REVIEW

Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease

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ABSTRACT: *Clostridium perfringens* uses its arsenal of >16 toxins to cause histotoxic and intestinal infections in humans and animals. It has been unclear why this bacterium produces so many different toxins, especially since many target the plasma membrane of host cells. However, it is now established that *C. perfringens* uses chromosomally encoded alpha toxin (a phospholipase C) and perfringolysin O (a pore-forming toxin) during histotoxic infections. In contrast, this bacterium causes intestinal disease by employing toxins encoded by mobile genetic elements, including *C. perfringens* enterotoxin, necrotic enteritis toxin B-like, epsilon toxin and beta toxin. Like perfringolysin O, the toxins with established roles in intestinal disease form membrane pores. However, the intestinal disease-associated toxins vary in their target specificity, when they are produced (sporulation vs vegetative growth), and in their sensitivity to intestinal proteases. Producing many toxins with diverse characteristics likely imparts virulence flexibility to *C. perfringens* so it can cause an array of diseases.

Background

Clostridium perfringens ranks among the most widespread bacteria, with an ubiquitous environmental distribution in soil, sewage, food, feces, and the normal intestinal flora of humans and animals [1]. However, this Gram-positive, anaerobic spore former is also one of the most common and important human and animal pathogens, causing a spectrum of diseases **(Table 1)**. For example, *C. perfringens* is the leading cause of traumatic gas gangrene [2]. It is also a major cause of foodborne illness, ranking as the second most common bacterial cause of food poisoning in the USA [3,4]. In addition, this bacterium is responsible for approximately 5–15% of all cases of antibiotic-associated diarrhea [5], which develops in 5–40% of all patients receiving antibiotic therapy [6]. It also causes an often-fatal human disease, enteritis necroticans [1]. As an animal pathogen, *C. perfringens* is responsible for several serious diseases, including avian necrotic enteritis, which drains approximately US$2 billion/year from the global agricultural system [7]. In addition, widespread vaccination is practiced to protect livestock from *C. perfringens*-induced enteritis and enterotoxemias, the latter characterized by intestinally produced toxins that are absorbed into the circulation and then affect other organs such as the brain [8].

The virulence of *C. perfringens* is mediated in large part by its intimidating toxin arsenal **(Table 2)**. Toxin production varies from strain to strain, permitting classification of *C. perfringens* isolates into five toxinotypes **(Table 3)** based upon the production of four typing toxins; that is,

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Table 1. Main diseases caused by the major toxins of *Clostridium perfringens*.		
Type of *Clostridium perfringens*[†]	Toxins produced	Most significant diseases[‡]
A	CPA[§] CPA, CPE[§] CPA, NetB[§]	Human and animal myonecrosis (gas gangrene) Human food poisoning and non-foodborne gastrointestinal disease; canine gastrointestinal disease Necrotic enteritis of poultry
B	CPA, CPB, ETX	Necrohemorrhagic enteritis of sheep (lamb dysentery)
C	CPA, CPB[§]	Human necrotic enteritis (enteritis necroticans, pigbel); necrotic enteritis of neonatal individuals of several animal species (horse, cattle, sheep, pigs)
D	CPA, ETX[§]	Enterotoxemia of sheep and goats
E	CPE, ITX	No known association with human disease; suspected, but no confirmed association with gastrointestinal disease of cattle, sheep and rabbits.

[†]All types of *Clostridium perfringens* may also produce several other toxins, including, but not limited to, CPB2, perfringolysin O and toxin perfringens large.
[‡]Only diseases that have been confirmed to be associated with each type of *C. perfringens*, and that are significant in terms of prevalence, are included in this table.
[§]Critical toxin for virulence. PFO also contributes to virulence during myonecrosis (see text).
CPA: *C. perfringens* alpha toxin; CPB: *C. perfringens* beta toxin; CPE: *C. perfringens* enterotoxin; ETX: *C. perfringens* epsilon toxin; ITX: *C. perfringens* iota toxin; NetB: Necrotic enteritis toxin B-like.
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alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX). Although historic and somewhat dated, this typing system is still useful since different toxinotypes, and often subtypes, of *C. perfringens* are associated with certain diseases **(Table 1)**.

For many years it remained unclear why *C. perfringens* produced so many different toxins, particularly since most of these toxins share a common target, that is, the plasma membrane of host cells. However, recent advances in molecular techniques for studying this bacterium, coupled with the development of appropriate animal models for *C. perfringens* diseases, are now providing insights into this question. Given this progress, it is timely to review our current understanding of the contributions of different *C. perfringens* toxins to disease.

Table 2. Properties of the most relevant toxins produced by *Clostridium perfringens*.				
Toxin	Location	Molecular mass (kDa)	LD$_{50}$ (mice[†])	Action
CPA	C	43	3 µg	Phospholipase C and sphingomyelinase activity; affects host signaling
CPB	P	35	<400 ng	Pore-forming toxin
ETX	P	33	100 ng	Pore-forming toxin
ITX	P	Ia 48; Ib 72	40 µg	Actin-specific ADP-ribosyltransferase
PFO	C	54	15 µg	Pore-forming toxin
CPE	C/P	35	81 µg	Pore-forming toxin
CPB2	P	28	160 µg	Pore-forming toxin
TpeL	P	191	600 µg?[‡]	Ras-specific monoglucosyltransferase
NetB	P	33	Unknown	Pore-forming toxin

[†]Per kg of mouse after intravenous injection.
[‡]Determined using a much less active TpeL variant.
C: Chromosomal; CPA: *C. perfringens* alpha toxin; CPB: *C. perfringens* beta toxin; CPE: *C. perfringens* enterotoxin; ETX: *C. perfringens* epsilon toxin; ITX: *C. perfringens* iota toxin; NetB: Necrotic enteritis toxin B-like; P: Plasmid; PFO: Perfringolysin O; TpeL: Toxin perfringens large.
Reproduced with modification from [8].

