Nontyping virulence factors of *Clostridium perfringens*

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Abstract

The anaerobic, rod-shaped, spore-forming bacterium *Clostridium perfringens* is known for its production of biochemically active substances. Most of them are proteins that have a pathogenic effect on a wide range of animal tissues and cause a specific syndrome or even a disease in humans or animals. Production of toxins is used to classify isolates of *C. perfringens* into 7 different toxin types (A–G). Other virulence factors (i.e. beta2-toxin, BEC toxin, sialidases, hyaluronidase etc.) only indirectly or partially participate in the development of the disease, and the function of some substances has not been fully elucidated. The article summarizes basic data on the non-typing virulence factors of *C. perfringens*.

Perfringolysin O, beta2-toxin, plasmid, sialidase, BEC, disease

Clostridium perfringens is a Gram positive, spore-forming, anaerobic, rod-shaped bacterium. This pathogen is associated with diverse environments including soils, sewage, food, and is a member of the gastrointestinal tract microbiota of both diseased, and non-diseased humans and animals (Kiu and Hall 2018).

The key feature of human and animal diseases is that they are mediated by the production of potent protein toxins, most of which are extracellular (Rood et al. 2018). There is a growing number of characterized virulence factors; to date, more than 20 toxins and hydrolytic enzymes have been identified (Rood et al. 2018; Abdelrahim et al. 2019). The virulence factors of *C. perfringens* can be classified according to their function as pore-forming toxins, intracellular toxins, membrane damaging enzymes, and hydrolytic enzymes (Abdelrahim et al. 2019; Revitt-Mills et al. 2019). The presence or absence of six major toxins is used for classification of *C. perfringens* isolates into seven different toxin types, A–G (Table 1) (Lacey et al. 2019).

Most of the toxins and hydrolytic enzymes produced by *C. perfringens* are encoded on large plasmids; some genes encoding virulence factors may be located on the chromosome. Almost all toxin plasmids and some tetracycline resistance plasmids are conjugative (Rood et al. 2018; Abdelrahim et al. 2019; Rewit-Mills et al. 2019). Therefore, the *C. perfringens* toxinotyping scheme is plasmid-based (Rood et al. 2018). By studying 464 genomes of *C. perfringens,* all the toxinotypes and a broad host range included, it was found that commensal strains are not phylogenetically distinct from pathogenic strains, and differ only in plasmid carriage (Gulliver et al. 2023).

Virulence factors located on the variable region of the chromosome include the *cpa* gene (encoding α-toxin), *colA* (κ-toxin), *nagH* (hyaluronidase or μ-toxin) and *pfoA* (θ-toxin). The *nanH*, *nanI*, and *nanJ* genes encoding different sialidases, and *cadA* encoding υ-toxin are located on a conserved region of the chromosome. The genes *cpb* (encoding β-toxin), *cpb2* (β2-toxin), *etx* (ε-toxin), *iap/ibp* (ι-toxin), *netB* (NetB toxin), *tpeL* (TpeL toxin), *ureABC* (ureases), *cpd* (δ-toxin), *lam* (λ-toxin), and *becA/becB* encoding the binary enterotoxin for *C. perfringens* are located on large plasmids of variable size ranging from 65 to 110 kb.

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Table 1. Toxins of *Clostridium perfringens*, gene location and mechanism of action.

	Toxin/Enzyme	Gene	Mechanism of action
Typing toxins	CPA toxin; α-toxin; alpha-toxin	*plc* or *cpa*	Membrane disrupting toxin
	CPB toxin; β-toxin; beta-toxin	*cpb*	Pore-forming toxin
	ETX toxin; ε-toxin; epsilon-toxin	*etx*	Pore-forming toxin
	ITX toxin; ι-toxin; iota-toxin	*iap; ibp*	Cytoskeleton disrupting toxin
	CPE toxin; enterotoxin	*cpe*	Pore-forming toxin; tight junction disintegrating toxin
	NetB toxin	*netB*	Pore-forming toxin
Other virulence factors	CPB2 toxin; β2-toxin; beta2-toxin	*cpb2*	Pore-forming toxin
	Theta-toxin; PFO toxin; perfringolysin O; θ-toxin	*pfoA*	Pore-forming toxin, cholesterol-dependent cytolysin
	Delta-toxin; δ-toxin	*cpd*	Pore-forming toxin
	BEC toxin; binary enterotoxin	*becA; becB*	Pore-forming toxin
	NetE	*netE*	Pore-forming toxin
	NetF	*netF*	Pore-forming toxin
	NetG	*netG*	Pore-forming toxin
	Sialidase	*nanH*	Mucolytic enzyme
	Sialidase	*nanI*	Mucolytic enzyme
	Sialidase	*nanJ*	Mucolytic enzyme
	Hyaluronidase; μ-toxin; Mu-toxin	*nagH*	Matrix-lysing enzyme
	TpeL toxin	*tpeL*	Apoptosis induction
	Lambda-toxin; λ-toxin	*lam*	Protease
	α-clostripain	*ccp*	Collagenase; cystein protease
	Kappa-toxin; κ-toxin	*colA*	Collagenase

Recently, three novel putative toxin genes encoding proteins related to the pore-forming leukocidin/haemolysin family were revealed. These putative genes were designated *netE*, *netF*, and *netG*. Whereas *netE* and *netF* are located together on a large conjugative plasmid, *netG* is located separately on another large plasmid. The *C. perfringens* enterotoxin gene (*cpe*) can be either chromosome or plasmid-located. A combination of chromosomal and plasmid-borne *cpe* gene has not yet been observed in any isolate (Mehdizadeh Gohari et al. 2016; Abdelrahim et al. 2019).

