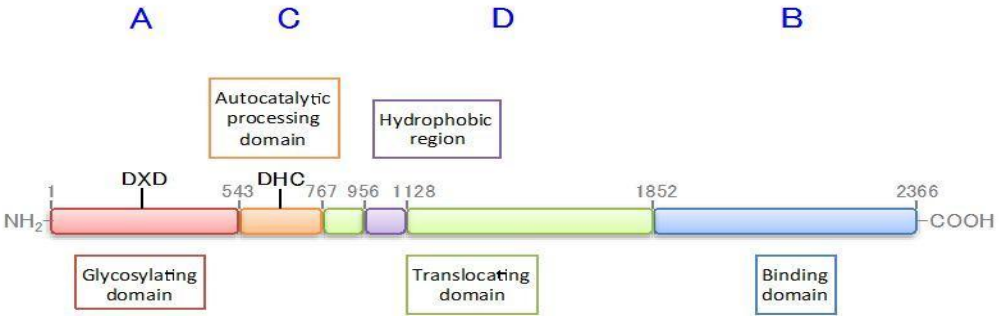

Fig. 1a: The structure of glycosylating toxins family (Nagahama et al., 2012).

Fig. 1b: (Alvin and Lacy, 2018).

Finally, Binding domain ($CROP_S$) (between residues 1852-2366) is located in the C-terminal region which is involved in binding to target cell surface receptor (Greco et al., 2006). C-terminal region consists of multiple amino acid (Long Repeat), separated by 31 amino acid (short repeats) (Pauillac et al., 2013) Fig. 2.

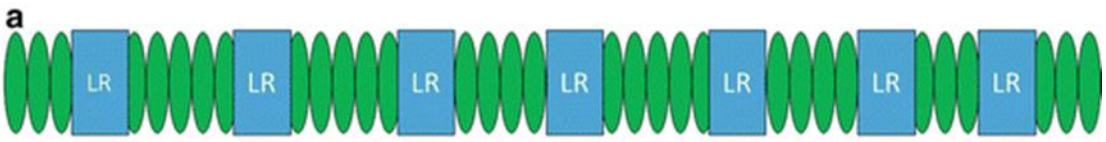

Fig. 2: Structural of C-terminal domain (Alvin and Lacy, 2018).

A domain which is harbored in the N-terminal region is very important for glycosyltransferase activity (Busch and Aktories, 2000), and this region has been highly conserved for long years, while C-terminal region which is responsible for the binding to the target cell, is not conserved. The full length of C-terminal (*TpeL*1-1779) is varies. Complete sequence of *C. perfringens* type A (strain 13) is available (Shimizu et al., 2002), but it was the lack of *TpeL* gene sequence. This suggests that the sequences of toxin gene which is carried by plasmid can eliminated during the passage of strains (Gibert et al., 1997).

2.2: Toxin function mechanism

TpeL binds to LDL receptor-related protein 1 (LRP1) in host cells through binding domain (Schorch et al., 2014) then enter target cell by receptor-mediated endocytosis (Papatheodorou et al., 2010). After endocytosis, TpeL toxin is entered into the endosomal membrane through D-domain. The acidic pH of the endosome induces primary conformational changes and leads to channel formation in the membrane. Inositol phosphate reacts with the C-domain cysteine protease and induces secondary conformational change and act as protease Fig. 3. (Nagahama et al., 2012).

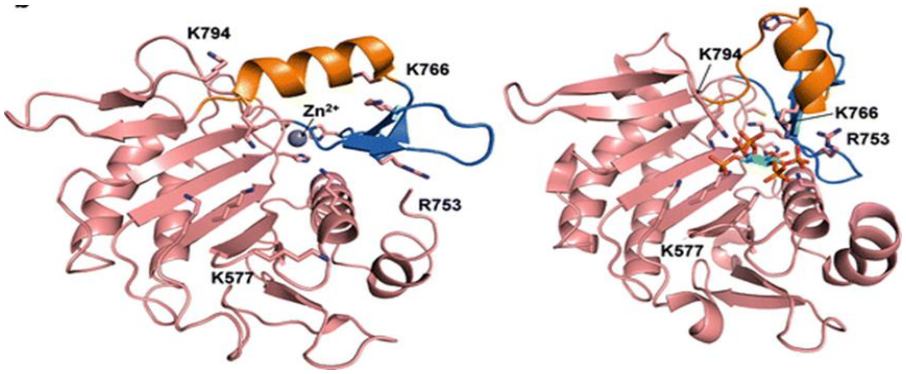

Fig. 3: LCT_S Conformational changes (the absence of InsP6 (left) and InsP6-bound structures (right) (Alvin and Lacy, 2018).