Table 3. Classification of *Clostridium perfringens* based on the production of the four major toxins.				
Type	**Toxin produced**			
	Alpha	*Beta*	*Epsilon*	*Iota*
A	+	−	−	−
B	+	+	+	−
C	+	+	−	−
D	+	−	+	−
E	+	−	−	+

Histotoxic infections

Clostridial myonecrosis, also known as gas gangrene, is a devastating histotoxic infection of humans and animals caused by *C. perfringens* type A. Although the incidence of gas gangrene in humans is relatively low, lethality rates remain generally high [9]. The successful invasion of traumatic wounds by vegetative cells or spores leads to rapid and extensive tissue damage and necrosis in the human host. If untreated, infection proceeds to shock, organ failure and ultimately death [9].

CPA and perfringolysin O (PFO) are quintessential to the pathogenesis of gas gangrene. The structural genes encoding these toxins, *cpa* (or *plc*) and *pfoA*, respectively, are located on the chromosome [10,11]. Production of both toxins is regulated by the Agr-like quorum sensing (QS) [12,13] system and also by the VirS/VirR two-component regulatory system [14–16]. However, PFO expression is directly regulated by the VirR response regulator, whereas CPA production is controlled by the VirR-regulated VR-RNA molecule [17].

CPA is a single polypeptide with two domains **(Figure 1)**: an N-terminal zinc-binding domain that has both phospholipase C and sphingomyelinase activity, and a C-terminal calcium-binding domain that has structural similarity to eukaryotic C2-lipid binding proteins and is responsible for the binding of the toxin to cell membranes, making it essential for toxic activity [18]. PFO is a member of the cholesterol-dependent cytolysin (CDC) family of pore-forming toxins [19], which have been shown in other organisms to play a role in virulence [20]. PFO has four structural domains **(Figure 1)**. Contact of domain 4 with the host cell membrane leads to two α-helical domains in domain 3 changing their conformation to form two amphipathic β-hairpins that insert into and span the membrane [21]. Up to 50 PFO monomers oligomerize to form a 15-nm membrane pore, which is much larger than the 1.5–1.7-nm pores formed by beta-pore-forming toxins such as NetB [22].

Early studies suggested a correlation between CPA production and disease [23]. For example, immunization with purified CPA or the C-terminal CPA domain protected mice from experimental gas gangrene [24]. Given its hemolytic nature and similarity to other CDCs, PFO also was proposed to contribute to the pathogenesis of gas gangrene. Definitive evidence for the essential role of CPA, and for an involvement of PFO in virulence only came about when an isogenic set of *C. perfringens* type A CPA and PFO null mutants was constructed by homologous recombination in type A strain 13 [23]. When the null mutants and complemented strains were tested in a mouse model, the results showed that CPA is essential for the development of myonecrosis, that is, the CPA mutants were avirulent in mice and virulence was restored upon complementation in trans by the wild-type *plc* gene [23]. Furthermore, these studies revealed that several features of gas gangrene, including tissue necrosis, thrombosis and the absence of a polymorphonuclear (PMN) leukocytic influx at the site of infection **(Figure 2)**, could be attributed to CPA activity [23].

Similar studies using an isogenic PFO null mutant of strain 13 and its complemented derivatives showed that PFO is not essential for disease, but that it does affect the host inflammatory response by contributing to the lack of PMN influx into the lesion and to the vascular accumulation of PMNs bordering the site of infection [25]. Furthermore, PFO appears to be the primary toxin responsible for killing mouse peritoneal macrophages *in vitro* and, together with CPA, enables *C. perfringens* to escape from the phagosomes of macrophages [25].

Subsequent studies involving virulence testing of a defined mutant deficient in both CPA and PFO production, and its complemented derivatives, showed that both toxins work
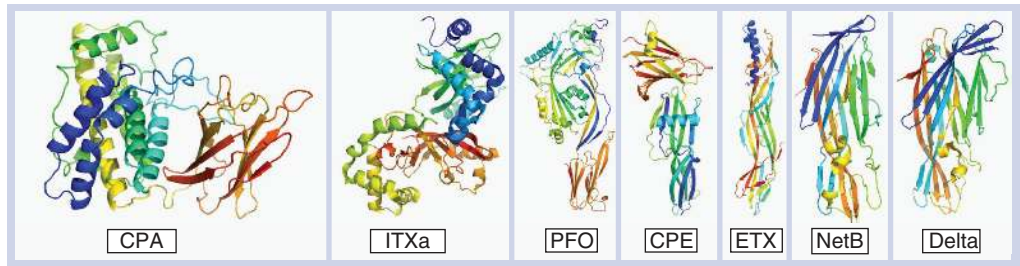
Figure 1. Crystal structures of *Clostridium perfringens* toxins. Graphic representations of all known toxin structures were prepared using Pymol [88]. Color coding is shown as a transition from N-terminal (blue) to C-terminal [35] residues. Note that delta toxin is a pore-forming toxin, but its role in disease has not yet been established.
CPA: *C. perfringens* alpha toxin (1CA1) [89]; CPE: *C. perfringens* enterotoxin (3AM2) [40,41]; Delta: Delta toxin (2YGT) [93]; ETX: *C. perfringens* epsilon toxin (1UYJ) [92]; ITXa: *C. perfringens* iota toxin a subunit (1GIR) [90]; NetB: Necrotic enteritis toxin B (4ION) [50]; PFO: Perfringolysin O (1PFO) [91].
For color images please see www.futuremedicine.com/doi/full/10.2217/fmb.13.168

synergistically to produce the overall clinical and pathological effects observed in *C. perfringens*-mediated gas gangrene [26]. It has been suggested that the mechanism of synergy may involve the initial membrane action of CPA facilitating the interaction of PFO with cholesterol in the cell membrane [27]. Both toxins contribute to the leukostasis observed in the peripheral blood vessels and the extracellular matrix of infected host tissues **(Figure 2)** [28].
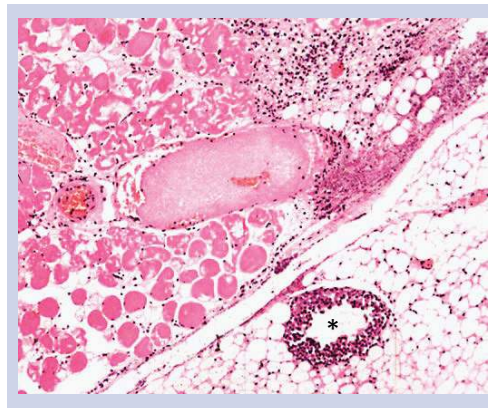