The emergence and course of the disease is influenced by the great resistance of *C. perfringens* spores, which survive for a long time in the soil, the external environment and on surfaces, and show significant resistance to physico-chemical factors such as the temperature. Spores can contaminate wounds or be ingested together with food/feed and germinate in the intestine, where the bacteria rapidly multiply into infectious doses. Considerable aerotolerance allows *C. perfringens* to survive even in an environment with the presence of oxygen. The progressive and very serious course of some infections (avian necrotic enteritis, bovine necro-haemorrhagic enteritis and gas gangrene) is primarily due to the rapid proliferation of vegetative cells. *Clostridium perfringens* is one of the fastest growing bacteria, at 37 °C the generation time in optimal media is 12–17 min (Kiu and Hall 2018). Several toxins are produced during *C. perfringens* proliferation, resulting in disease onset; the mechanisms and stimuli of toxin production are not fully understood (Verherstraeten et al. 2015). Most toxins are maximally expressed within ~10 h, i.e., during late-log or early-stationary growth phases (Chen and McClane 2015).

The overall pathogenic effect of *C. perfringens* is not caused by the toxins alone but rather by a complex action. In the intestine, virulence factors act in individual layers. Spore germination, rapid proliferation of vegetative cells and colonization of the intestine occur in the intestinal lumen. Mucolytic enzymes (sialidases) help the entry of bacteria into the enterocytes by dissolving the intestinal mucus layer. In the intestinal epithelium, both main and secondary virulence factors (membrane disrupting toxins, pore forming toxins, cytoskeleton disrupting toxins) act, thereby enabling the penetration of bacteria up to the basement membrane and lamina propria. Enterotoxin, collagenase, and matrix-lysing enzymes act pathogenically at this level (Kiu and Hall 2018).

Toxin typing is very important for estimation of the pathogenic potential, as some toxins are strongly associated with disease in certain animal hosts, such as NetB (type G) and necrotic enteritis in chickens, and enterotoxin (type F) in food poisoning. However, toxin typing based on the six major toxins does not account for the full toxigenic potential that a strain may be capable of producing and therefore lacks the high resolution provided by whole genome sequencing (WGS) (Lacey et al. 2019).

The aim of this study was to summarize basic data about non-typing virulence factors of *C. perfringens*.

Beta2-toxin

Beta2-toxin (β2-toxin, CPB2 toxin), a cytolytic pore-forming toxin shares < 15% sequence homology with β-toxin, and is considered as a novel toxin produced by *C. perfringens* (Kiu and Hall 2018). Although genetically distinct, the CPB2 shows cytotoxic activity similar to that of *C. perfringens* β-toxin (CPB; also called beta-1 toxin) (Simpson et al. 2018). CPB2 toxin is expressed as a 31 kDa prototoxin that is subsequently cleaved during secretion into the mature 28 kDa toxin. In *C. perfringens* strains, CPB2 toxin is encoded on plasmids ranging in size from 45–97 kb. This toxin is active *in vitro*, causing cell rounding and death of both CHO and I407 cell lines at CPB2 toxin concentrations > 20 μg/ml (Freedman et al. 2015). It is not clearly known how CPB2 toxin contributes to pathogenesis, but it has been proven that CPB2 toxin is able to form highly cation-selective channels in lipid bilayers (Benz et al. 2022) and to induce apoptosis and inflammatory response in intestinal porcine epithelial (JPEC-J2) cells (Gao et al. 2020). Many type A strains encode CPB2 toxin; the *cpb2* gene has also been found in types B, C, D and E of *C. perfringens* isolates (Freedman et al. 2015).

The role of CPB2 toxin in pathogenesis of clostridium-related diseases has not been entirely understood, although some researches discussed the correlation between the presence of CPB2 toxin and the disease (Fahimeh et al. 2018). The CPB2 toxin was detected with high frequency among strains from all sources except those from canine and ovine sources (Park and Rafii 2019). Many authors (Finegold et al. 2017; Fahimeh et al. 2018; Kiu and Hall 2018; Park and Rafii 2019) published data about the existence of CPB2 toxin producing *C. perfringens* strains which are associated with gut diseases such as necrotic enteritis in piglets, and enterocolitis in foals and calves. The study by Park and Rafii (2019) showed a higher prevalence of the *cpb2* gene in diseased swine and chickens than in strains from other animals, but no information was available on the health status of the chickens.

Clostridium perfringens isolates carrying the *cpb2* gene have been described in human gastrointestinal diseases, including food poisoning (FP), sporadic diarrhoea (SD), and antibiotic associated diarrhoea (AAD). The *Cpb2* gene was also found in higher extent in *C. perfringens* strains isolated from autistic children (Finegold et al. 2017). As described by Kiu and Hall (2018) CPB2 toxin plays an important role in pre-term necrotic enteritis in potential synergistic effects with aminoglycoside antibiotic gentamicin. Other studies

are also required to determine whether the strains identified as positive for the *cpb2* toxin genes are capable of producing a biologically active toxin (Finegold et al. 2017).