This process leads to breakdown of toxin and release of A-domain (DXD fragment) into cytosol (Genisyuerek et al., 2011). DXD fragment acts small GTPases such as (Rac1, Ras, Rap and Ral) (Nagahama et al., 2011) which are involved the regulation of the actin cytoskeleton. The inactivation of the proteins leads to extensive morphological changes, such as loss of actin fibers and disaggregation of the intercellular connections and finally cell death Fig. 4 (Nagahama et al., 2012).

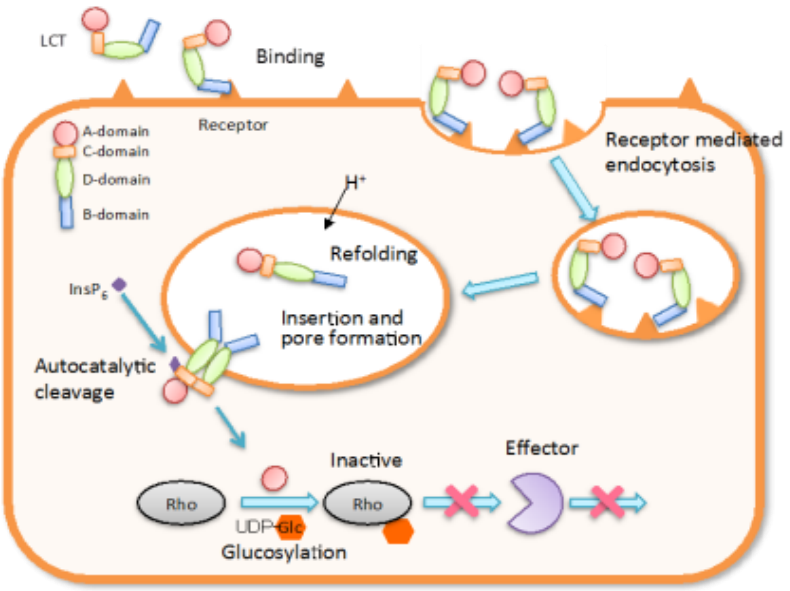

Fig. 4: Entry, activation and modification of LCT_S (large cytotoxin) (Nagahama et al., 2012).

***2.3. TpeL* gene**

TpeL toxin encoded by plasmid and the length of gene encoding *TpeL* in *C. perfringens* type A and C is 5340 and 1651 respectively. This gene encoded 1651 amino acids residue and the TpeL molecular mass was estimated 191 kDa using SDS- Page analysis. There is no signal peptide sequence (Nagahama et al., 2012). Sayeed et al., Gurjar et al. reported that *TpeL* gene is located approximately 3 kb downstream of the *cpb* gene from ~90 or 65-60 kb plasmids (Gurjar et al., 2010; Sayeed et al., 2010). (Table 1).

Table 1: Size diversity *TpeL* gene (Li et al., 2013).

Type	*TpeL*
A	ND
B	65 90
C	65/90 65
D	-
E	-

2.4. TpeL Characterization

This toxin uses UDP-N-acetylglucosamine as sugar donors (donor cosubstrate). Change of alanine 383 to isoleucine converts UDP-N-acetylglucosamine into UDP-glucose (Guttenberg et al., 2012). TpeL toxin acts Rac1, H-Ras, Rap, Ral and modifies the Rac1 and Ras subfamily by glycosylation. Thr-35 changes in Rac1 induce cytotoxicity in Vero cell (Nagahama et al., 2012).

2.5. TpeL toxicity

TpeL in the presence of SLO, is cause of glycosylation of Rac1 and the transfer of Rac and Ras proteins to the membrane in Vero cells (Nagahama et al., 2011), which leads to cytotoxic effects (morphological changes such as enlargement and the rounding of Vero cells). The cytotoxicity was neutralized by 4 CU of the purified TpeL toxin versus 0.5 µg mAb ml $^{-1}$. Cytotoxic effects neutralized in the presence of antitoxin and heat. TpeL is also cause of death to mice by intravenous injection. Studies showed that the lethality in mice was 62 MLD mg $^{-1}$ (one MLD was 16 µg) and 91 LD_{50} mg $^{-1}$ (one LD_{50} was 11 µg) (Amimoto et al., 2007).