Figure 2. Microscopic lesions in mice inoculated with *Clostridium perfringens* type A (mouse gas gangrene model). The inner tight muscle shows severe necrosis of myofibers characterized by flocculation and fragmentation of the sarcolemma, hypereosinophilia and nuclear pyknosis. A mid-size venule (*) shows marked margination of neutrophils, which are mostly absent in the extravascular tissue. Sections were stained with hematoxylin and eosin and photographed at 100× magnification. Modified with permission from [26].

The genetic studies outlined above provided insight into the pathogenesis of *C. perfringens*-mediated gas gangrene, but *C. perfringens* type A produces >ten extracellular toxins and/or enzymes. Studies involving the construction of other isogenic mutants have shown that collagenase [29], the NanI and NanJ sialidases [30], and alpha-clostripain [31], are not essential for disease in the mouse myonecrosis model. However, these studies are of necessity limited given that the mouse gas gangrene model does not enable conclusions to be drawn about the early stages of a histotoxic infection.

Diseases originating in the intestine
• Infections caused by *C. perfringens* enterotoxin-positive strains of *C. perfringens* type A

As mentioned in the Introduction, Type A strains producing *C. perfringens* enterotoxin (CPE) are the second most common cause of bacterial food-borne illness in the USA, where they cause nearly 1,000,000 cases per annum, resulting in a net economic burden of US$382 million [3,4]. Symptoms of *C. perfringens* type A food poisoning include abdominal cramping, nausea and diarrhea, which usually begin 8–18 h after ingestion of contaminated food and then persist for 12–24 h [1]. Classic CPE-mediated *C. perfringens* food poisoning occurs when contaminated meat and/or poultry products are undercooked and/or held at improper temperature, allowing for spore germination and growth of CPE-producing type A strains in foods [1]. When that contaminated food is ingested, the bacteria sporulate in the small intestine, at which

time CPE is produced. At the completion of sporulation, the toxin is released into the intestinal lumen when the mother cell lyses to release the mature spore [1].

In addition to food poisoning, type A strains producing CPE cause 5–15% of all cases of non-food-borne human gastrointestinal (GI) diseases, including antibiotic-associated diarrhea and sporadic non-foodborne diarrhea [5]. These non-food-borne GI illnesses tend to be more severe and longer lasting than most *C. perfringens* type A food poisoning cases [5].

Only approximately 1–5% of *C. perfringens* type A strains carry the *cpe* gene, which is associated with insertion sequences [1]. The *cpe* gene can have a chromosomal or plasmid location in type A strains [32], but most type A food poisoning isolates carry a chromosomal *cpe* gene, whereas non-food poisoning type A isolates typically carry a plasmid-encoded *cpe* gene [32]. Type A strains carrying a chromosomal *cpe* gene comprise a distinct sublineage of *C. perfringens* that appears to be well-adapted for food-borne transmission [33], due in part to their production of spores with exceptional resistance against heating, low temperatures and certain food preservatives [34]. This resistance phenomenon involves, in large part, production of a variant small acid-soluble protein (Ssp4) that provides enhanced protection to the spores of chromosomal *cpe* type A strains [34,35].

CPE, an approximately 35-kDa protein belonging to the aerolysin family of β-pore-forming toxins, is comprised of a C-terminal receptor binding domain (Domain I) and an N-terminal domain (Domain II) that mediates toxin oligomerization and membrane insertion/pore formation **(Figure 1)**. The action of CPE starts with binding to claudin receptors on enterocytes [36–38]. Claudins are a 24-member protein family that play critical structural and functional roles in the mammalian tight junction [39]. Only certain claudins (e.g., claudins-3, -4, -8 and -14) can function as CPE receptors [36]. CPE binding to claudins results in the formation of an approximately 90-kDa CPE 'small complex' that contains CPE, a receptor claudin, and nonreceptor claudins, likely due to interactions of those claudins with the claudin receptors [1]. Following small complex formation, CPE hexamerizes on the membrane surface to form large (>450 kDa), heterologous prepore complexes that contain CPE, claudins and sometimes occludin, another tight junction

protein. Once formed, the CPE prepore inserts amphipathic, transmembrane β-hairpins from Domain II of each toxin monomer into the host membrane to form an active pore [40,41]. The resultant plasma membrane permeability alterations then allow a strong influx of calcium, causing downstream cell death due to apoptosis or oncosis [1]. Enterocyte death disrupts villus integrity, resulting in intestinal damage and electrolyte disregulation [1,42].

Animal model studies and human disease observations suggest that, when diarrhea is impaired in *C. perfringens* food poisoning victims due to medication side effects, CPE can enter the circulation and damage internal organs such as the kidney or liver, leading to excessive serum potassium. In animal models, and presumably people, this can result in hyperkalemia-associated heart failure and death [43].

A wealth of data [1] supports a critical role for CPE in the pathogenesis of food poisoning caused by CPE-positive type A strains. First, while absent from the feces of healthy individuals, CPE is found in the feces of 80–100% of individuals with *C. perfringens* type A food poisoning. Second, peroral administration of purified CPE to human volunteers caused the development of classical *C. perfringens* type A food poisoning symptoms. Third, CPE is present in fecal specimens from patients of *C. perfringens* type A food poisoning outbreaks at levels known to cause pathogenic effects in animal models. Fourth, CPE-specific antisera can neutralize the intestinal pathology caused by CPE-positive type A isolates in animal models. Fifth, purified CPE was shown to be sufficient to cause morphological damage in human ileal tissue *ex vivo*. Finally, isogenic *cpe* null mutants and complemented strains have been used to fulfill molecular Koch's postulates by demonstrating that CPE is essential for the type A food poisoning strain SM101 to cause fluid accumulation and histological damage in rabbit small intestinal loops **(Figure 3)**. Similar analyses indicated that CPE is also essential for the non-foodborne human GI disease isolate F4969 to cause pathology in rabbit intestinal loops [44].