Perfringolysin O, theta-toxin

Perfringolysin O (PFO; also θ-toxin), encoded by gene *pfoA* or *pfo*, is a pore-forming toxin and a member of the cholesterol dependent cytolysins (Bryant et al. 2015; Kiu and Hall 2018). PFO shares structural homology with similar pore-forming toxins identified in *Streptococcus*, *Bacillus*, *Listeria* and many other genera (Kiu and Hall 2018). The common feature of this group of toxins is that they specifically interact with cholesterol-containing membranes (Kulma et al. 2017). PFO is secreted as a water-soluble monomer that binds to membranes via cholesterol. PFO is expressed in nearly all *C. perfringens* strains and has interesting properties that suggest a potential undefined role for PFO in disease development. The *pfoA* gene is suspected to be encoded by nearly all *C. perfringens* strains, although genome comparisons revealed that most of the strains responsible for food poisoning lack *pfoA* (Verherstraeten et al. 2015). PFO is also known for its capacity to induce tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) expression in the host, and it could activate apoptosis through p38 MAPK (mitogen-activated protein kinase) pathway as demonstrated in *in vitro* models (Kiu and Hall 2018). PFO causes macrophage cytotoxicity in the early stages of myonecrosis and is important for thrombus formation in the later stages of infection. The latter is caused by effects on the expression of adhesion factors and chemokines by endothelial cells and leukocytes (Verherstraeten et al. 2015). Although PFO can lyse mammalian cells, genetic studies have shown that PFO by itself is not essential in causing mortality since an insertionally inactivated chromosomal structural gene (*pfoA*) mutant is still lethal in the mouse myonecrosis model (Bryant et al. 2015). This toxin has been shown to be involved in the pathogenesis of *C. perfringens* myonecrosis (also called gas gangrene), and haemorrhagic enteritis in calves (Bryant et al. 2015; Kiu and Hall 2018). Clostridial myonecrosis is reported in several animal species, such as dogs, cats, cattle, sheep, goats and horses, but also occurs in humans. The disease is characterized by rapid spreading of tissue necrosis within the muscle (thus the term myonecrosis), which can lead to death caused by systemic toxaemia and shock, with very high mortality rates, if not promptly treated. The role of PFO in gas gangrene is less elucidated. It was suggested that PFO works synergistically with α-toxin to effect peripheral to myonecrosis leukostasis and intravascular coagulopathy, whereas the majority of the myonecrosis can be attributed to α-toxin alone. The involvement of PFO in the pathogenesis of bovine necrohaemorrhagic enteritis or enterotoxaemia has been demonstrated due to synergistic cytotoxic effects with α-toxin on bovine epithelial cells. Necrohaemorrhagic enteritis is a major cause of mortality in calves (Verherstraeten et al. 2015; Kiu and Hall 2018). PFO is able to induce a potent proinflammatory cytokine production and since human erythrocyte haemolysis has been found to be correlated with PFO, but not with α-toxin concentration, PFO has been suggested a major virulence factor of human sepsis with massive intravascular haemolysis (Suzaki et al. 2021).

Delta-toxin

Delta-toxin (δ-toxin) is a β-pore-forming-toxin (β-PFT) produced by *C. perfringens* strains B and C. *Clostridium perfringens* type B and type C strains cause necrotizing enteritis in domestic animals and humans. The role of δ-toxin in infectious disease has remained poorly characterized but it is thought that there may be a synergistic effect between δ- and β toxins of *C. perfringens* (Rewitt-Mills et al. 2019; Seike et al. 2019). Not much information regarding the genetics of δ-toxin is currently available. Delta-

toxin is encoded by the *cpd* gene which is likely carried on a plasmid (Rewitt-Mills et al. 2019). However, in one type A strain the *cpd* gene was found located chromosomally, indicating that it may be present on a chromosomally inserted, transposable element (Gulliver et al. 2023). The toxin has been assigned to the β-PFT family, including also β-toxin and NetB toxin from *C. perfringens* and α-toxin from *Staphylococcus aureus*. Delta-toxin forms mushroom-shaped heptameric pores in eukaryotic cell membranes and has the same mechanism of action as α-toxin of *S. aureus* (Seike et al. 2019). It induces rapid necrosis via pore formation in the lipid rafts of sensitive cells. The toxin is lethal for mice and cytotoxic to many eukaryotic cells, including rabbit macrophages, human monocytes and platelets derived from humans, guinea pigs, rabbits and goats. The study by Seike et al. (2019) demonstrated that δ-toxin has enterotoxic activity. The toxin induces elevation of intestinal permeability which is accompanied by a histological change in the intestinal epithelia. Delta-toxin at a dose of 250–500 ng causes villus shortening coincident with the histological damage. Villus shortening is associated with the shedding of intestinal epithelial cells. Moreover, the toxin impaired permeabilization of mitochondrial membranes and the release of cytochrome C. The study by Seike et al. (2019) reports that δ-toxin caused more damaging efects than β-toxin and plays significant role in necrotic enteritis in humans.