3. Studies in Iran

In 1392, the study was conducted to detect of *NetB* (*C. perfringens* necrotic enteritis beta-like toxin) and *Tpel* genes in thirty six isolates with necrotic and healthy in Khorasan-e-Razavi province. The results of this study showed that all isolates were confirmed as type A and C. *TpeL* gene, which is virulence factor, was identified in 37.5% of the cases and 10% of healthy cases. Statistical analysis showed that there is no significant difference between healthy and diseased flocks (p=0.103) (Mirzazadeh, 1392).

In 2012, another study was carried out to detect of *CPA*, *CPB2*, *Cpe*, *netB* and *TpeL* genes in horses fecal with colitis using enzyme-linked immunosorbent assays. This study determined that C. *perfringens* were identified from 40% of cases. All of the cases were negative for this gene (Mehdizadeh Gohari, 2012).

In 2014, the study was performed to determine the biotypes of *C. perfringens* in the ostrich with necrotic enteritis and healthy for *cpa*, *cpb*, *etx*, and *iA*, *cpb2*, and *cpe* gene in Khorasan-e-Razavi province. There was evidence that TpeL -positive strains cause more severe disease than TpeL -negative strains (Unpublished data) (Razmyar et al., 2014).

In 2014, another study was carried out to determine the biotypes of *C. perfringens* in packed chicken using PCR by PourSoltani. The results of this study reported that *C. perfringens* were identified from 3.33% of cases. The *TpeL* gene was identified from 50% of cases (Poursoltani et al., 2014).

In 2015, another study was conducted to detect of *C. perfringens* in 131 healthy dog and 20 samples with clinical signs using culture and PCR in northwest of Iran. This study determined that *C. perfringens* were identified from 25% of cases with clinical signs and 23.8% of cases healthy dog. All of the cases had cpa+ TpeL - toxin profile (Salari Sedigh et al., 2016).

In the year 2014, Immunoinformatic Analysis was performed on alpha and TpeL toxins of *C. perfringens*. Alpha and TpeL toxins complete sequences were analyzed and tertiary structure were designed. Then conformational B cell epitopes were analyzed using Immunoinformatic tools including ElliPro, DiscoTope 2.0, SEPPA, CBTOPE, BCEP, and B-pred servers. Totally, 3 and 2 unique B-cell antigenic sequence was showed for these toxins respectively. The amino acids were identified including 48-122, 160-210 and 270-370 for Alpha toxin and 80-265 and 1130-1500 for TpeL toxin of *C. perfringens*. In-silico epitopes prediction is essential for designing new generation vaccines (Tolooe et al., 2014).

In another study, the NetB, alpha toxin and TpeL sequences were analyzed using bioinformatics software for production of protein with high immunogenicity. Different regions of the gene were selected, and evaluated for making the fusion protein. Fusion gene was synthesized and recombinant plasmid was transformed into the competent *E. coli* BL21 (DE3). Then fusion protein was purified by Ni-NTA, SDS-PAGE and western blotting was used for confirmation. Finally, Serum neutralization test was used for measuring of immunity in rabbit. The results of this study reported that chimeric vaccine could be used as suitable vaccine against avian necrotic enteritis (Rostami et al., 2016).

In 2019, the study was conducted on cloning of *TpeL* gene of *C. perfringens* type B in *E. coli*. For this purpose, genomic DNA was extracted by phenol-chloroform and PCR was done by specific primers. Then PCR product was added to PTZ57R / T vector and became cloned in competent *E. coli* TOP10. Then colony PCR method carried out for screening the colonies. There was evidence that the *TpeL* gene in *E. coli* strain TOP10 have be cloned (Mamandi et al., 2019).

4. Studies In the world

In another study was performed to detect *TpeL* gene using PCS by Katsuhiko on 14 , isolated from bovine and swine in Japan and 4 reference *C. perfringens* strain, all of *C. perfringens* type B (except one case) and all types of *C. perfringens* type C strains contained *TpeL* gene. While 12 strains of *C. perfringens* type A was lack of this gene. The dot blot technique was also used to detect this gene with anti- TpeL mAb and anti-mouse IgG. The results of this study showed all cases had this gene (except one strain, ATCC 3626). But the intensity of the reaction was different among the cases. The MC18 and Chi8 strains showed strong signals, and Shi2 and 3511-2 strains showed slightly weaker signals than them. The CP46 strains showed a very weak reaction (Amimoto et al., 2007).