• Infections caused by NetB-positive strains of *C. perfringens* type A

NetB-positive type A strains of *C. perfringens* cause necrotic enteritis in chickens [45]. This debilitating disease of the gastrointestinal tract
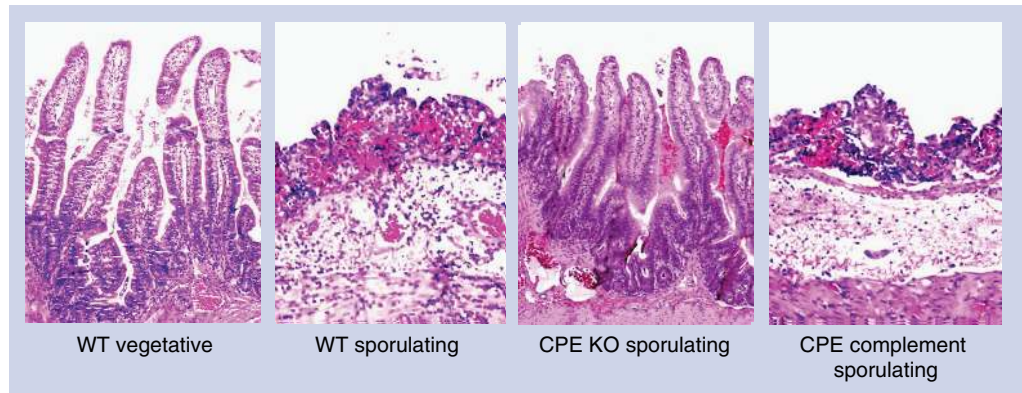
Figure 3. Histological damage in rabbit ileum treated with lysates from *Clostridium perfringens* enterotoxin-positive *C. perfringens* type A strain SM101. Loops inoculated with WT SM101 vegetative culture (WT vegetative) or an isogenic CPE KO sporulating culture lysate (CPE KO sporulating) show normal, full-length intestinal villi with a well-preserved epithelium and lamina propria. Loops inoculated with sporulating culture lysate of WT SM101 (WT sporulating) or the isogenic CPE complement (CPE complement sporulating) show histological damage consisting of necrosis and loss of epithelium, necrosis of lamina propria, villous blunting, and hemorrhage and edema of the mucosa and submucosa. Sections were stained with hematoxylin and eosin and photographed at 250× magnification.
CPE: *C. perfringens* enterotoxin; KO: Knockout; WT: Wild-type.
Figure and legend modified with permission from [44].

is found in chickens in all poultry-producing regions of the world and, as indicated in the Introduction, imposes a significant economic burden because of reduced production efficiency and the cost of control measures [46]. The disease is most common in broilers, but layer hens and turkeys can also be affected. Liver lesions and erosive lesions of the gizzard have also been associated with *C. perfringens* infection, but little work has been reported on these syndromes and the involvement of toxins remains unknown [47]. Subclinical disease, which adversely affects feed conversion ratios without causing significant mortality, is more of a problem because it is less obvious and may remain untreated, thus leading to longer grow-out periods and negatively impacting productivity and profitability [46].

The intestinal lesions of avian necrotic enteritis are characterized by a massive infiltration of granulocytes, a finding that was at odds with the previously held theory that CPA was the key virulence factor in necrotic enteritis [48,49]. Furthermore, CPA null mutants retained full virulence in a chicken disease induction model. These findings provided a strong indication that another toxin was involved, which led to the discovery of a new *C. perfringens* toxin, NetB [49]. Bioinformatic analyses of the NetB sequence suggested it is a β-pore-forming toxin, which

was confirmed when NetB was shown to form a heptameric pore that affects membrane ion permeability [50,51]. In addition, structural studies showed that NetB is similar in structure to several other pore-forming toxins, including *C. perfringens* delta toxin **(Figure 1)** and α-hemolysin from *Staphylococcus aureus* [50,51].

Expression of NetB is under the control of the VirSR two-component signal transduction system; *virR* mutants produce reduced levels of NetB and complementation restores NetB production [52]. Strain surveys have generally found that most *C. perfringens* isolates from lesions of diseased birds carry the *netB* gene, whereas carriage of the gene is less frequent in isolates from healthy birds [45,53]. The isolation of NetB-negative strains from diseased birds has called into question the importance of the NetB toxin. However, in most studies in which the virulence of disease isolates has been directly studied, only NetB-positive strains reliably reproduce disease [45,54]. One report did find some disease from NetB-negative strains, but since the disease-causing strains were not re-isolated from diseased birds the exact etiology of the disease in these birds is not clear [47]. The *netB* gene is carried on a large conjugative plasmid [55–57], which can be lost during culturing, which appears to be an issue particularly when initially culturing

from clinical material. Loss of plasmids when the strains are under severe stress, such as moving from the *in vivo* environment to *in vitro* culture conditions, may explain why many NetB-negative strains have been isolated from diseased birds. Alternatively, mixed colonization with pathogenic and nonpathogenic strains could give rise to the isolation of NetB-negative strains from diseased birds.

Studies using *netB* null mutants have provided convincing evidence for the essential role of NetB in the pathogenesis of avian necrotic enteritis **(Figure 4)**. Wild-type Culture supernatant from a *netB*-positive strain is toxic to chicken leghorn male hepatoma (LMH) cells, but the supernatant from *netB* null mutants is not toxic. When a functional wild-type *netB* gene was reintroduced to complement the mutation, wild-type virulence was restored. This cellular toxicity can be neutralized by anti-NetB polyclonal antibodies [49]. These *netB* null mutants are also completely avirulent in the chicken disease induction model **(Figure 4)**. Finally, recent studies have shown that NetB is efficacious as a vaccine antigen [58–61].