BEC toxin

Binary enterotoxin of *C. perfringens* (BEC) also named as *C. perfringens* iota-like enterotoxin (CPILE) is a novel toxin that causes acute gastroenteritis in humans (Freedman et al. 2015; Matsuda et al. 2019). Genome sequence analysis revealed that BEC is comprised of two proteins, BECa (~47 kDa) and BECb (~80 kDa). BEC has meaningful sequence homology with members of the binary ADP-ribosylating toxin (ADPRT)-family proteins that also includes *C. perfringens* iota-toxin, *Clostridium spiroforme* CST toxin, *Clostridioides difficile* CDT toxin, *Clostridium botulinum* C2 toxin and vegetative insecticidal protein (VIP) from *Bacillus cereus* (Kawahara et al. 2016; Yonogi et al. 2016). BEC was found to be encoded on large plasmids of ~54 kb suggesting its potential for horizontal gene transfer (Freedman et al. 2015; Kiu et al. 2019). BEC consists of two proteins, of which the smaller component (BECa) has ADP-ribosylating activity on actin and BECb causes fluid accumulation in tissues (Matsuda et al. 2019). More recently it was reported that BEC was linked to food poisoning outbreaks in Japan, with its enterotoxic activity confirmed experimentally. This toxin was identified in *C. perfringens* strains lacking the *cpe* gene (Kiu et al, 2019; Matsuda et al. 2019). Little is known about the prevalence of BEC in *C. perfringens* isolates, but it seems that BEC positive strains are rare (Rood et al. 2018). A survey made by Matsuda et al. (2019) indicated 0.78% positivity of BEC among isolates from diarrhoeal patients, which was much lower than that of *cpe* (7.8%). Similarly, a more recent survey of Aung et al. (2021) reported the *bec/cpile* gene positivity in 0.17% out of 585 clinical specimens.

NetE, NetF, NetG

Recently, three novel putative toxin genes encoding proteins related to the pore-forming leukocidin/haemolysin superfamily were identified. These were designated *netE*, *netF*, and *netG*, but only *netF* was associated with the cytotoxicity (Mehdizadeh Gohari et al. 2015; Mehdizadeh Gohari et al. 2016). The *netE* and *netF* genes were found on a large *tcp*-conjugative plasmid, and netG was based on another large *tcp*-conjugative plasmid (Sindern et al. 2019). NetF is an extracellular β-pore-forming toxin that belongs to the same toxin superfamily as netB, β-toxin and *C. perfringens* δ-toxin.

NetEF-positive strains also harbour a plasmid that encodes CPE and β2-toxin and a proportion also carry a netG plasmid (Rood et al. 2018). NetF has been implicated as the primary virulence factor of foal necrotizing enteritis and canine haemorrhagic gastroenteritis. *Clostridium perfringens* strains encoding for *netE* and *netF* genes may play a role in dogs with acute haemorrhagic diarrhoea syndrome (AHDS). Almost 75% of isolates from foal necrotizing enteritis and canine AHDS were NetF-positive, whereas the isolates from undifferentiated diarrhoeal disease were mostly negative. Possible synergism between NetF and CPE was suggested (Mehdizadeh Gohari et al. 2015). Similarly, significantly higher prevalence of *netE* and *netF* genes was found in AHDS in comparison to healthy dogs; however, no differences in duration of hospitalization, time to recovery, or outcome were found between NetF-positive and NetF-negative dogs with AHDS (Sindern et al. 2019).

Sialidases

Clostridium perfringens can produce many mucin-degrading enzymes which include sialidases. These enzymes cleave terminal sialic acids from sugar chains of glycoproteins, glycolipids, oligosaccharides, gangliosides and other sialoglycoconjugates. Sialic acids are especially abundant in the intestinal tract, where they are major constituents of mucins (Goossens et al. 2017). Thus sialidases may possibly contribute to pathogenesis of necrotic enteritidis by degradation of the intestinal mucus layer (Van Damme et al. 2022). Sialic acids are also important components of the serum and represent the terminal sugar residue of many glycan chains on host cell surfaces, where they are involved in cell-cell recognition. Sialic acids can also stabilize enzymes or cell membrane proteins and can mediate binding and transport of positively-charged molecules. Sialidases, also referred to as neuraminidases, are key enzymes that hydrolyze the linkage of terminal sialic acids on various sialoglycoconjugates to generate free sialic acid (Li et al. 2016). It has been proven that at least some pathogenic strains of *C. perfringens* are able to use sialic acid as a carbon source for bacterial growth (Van Damme et al. 2022). Sialidases are produced by some viruses, microorganisms, and vertebrates, but cannot be found in plants (Li et al. 2016). In *C. perfringens* three sialidase enzymes have been reported, the large exo-sialidases NanI and NanJ, and a smaller intracellular NanH enzyme (Goossens et al. 2017; Kiu and Hall 2018). NanH (43 kDa) lacks a secretion signal peptide and thus has a cytoplasmic location in log-phase cultures. In contrast, NanI (77 kDa) and NanJ (129 kDa) are secreted exosialidases. Majority of *C. perfringens* strains produce all three sialidases, with NanI usually being responsible for most of the sialidase activity in culture supernatants of those *C. perfringens* strains (Li et al. 2016). Recently, 372 *C. perfringens* genomes from multiple locations and sources were studied and the *nanH* gene was found at 99% of the genomes (Camargo et al. 2022). Interestingly, the *nanI* gene is consistently absent from the type F *C. perfringens* strains carrying a chromosomal *cpe* gene, as well as the genetically related C Darmbrand strains (Li et al. 2016). Some *C. perfringens* strains produce only one or two of the three sialidases, NanJ being the least common (Camargo et al. 2022). For strains producing all three sialidases, NanI is usually responsible for ~70% of total exosialidase activity. The genes encoding the three *C. perfringens* sialidases are located on chromosome, although in different regions (Li et al. 2016). *In vitro* studies have also demonstrated that α-toxin associated with NanI (exoalpha-sialidase) increased the virulence of *C. perfringens*. Furthermore, NanI was shown to potentiate the virulence of ε-toxin, β-toxin and CPE, via binding-enhancing (ε-toxin) and proteolytic activation (β-toxin and CPE) mechanisms, potentially enhancing *C. perfringens* pathogenesis. However, in a gas gangrene mouse model, NanI and NanJ were not essential for virulence (Kiu and Hall 2018).