In 2019, the study was performed to detect of *C. perfringens* in chickens using PCR in Egypt. The results reported that *C. perfringens* were identified from 49.1%, 82.5%, 60% and 50% of the healthy birds, diseased birds, feed and litter, respectively. The *TpeL* gene was identified from 27.77% of total cases using single PCR. None of the healthy birds were positive for the *TpeL* gene. *C. perfringens* type A was identified from 27.77% of cases by neutralization test in mice and dermonecrotic test in guinea pigs. Additionally, the effect of trypsin on this toxin in guinea pigs was inhibitory effects (Galal et al., 2018).

In 1995, postmortem examination of Guinea pigs infected with *C. perfringens* was carried out by EL Bardisy et al. The result showed pulmonary edema, vacuolar degeneration, hepatocytes necrosis, necrotic enteritis, glomeruli atrophy (El-Bardisy et al., 1995).

In a study, 95 isolates of *C. perfringens* type A (including 88 samples from Sweden, 7 Norway samples), 5 references strains (Culture Collection of Institute Pasteur, Paris, France) (*C. perfringens* type A to E) and one *C. perfringens* type A strain (Culture Collection of the University of Gothenburg, Sweden) was collected and analyzed for genetic diversity using PFGE. This study has reported there was high genetic diversity among isolated from different sources, whereas there was low genetic diversity among isolated from the same sources (Johansson et al., 2006).

Another study was carried out to determine of characterization of the enzymatic activity of TpeL toxin. So N-terminal part of TpeL was synthesized from *C. perfringens* type B by specific primers. Then PCR product was cloned into pCR2.1 vector and became subcloned into pET28a. The recombinant protein was ligated to an N-terminal region from pET28. Recombinant plasmid was transformed into the competent *E. coli* BL21. The expression was induced with IPTG. Then recombinant bacteria were purified using cobalt column and dialyzed. Finally further studies were done to better understand the structure and function of TpeL toxin (Pauillac et al., 2013).

In 2015, another study was performed to detect of toxin genes in 88 chickens with necrotic enteritis in Korea. Then was determined genetic diversity using pulsed-field gel electrophoresis in 17 dead chickens with necrotic enteritis. The results showed the rate of TpeL -positive strains from dead chicken was 2 of 18 and the rate of TpeL -positive strains from healthy chicken was 0 of 50. Therefore, there was no significant difference between dead chicken and healthy chickens. Furthermore 3 genetic cluster were identified which is showed that high genetic diversity among strains (Park et al., 2015).

In 2015, the study was performed to detect of *TpeL* toxin genes in 22, 10 and 10 isolate of *C. perfringens* type A, B and C, respectively. Totally, *TpeL* gene was detected in 2, 8 and 8 case respectively. However, none of isolate of *C. perfringens* type D and E was positive for *TpeL* gene. This study reported that in 18 disease sample and 4 healthy samples from North America, all healthy cases were negative for the *TpeL* gene. The TpeL -positive type strains were related to avian necrotic enteritis. Also, the results of southern blot using a *TpeL* -specific probe confirmed the PCR results among all of the *C. perfringens* strains. On the other hand, comparison of different culture medium on TpeL production and release for *C. perfringens* type A and C was done using western blot with polyclonal antiserum. The results showed that the highest and lowest amount of this toxin was produced in TH and TGY medium, respectively. The TGY medium had a higher glucose concentration than TH medium. So glucose and sucrose have inhibitory effects on the production and release of TpeL toxin. In addition to, the effect of trypsin on this toxin was decreased the effects of TpeL toxin (similar to beta toxin) (Chen and McClane, 2015).

5. CONCLUSION

TpeL is one of the important virulence factors of *C. perfringens* type A, B and C, which participate to the development of various diseases, including necrotic intestine. *C. perfringens* strains which are carried of *TpeL* gene can lead to the mortality rate. Therefore, *TpeL* can enhance other virulence characteristics. This paper reviews the studies on molecular working, determination of the biotypes of *C. perfringens*, analyze for genetic diversity using PFGE. Furthermore, Immunoinformatics Analysis was performed on TpeL toxin. TpeL sequences were analyzed using bioinformatics software for production of protein with high immunogenicity. Different regions of the gene were selected, and evaluated for making the fusion protein. Fusion gene was synthesized and recombinant plasmid was transformed into the competent *E. coli* BL21 (DE3). Then fusion protein was purified by Ni-NTA, SDS-PAGE and western blotting was used for confirmation. Finally, Serum neutralization test was used for measuring of immunity in rabbit. The results of this study reported that chimeric vaccine could be used as suitable vaccine against avian necrotic enteritis.

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