• **Infections caused by *C. perfringens* type C strains**

C. perfringens type C strains are capable of causing fulminant disease in both humans and animals. In humans, type C infections cause enteritis necroticans (also known regionally as Darmbrand or Pigbel), an intestinal infection resulting in vomiting, bloody stool, abdominal pain and, in severe cases, toxemia leading to rapid death [62]. These infections are endemic in Southeast Asia, where poor diet and consumption of trypsin inhibitor-rich foods, such as sweet potatoes, provide ideal conditions for disease, suggesting that trypsin is a key host defense against disease. Disease is also occasionally observed in developed countries, where it is usually confined to individuals with reduced pancreatic function, such as diabetics [63,64].

Type C infections also occur in most livestock species, producing a hemorrhagic necrotic enteritis and enterotoxemia similar to human infection. Although adult animals can be infected and killed rapidly by this organism, most disease is observed in neonates. The propensity for type C strains to cause disease in young animals is likely due to trypsin inhibition by colostrum. Colonization of animals occurs within hours of birth, most likely originating from contact with contaminated fecal material. Infection in animals

typically involves outbreaks in unvaccinated herds, with lethality rates >50% [62].

C. perfringens type C disease begins with multiplication of the bacterium in the host intestine, followed by CPB production. CPB then affects the epithelium of the jejunum, ileum and, to a lesser extent, the duodenum and colon, where it causes fluid accumulation and necrosis of the intestine with variable hemorrhage [65]. Mucosal damage is followed by absorption of CPB into the circulation, from which it acts at distal sites, including the brain [62,66]. At the cellular level, the action of CPB remains incompletely defined. However, CPB is a pore-forming toxin that uses an unidentified receptor for binding to host cells [8]. Once bound, this toxin oligomerizes into hexamers or heptamers on host cells, creating transmembrane pores in susceptible cells [67]. These pores cause K^+ efflux and Ca^{2+}, Na^+ and Cl^- influx, leading to cellular swelling and necrosis of intoxicated cells [68]. Interestingly, several studies using cell culture and tissue samples
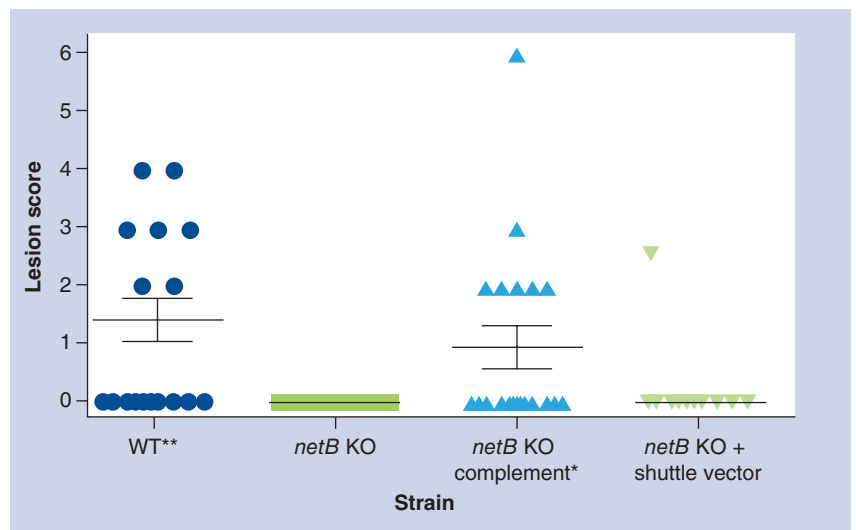

Figure 4. Virulence of *Clostridium perfringens* type A strains in 24-day-old broiler chickens challenged with different *C. perfringens* strains are shown.
Solid horizontal bars depict the mean small intestinal lesion score in each group (n = 10). Lesion scores correspond to 0: no gross lesions; 1: thin and/or friable walls; 2: focal necrosis or ulceration (1–5 foci); 3: focal necrosis or ulceration (6 to 15 foci); 4: focal necrosis or ulceration (>16); 5: patches of necrosis 2–3 cm long; 6: diffuse necrosis typical of field cases. The strains tested included: WT; *netB* KO, *netB* KO complemented (*netB* KO complement); *netB* knockout + shuttle vector (*netB* KO + shuttle vector). One tailed, nonparametric t-test analysis of the wild-type and complemented mutant derivatives against *netB* KO showed a statistical difference (*p = 0.05 and **p = 0.01), but no statistical significance was observed between the *netB* KO and the *netB* KO + shuttle vector.
KO: Knockout; WT: Wild-type.
Figure and legend modified from [49].

from infected humans and piglets have shown that CPB binds to endothelial cells, although the contribution of those CPB interactions to disease remains to be delineated [68,69].

The *cpb* gene is carried on large virulence plasmids of approximately 65–110 kb that can contain additional toxin genes encoding CPE or TpeL [70,71]. CPB is expressed as a 336 amino acid protein containing a 27 amino acid leader sequence that is cleaved to yield an approximately 35-kDa functional protein sharing 20–28% identity with some staphylococcal pore-forming toxins and 38% identity with *C. perfringens* NetB toxin [8]. Investigations into the regulatory mechanism of CPB production by type C strains have demonstrated the involvement of the Agr-like QS system in regulating the production of CPB during intestinal disease. An AgrB (membrane-bound endopeptidase) null mutant of strain CN3685 [72] exhibited sharply reduced *in vivo* CPB production, resulting in the mutant being unable to induce necrotizing enteritis in rabbit ileal loops or lethality in mice. In addition to the Agr-like QS system, the VirS/VirR system is also involved in regulating *in vivo* CPB production and type C virulence [73].