Hyaluronidases

Hyaluronate can form highly viscous solutions and is a major constituent of the extracellular matrix, especially in soft connective tissues. The viscous consistency usually provides resistance to penetration of infectious agents and their extracellular products (Goossens et al. 2017). The ultimate products of hyaluronidase degradation of hyaluronate are disaccharides. These disaccharides can be utilized as nutrients for a pathogen as it replicates and spreads (Hynes and Walton 2000). Hyaluronidases are produced by a number of bacteria that cause infections at mucosal surfaces. In *C. perfringens* 5 hyaluronidase genes are described (*nagH, nagI, nagJ, nagK* and *nagL*), which encode secreted enzymes. Not much research has been done on the *C. perfringens* hyaluronidases. The best studied enzyme is μ-toxin or NagH. By itself, μ-toxin is a non-lethal toxin but it is thought to contribute to the pathogenesis of *C. perfringens* infections through the degradation of mucins and connective tissue (Goossens et al. 2017). Thanks to the decreased viscosity, the pathogen is allowed to spread, although the magnitude of such an effect on proliferation is currently unknown. Secondly, the clostridial hyaluronidase may degrade hyaluronate cell surface coatings, thereby allowing direct contact between the bacterium and specific cell surface receptors (Hynes and Walton 2000). Furthermore, μ-toxin facilitates the spread of α-toxin, thereby potentiating its activity (Goossens et al. 2017).

TpeL toxin

TpeL, a recently discovered toxin produced by some *C. perfringens* strains, is a member of the large clostridial toxin (LCT) family. Other LCTs include toxins A (TcdA) and B (TcdB) from *Clostridioides difficile*, the haemorrhagic toxin TcsH and the lethal toxin TcsL from *C. sordellii*, and alpha-toxin (TcnA) from *C. novyi* (Chen and McClane 2015). This toxin group mediate cytotoxic effects through the glycosylation of host cell proteins (Revitt-Mills et al. 2015). The LCT family includes proteins ranging in size from ~195 to 310 kDa with 36% to 90% identical sequences of primary amino acids. TpeL is the largest toxin among the >17 toxins produced by *C. perfringens*, with a typical size of ~205 kDa (Chen and McClane 2015). The *tpeL* toxin gene is located on a plasmid; it was detected from isolates from all sources, but in general the frequency of *tpeL* was higher in strains isolated from animals and soil. The *tpeL* toxin gene was detected only in a few strains isolated from foods and humans (9.5% and 11%, respectively) (Park and Rafii 2019). In a recent study, the *tpeL* gene was not detected in type D or F isolates carrying the *cpe* and *itx* toxin genes. It has been suggested that plasmids carrying these genes may be potentially incompatible (Camargo et al. 2022). TpeL displays obvious cytotoxicity in Vero cells. The cytopathic effect is characterized by the enlargement of cells and appearance of rounded cells (Nagahama et al. 2015). TpeL production and release by early TpeL-producing strains, as well as production of alpha-toxin and perfringolysin O, appears to be inhibited by the presence of glucose and sucrose. TpeL is trypsin sensitive (Chen and McClane 2015).

There is no definitive evidence that TpeL is involved in disease; however, it is postulated that this toxin may make a contribution to virulence (Revitt-Mills et al. 2015). *Clostridium perfringens* carrying the *tpeL* gene were highly virulent for chickens (Park and Rafii 2019). The study of Chen and McClane (2015) surveyed the presence of the *tpeL* gene in wild-type strains, including representatives of all *C. perfringens* toxigenic types. TpeL-positive strains often are associated with avian necrotic enteritis, although the NetB toxin is considered to play a major role in pathogenesis. The presence of TpeL-encoding plasmid may increase the severity of necrotic enteritidis in broilers but the evidence is circumstantial (Chen and McClane 2015; Lacey et al. 2016).

Lambda-toxin

Lambda-toxin (λ-toxin) is a ~35 kDa thermolysin-like zinc metalloprotease produced by *C. perfringens*, specifically by some type B, D and E strains. Enzyme activity was detectable over a broad range of pH 5 to 8, and the pH for optimum activity was 7.5. Lambda toxin is sensitive to chelating agents (Harkness et al. 2012). The *lam* gene is located on a plasmid. Included in the thermolysin family are metalloproteases of pathogenic bacteria, which have been suggested to play roles in their virulence (Jin et al. 1996). Lambda-toxin contributes to the pathogenicity by degrading certain protein components of host cells (e.g. components of connective tissue and host defense system cells) (Minami et al. 1997; Harkness et al. 2012). *In vitro*, purified lambda-toxin can cleave a variety of biologically important substances, such as immunoglobulins, the complement C3 component, fibrinogen, various collagens, and fibronectin (Jin et al. 1996). Lambda-toxin activates epsilon-prototoxin in the same manner as trypsin does, but to a greater extent than trypsin, and to almost the same extent as trypsin plus chymotrypsin (Minami et al. 1997).

Alpha-clostripain

Alpha-clostripain is a cysteine endopeptidase produced by *Clostridium histolyticum*, one of the histolytic clostridia causing fulminant clostridial myonecrosis (Manabe et al. 2010). The homologue protease was later identified in *C. perfringens*. Both the *C. perfringens* and *C. histolyticum* enzymes are heterodimeric and consist of two polypeptide chains, where the heavy and light chains are held together by strong, non-covalent forces. They are encoded by a single gene (Chakravorty et al. 2011). This gene (*ccp*) was found in 99% out of 372 *C. perfringens* genomes (Camargo et al. 2022). The expression of α-clostripain is regulated by the two-component system VirR/VirS, like that of other virulence factors (perfringolysin O, α-toxin and collagenase) (Manabe et al. 2010; Chakravorty et al. 2011). Functionally, α-clostripains cleave peptide bond of arginine; the maximum enzymatic activity is in the presence of calcium and under reducing conditions. Clostripain of *C. perfringens* (Clp) exhibits stronger peptidase activity toward natural protein substrates than clostripain of *C. histolyticum* (Clo). Thus Clp possesses vascular permeability enhancement activity. The involvement of these clostridial proteases in myonecrosis has remained unknown; α-clostripain is not necessary for development of disease (Chakravorty et al. 2011). The caseinolytic activity of Clp is 2.3-fold higher than that of Clo, as Clp can also slightly hydrolyse the peptide bond of lysin. Clp increased vascular permeability in a mouse model after subcutaneous aplication, thus may contribute to pathogenesis of clostridial myonecrosis (Manabe et al. 2010). Alpha-clostripain with its proteolytic activity affects the processing and degradation of other extracellular proteins produced by *C. perfringens* (Chakravorty et al. 2011).

Microbial collagenase

Microbial collagenase (also known as kappa-toxin; κ-toxin) is a key toxin produced by *C. perfringens* that hydrolyses collagen (Kiu and Hall 2018). The chromosomal *colA* gene coding this enzyme was one of the most frequent virulence factors, present in 98% out of 372 *C. perfringens* genomes (Camargo et al. 2022). Collagen disruption by bacterial collagenases may result in the loss of tissue integrity and subsequent tissue necrosis. *Clostridium perfringens* can produce various collagenolytic enzymes with molecular masses ranging from ~80 to ~120 kDa. Collagenases could play a role in clostridial virulence in terms of spreading toxins and bacterial cells to host tissue and in tissue necrosis. The role of *C. perfringens* collagenases in intestinal diseases is not yet explored

(Goossens et al. 2017). Kappa-toxin does not play an important role in a clostridial myonecrosis mouse model (gas gangrene), despite its capacity to degrade collagen (Kiu and Hall 2018). Collagenases are likely involved in various phases of necro-haemorrhagic enteritis: its action on the basal membrane might induce epithelial sloughing, and later it may degrade the collagen to cause massive tissue necrosis; moreover, it plays a role in development of haemorrhagic lessions. Haemorrhagic and dermonecrotic activities with extensive connective tissue destruction were reported by Goossens et al. (2017).

Alveolysin

In addition to well described toxins, Kiu et al. (2019) introduced alveolysin, a thiol-activated (cholesterol-binding) pore-forming toxin first identified in *Paenibacillus alvei* (a beehive-colonising bacterium). This toxin is able to damage cell membranes in a way similar to the homologous thiol-activated toxin perfringolysin O, which is typically produced by *C. perfringens*. Sequence analysis revealed that there are sequence homologies common to both alveolysin and other pore-forming toxins secreted by other Gram-positive bacteria, e.g. listeriolysin O, pneumolysin and streptolysin O. Despite the potential significance of alveolysin in *C. perfringens*, this toxin has received very limited research attention so far. Its connection with canine/foal enteritis, caused by both D and E types of *C. perfringens*, emphasizes the need for further research, particularly in relation to intestinal diseases in domestic animals (Kiu et al. 2019).

Conclusion

The six major toxins used for toxin typing of *C. perfringens* represent but a small part of virulence factors contributing to the complex pathogenesis. This review aims to summarize the knowledge on the most important non-typing representatives of more than 20 toxins and hydrolytic enzymes that have been identified up to date. The exact role in pathogenesis and specific mode of action of some of the toxins and enzymes have not been fully elucidated yet. As majority of *C. perfringens* toxin genes are located on conjugative plasmids, these virulence genes may be disseminated via horizontal gene transfer. A recent extensive genome analysis revealed that *C. perfringens* isolates usually harbour two plasmids, but presence of up to ten plasmids per isolate is possible. The authors studied in detail 55 plasmids encoding 2,852 genes, of which at least 1,162 (40.7%) were hypothetical or had an unknown function (Gulliver et al. 2023). This leaves much room for further studies on possible virulence factors, the coding genes and their dissemination within the *C. perfringens* strains and between related species.