Type C strains must carry genes encoding CPA and CPB, and may also possess genes for additional accessory toxins. However, several recent studies provided direct evidence identifying CPB as the primary toxin responsible for development of disease by type C strains. Initial studies utilizing monoclonal antibodies indicated that neutralization of CPB, but not CPA, protects mice from a lethal IV challenge with type C culture supernatants [62]. More recently, purified CPB was shown to induce necrotizing enteritis in rabbit small intestinal loops **(Figures 5 & 6)** or lethality in a mouse enterotoxemia assay [65]. The most convincing support for CPB involvement in type C disease was provided by studies using toxin null mutants, where a CPB null mutant of type C strain CN3685 was essentially avirulent in mouse, rabbit **(Figures 5 & 6)** and goat models of necrotizing enteritis or enterotoxemia [74–76]. Reversing this *cpb* null mutation restored virulence in all three animal models, ruling out secondary mutation effects. In contrast, isogenic CPA, PFO or double CPA/PFO null mutants retained near full virulence in the rabbit small intestinal loop model **(Figures 5 & 6)** and the mouse enterotoxemia model [76].
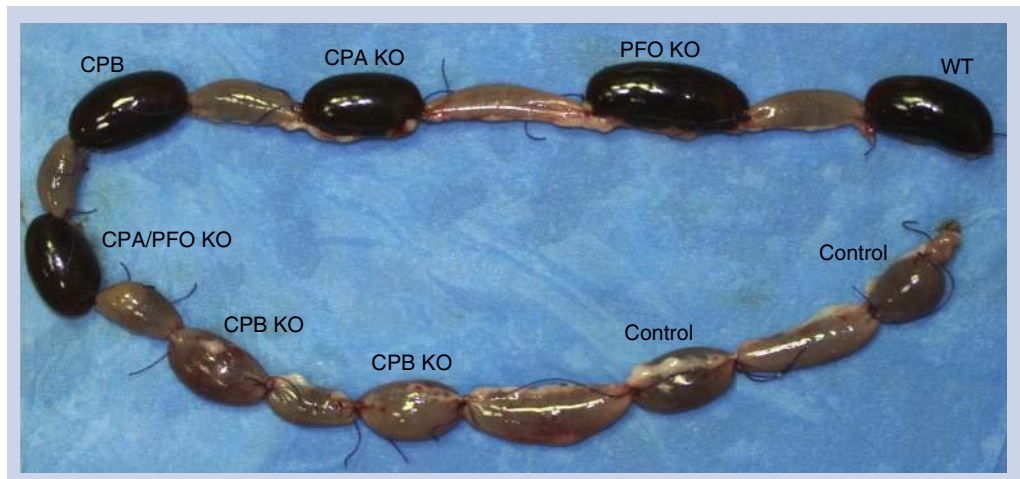

Figure 5. Gross pathology of rabbit-ligated small intestinal loops challenged for 6 h with *Clostridium perfringens* **type C strain CN3685 WT, isogenic single- and double-toxin mutants (PFO KO, CPA KO, CPB KO, CPA/PFO KO), purified CPB (CPB) or sterile tryptic soy broth–glucose–yeast extract broth (control).** Note that loops inoculated with the WT, PFO KO, CPA KO, CPA/PFO KO or purified CPB are severely hemorrhagic and distended with fluid. No significant gross abnormalities are observed in the loops inoculated with CPB KO or sterile tryptic soy broth–glucose–yeast extract broth.
CPA: *C. perfringens* alpha toxin; CPB: *C. perfringens* beta toxin; KO: Knockout; PFO: Perfringolysin O; WT: Wild-type.
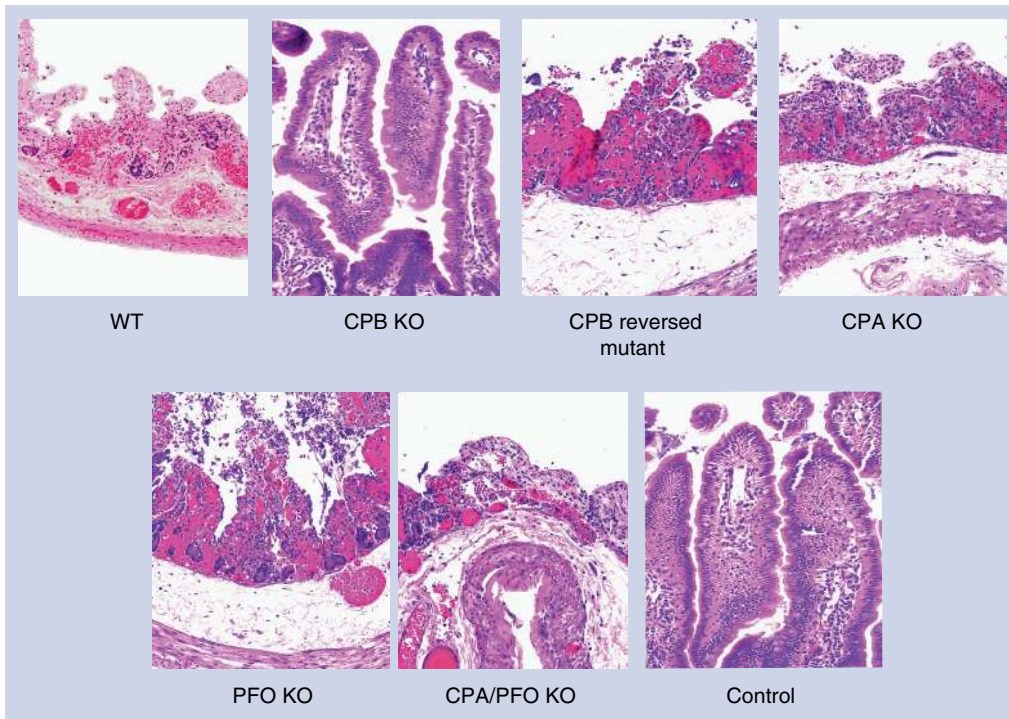Modified with permission from [76].

Figure 6. Histological damage in rabbit small intestine treated for 6 h with an 8-h culture of WT strain CN3685 (WT), single- and double-toxin isogenic mutants (PFO KO, CPA KO, CPB KO, CPA/PFO KO), or sterile tryptic soy broth–glucose–yeast extract broth medium (control). Control loops and loops inoculated with the CPB KO showed normal, full-length intestinal villi with a well-preserved epithelium and lamina propria. Loops inoculated with WT, CPB reversed mutant, PFO KO, CPA KO, or CPA/PFO KO all showed microscopic changes, including mucosal necrosis, hemorrhage and blunting of the villi, together with neutrophilic infiltration of mucosa and submucosa. Tissue sections were stained with hematoxylin and eosin, and photographed at 200× magnification.
CPA: *C. perfringens* alpha; CPB: *C. perfringens* beta; KO: Knockout; PFO: Perfringolysin O; WT: Wild-type.
Figure and legend modified with permission from [76].

• Infections due to *C. perfringens* type D strains

C. perfringens type D produces enterotoxemia in sheep and goats [77]. Less frequently, these strains are responsible for enterotoxemia in cattle and other animal species. ETX increases vascular permeability in the brain and other organs. In the brain, this toxin affects endothelial cells, which is followed by swelling and rupture of the perivascular astrocyte feet. The immediate consequence of this effect is perivascular edema, with an increase of the intracerebral pressure and occasionally parenchymal brain necrosis (focal symmetrical encephalomalacia). Owing to these effects, type D disease is mainly characterized by neurological clinical signs in the most affected animal species, although goats can also suffer intestinal damage [77].

Type D strains by definition must produce ETX, which is encoded by the plasmid-borne *etx* gene [32]. The regulation of ETX production is more variable than that of many other toxins discussed in this review. For example, while ETX production is regulated by the Agr-like QS system in type D strain CN3718, this is not true for two type B strains [78,79]. In addition, ETX production by type D strain CN3718 is not dependent upon a functional VirS/VirR system [78].

ETX is secreted as a 33-kDa prototoxin that must be proteolytically activated by cleavage of the C-terminal 29 amino acids to achieve full potency [80]; activating ETX with trypsin leads to a 1000-fold increase in toxicity [80]. Structurally, the ETX protein belongs to the same aerolysin pore-forming toxin family that includes CPE **(Figure 1)**. Furthermore, ETX is able to bind to glutamatergic neuronal cells, where it causes release of the neurotransmitter glutamate, which is thought to contribute to the
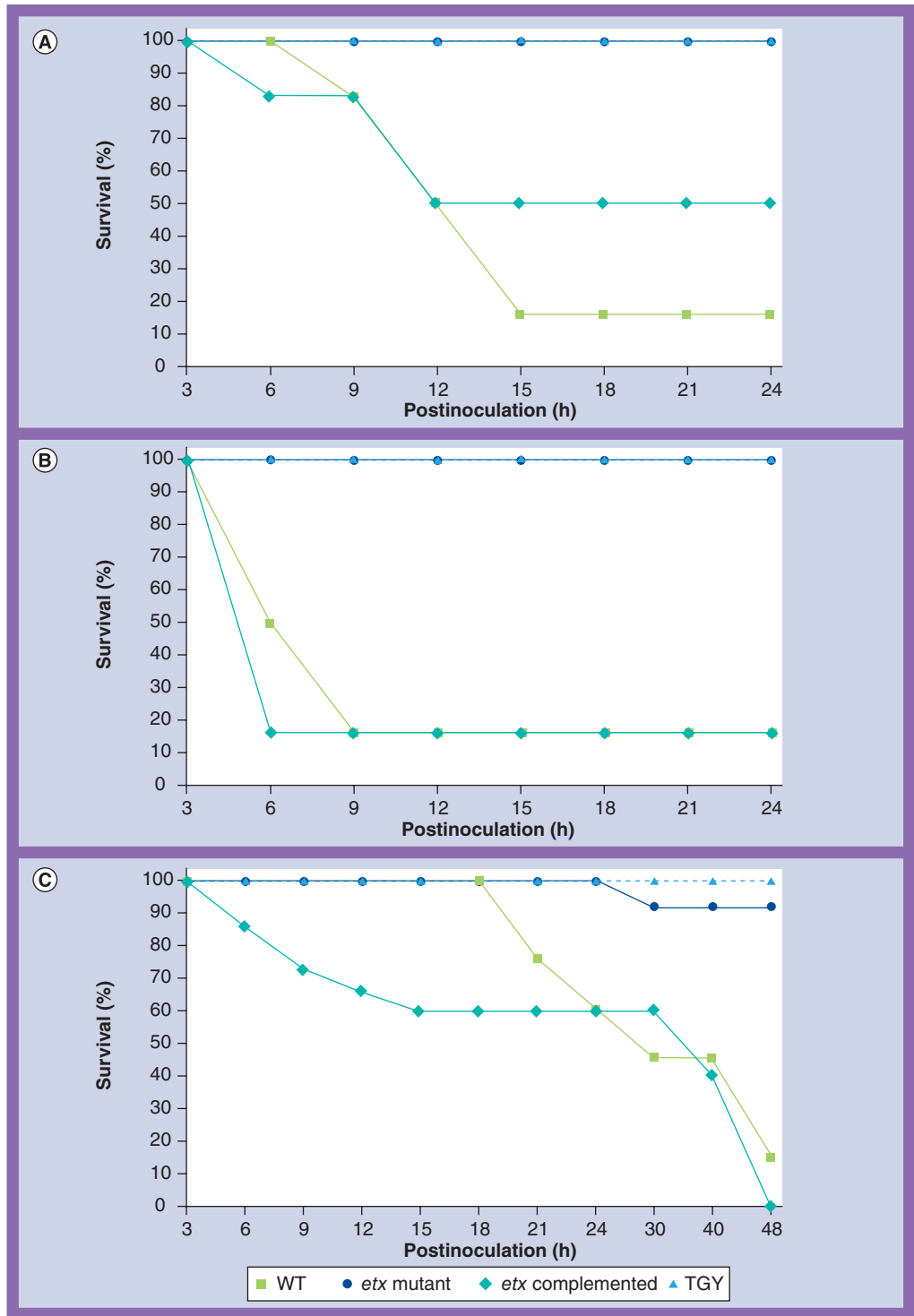
Figure 7. Kaplan–Meier survival curves for sheep, goats and mice *Clostridium perfringens* type D infection models. Survival over 24 h in **(A)** sheep and **(B)** goats or 48 h **(C)** in mice after intraduodenal treatment with the WT strain CN1020 (WT), the *etx* mutant JIR4981 (ETX knockout), its complemented derivative JIR12604 (ETX complemented), or TGY. Each inoculum was administered to six sheep, five goats and 15 mice.