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References

Abdelrahim MA, Radomski N, Delannoy S, Djellal S, Le Négrate M, Hadjab K, Fach P, Hennekinne JA, Mistou MY, Firmesse O 2019: Large-Scale genomic analyses and toxinotyping of *Clostridium perfringens* implicated in foodborne outbreaks in France. Front Microbiol **10**: 777

Aung MS, Matsuda A, Urushibara N, Kawaguchiya M, Ohashi N, Matsuda N, Nakamura M, Ito M, Habadera S, Matsumoto A, Hirose M, Kobayashi N 2021: Clonal diversity of *Clostridium perfringens* human clinical isolates with various toxin gene profiles based on multilocus sequence typing and alpha-toxin (PLC) typing. Anaerobe **72**: 102473

Benz R, Piselli C, Hoxha C, Koy C, Glocker MO, Popoff MR 2022: *Clostridium perfringens* Beta2 toxin forms highly cation-selective channels in lipid bilayers. Eur Biophys J **51**: 15-27

Bryant A, Aldape MJ, Stevens DL 2015: *Clostridium Perfringens* and Other Life-Threatening Clostridial Soft Tissue Infections. In: Tang Y-W, Sussman M, Jiu D, Poxton I, Schwartzman D (eds): Molecular Medical Microbiology, second edition, Academic Press, London, UK, pp 899-907

Camargo A, Guerrero-Araya E, Castañeda S, Vega L, Cardenas-Alvarez MX, Rodríguez C, Paredes-Sabja D, Ramírez JD, Muñoz M 2022: Intra-species diversity of *Clostridium perfringens*: A diverse genetic repertoire reveals its pathogenic potential. Front Microbiol **13**: 952081

Chakravorty A, Awad MM, Hiscox TJ, Cheung JK, Carter GP, Choo JM, Lyras D, Rood JI 2011: The cysteine protease α-clostripain is not essential for the pathogenesis of *Clostridium perfringens*-mediated myonecrosis. PloS ONE **6**: e22762

Chen J, McClane BA 2015: Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. Infect Immun **83**: 2369-2381

Fahimeh Y, Peyman N, Gholamreza H, Gholamali K, Mohammad R, Jamshid R 2018: Major and minor toxins of *Clostridium perfringens* isolated from healthy and diseased sheep. Small Rumin Res **168**: 1-5

Finegold SM, Summanen PH, Downes J, Corbett K, Komoriya T 2017: Detection of *Clostridium perfringens* toxin genes in the gut microbiota of autistic children. Anaerobe **45**: 133-137

Freedman JC, Theoret JR, Wisniewski JA, Uzal FA, Rood JI, McClane BA 2015: *Clostridium perfringens* type A–E toxin plasmids. Res Microbiol **166**: 264-279

Gao X, Yang Q, Huang X, Yan Z, Zhang S, Luo R, Wang P, Wang W, Xie K, Jiang T, Gun S 2020: Effects of *Clostridium perfringens* beta2 toxin on apoptosis, inflammation, and barrier function of intestinal porcine epithelial cells. Microb Pathog **147**: 104379

Goossens E, Valgaeren BR, Pardon B, Haesebrouck F, Ducatelle R, Deprez PR, Van Immerseel F 2017: Rethinking the role of alpha toxin in *Clostridium perfringens*-associated enteric diseases: a review on bovine necro-haemorrhagic enteritis. Vet Res **48**: 9-26

Gulliver EL, Adams V, Marcelino VR, Gould J, Rutten EL, Powell DR, Young RB, D'Adamo GL, Hemphill J, Solari SM, Revitt-Mills SA, Munn S, Jirapanjawat T, Greening C, Boer JC, Flanagan KL, Kaldhusdal M, Plebanski M, Gibney KB, Moore RJ, Rood JI, Forster SC 2023: Extensive genome analysis identifies novel plasmid families in *Clostridium perfringens*. Microb Genom **9**: 000995

Harkness JM, Li J, McClane BA 2012: Identification of a lambda toxin-negative *Clostridium perfringens* strain that processes and activates epsilon prototoxin intracellularly. Anaerobe **18**: 546-552

Hynes WL, Walton SL 2000: Hyaluronidases of Gram-positive bacteria. FEMS Microbiol Lett **183**: 201-207

Jin F, Matsushita O, Katayama S, Jin S, Matsushita C, Minami J, Okabe A 1996: Purification, characterization, and primary structure of *Clostridium perfringens* lambda-toxin, a thermolysin-like metalloprotease. Infect Immun **64**: 230-237

Kawahara K, Yonogi S, Munetomo R, Oki H, Yoshida T, Kumeda Y, Matsuda S, Kodama T, Ohkubo T, Iida T, Nakamura S 2016: Crystal structure of the ADP-ribosylating component of BEC, the binary enterotoxin of *Clostridium perfringens*. Biochem Biophys Res Commun **480**: 261-267

Kiu R, Hall LJ 2018: An update on the human and animal enteric pathogen *Clostridium perfringens*. Emerg Microbes Infect **7**: 1-15

Kiu R, Sim K, Shaw A, Cornwell E, Pickard D, Kroll JS, Hall LJ 2019: Genomic analysis of *Clostridium perfringens* BEC/CPILE-positive, toxinotype D and E strains isolated from healthy children. Toxins **11**:543-557

Kulma M, Kacprzyk-Stokowiec A, Kwiatkowska K, Traczyk G, Sobota A, Dadlez M 2017: R468A mutation in perfringolysin O destabilizes toxin structure and induces membrane fusion. Biochim Biophys Acta Biomembr **1859**: 1075-1088

Lacey JA, Johanesen PA, Lyras D, Moore RJ 2016: Genomic diversity of necrotic enteritis-associated strains of *Clostridium perfringens*: a review. Avian Pathol **45**: 302-307

Lacey JA, Johanesen PA, Lyras D, Moore RJ 2019: In silico identification of novel toxin homologs and associated mobile genetic elements in *Clostridium perfringens*. Pathogens **8**: 16

Li J, Uzal FA, McClane BA 2016: *Clostridium perfringens* Sialidases: potential contributors to intestinal pathogenesis and therapeutic targets. Toxins **8**: 341-356

Manabe S, Nariya H, Miyata S, Tanaka H, Minami J, Suzuki M, Taniguchi Y, Okabe A 2010: Purification and characterization of a clostripain-like protease from a recombinant *Clostridium perfringens* culture. Microbiol **156**: 561-569

Matsuda A, Aung MS, Urushibara N, Kawaguchiya M, Sumi A, Nakamura M, Horino Y, Ito M, Habadera S, Kobayashi N 2019: Prevalence and genetic diversity of toxin genes in clinical isolates of *Clostridium perfringens*: Coexistence of alpha-toxin variant and binary enterotoxin genes (*bec/cpile*). Toxins **11**: 326-341

Mehdizadeh Gohari I, Parreir, VR, Nowell VJ, Nicholson VM, Oliphant K, Prescott JF 2015: A novel pore-forming toxin in type A *Clostridium perfringens* is associated with both fatal canine hemorrhagic gastroenteritis and fatal foal necrotizing enterocolitis. PLoS One **10**: e0122684

Mehdizadeh Gohari I, Kropinski AM, Weese SJ, Parreira VR, Whitehead AE, Boerlin P, prescott JF 2016: Plasmid characterization and chromosome analysis of two netF+ *Clostridium perfringens* isolates associated with foal and canine necrotizing enteritis. PLoS ONE **11**: e0148344

Minami J, Katayama S, Matsushita O, Matsushita C, Okabe A 1997: Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N-and C-terminal peptides. Microbiol Immunol **41**: 527-535

Nagahama M, Oda M, Tsuge H, Kobayashi K 2015: Enteric Toxins of *Clostridium perfringens*: Beta Toxin, TpeL, Epsilon Toxin and Iota Toxin. In: Tang Y-W, Sussman M, Jiu D, Poxton I, Schwartzman D (Eds): Molecular Medical Microbiology, second edition, Academic Press, London, UK, pp. 997-1013

Park M, Rafii F 2019: The prevalence of plasmid-coded *cpe* enterotoxin, *b2* toxin, *tpeL* toxin, and tetracycline resistance in *Clostridium perfringens* strains isolated from different sources. Anaerobe **56**: 124-129

Revitt-Mills SA, Rood JI, Adams V 2015: *Clostridium perfringens* extracellular toxins and enzymes: 20 and counting. Microbiol Aust **36**: 114-117

Revitt-Mills SA, Vidor CJ, Watts TD, Lyras D, Rood JI, Adams V 2019: Virulence plasmids of the pathogenic *Clostridia*. Microbiol Spectr **7**: 10

Rood JI, Adams V, Lacey J, Lyras D, McClane B, Melville S, Van Immerseel F 2018: Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe **53**: 5-10

Seike S, Takehara M, Kobayashi K, Nagahama M 2019: *Clostridium perfringens* delta-toxin damages the mouse small intestine. Toxins **11**: 232

Simpson KM, Callan RJ, Van Metre, DC 2018: Clostridial abomasitis and enteritis in ruminants. Vet Clin Food Anim **34**: 155-184

Sindern N, Suchodolski JS, Leutenegger CM, Mehdizadeh Gohari I, Prescott JF, Proksch A-L, Mueller RS, Busch K, Unterer S 2019: Prevalence of *Clostridium perfringens* netE and netF toxin genes in the feces of dogs with acute hemorrhagic diarrhea syndrome. J Vet Intern Med **33**: 100-105

Suzaki A, Ohtani K, Komine-Aizawa S, Matsumoto A, Kamiya S, Hayakawa S 2021: Pathogenic characterization of *Clostridium perfringens* strains isolated from patients with massive intravascular hemolysis. Front Microbiol **12**: 713509

Van Damme L, Callens C, Dargatz M, Flügel M, Hark S, Thiemann F, Pelzer S, Ducatelle R, Van Immerseel F, Goossens E 2022: NanI sialidase contributes to toxin expression and host cell binding of *Clostridium perfringens* type G strain CP56 *in vitro*. Vet Microbiol **266**: 109371

Verherstraeten S, Goossens E, Valgaeren B, Pardon B, Timbermont L, Haesebrouck F, Ducatelle R, Deprez P, Wade KR, Tweten R, Van Immerseel F 2015: Perfringolysin O: The underrated *Clostridium perfringens* toxin? Toxins **7**: 1702-1721

Yonogi S, Kanki M, Ohnishi T, Shiono M, Iida T, Kumeda Y 2016: Development and application of a multiplex PCR assay for detection of the *Clostridium perfringens* enterotoxin-encoding genes *cpe* and *becAB*. J Microbiol Methods **127**: 172-175