etx: *C. perfringens* epsilon; TGY: Tryptic soy broth–glucose–yeast extract broth; WT: Wild-type. Figure and portions of the legend used with permission from [83].

neurological signs of type D disease [80]. ETX is the third most potent clostridial toxin, that is, ETX is 100-times more potent than other members of the aerolysin pore-forming toxin family, suggesting that pore formation may not be the only mechanism of action utilized by ETX [80]. As a result of this potency, ETX has been of interest as a potential bioterrorism agent [80] and was included until recently on the CDC list of select toxins. We have shown that the *etx* gene is located on large plasmids [32] and, for two type D isolates, the plasmids were shown to be conjugative [81].

Most clinical and pathological changes in type D disease were reproduced by inoculation of ETX in sheep, goats and cattle. Recent studies have confirmed that ETX is necessary for the virulence of type D isolates. Assaying a large number of type D culture supernatants using an intravenous injection mouse model revealed a strong correlation between ETX levels and lethality, suggesting this is the major toxin contributor to mouse lethality [82]. An *etx* null mutant was later produced by allelic exchange, which resulted in its insertional inactivation by an antibiotic resistance cassette. This process allowed for the tracking of the plasmid encoding the deleted *etx* gene and led to the discovery that these plasmids could be transferred to other *C. perfringens* strains at a very high frequency [81]. This *etx* null mutant was also used to demonstrate the role of ETX in *C. perfringens* type D virulence. Clinical disease followed by death was observed in sheep, goats and mice challenged intraduodenally with a wild-type isolate of a type D strain **(Figure 7)**. Gross and/or microscopic changes observed in affected sheep included edema of the brain **(Figure 8)**, lungs and heart and hydropericardium. Necrotizing colitis, pulmonary edema and hydropericardium were observed in affected goats. Mice challenged with the wild-type strain died, but presented no significant gross or histological abnormalities [83]. No clinical disease, gross abnormalities, or microscopic damage were observed in any of the sheep, goats and mice treated with the isogenic *etx* null mutant. Clinical and pathological changes similar to those observed in animals infected with the wild-type strain were observed in most goats, sheep and mice challenged with the complemented mutant **(Figure 7)**. Based on these results, it was concluded that ETX is necessary for type D isolates to cause disease.

Conclusion

Research is now providing substantial insights as to why *C. perfringens* produces so many different toxins. The emerging evidence is that some of these toxins (CPA and PFO) are utilized mainly when this bacterium causes histotoxic infection, including gas gangrene **(Figure 9)** [9]. During gas gangrene, these toxins interfere with the immune response and cause localized necrosis to facilitate the growth of *C. perfringens*, an anaerobe. These toxins also induce host cell leakage and lysis, which may provide nutrients (particularly many required amino acids) for *C. perfringens* growth. The combination of regional damage and, especially, distant toxin effects soon kills the host, providing a significant food source for further *C. perfringens* multiplication.

When causing diseases originating in the intestine, *C. perfringens* generally relies upon other toxins, namely CPE, NetB, CPB or
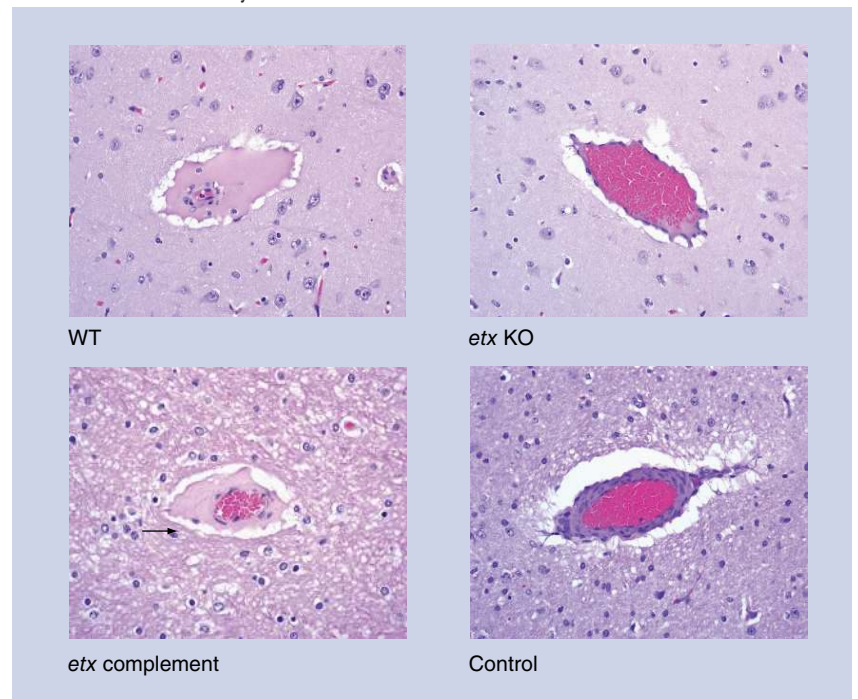

WT   *etx* KO

etx complement   Control

Figure 8. Microscopic lesions in sheep after intraduodenal treatment with ***Clostridium perfringens*** **type D WT strain CN1020 (WT) or isogenic derivatives including an** ***etx*** **null mutant (** ***etx*** **KO), its complemented derivative (** ***etx*** **complement), or sterile, nontoxic culture medium (control) are shown.** Brains from sheep challenged intraduodenally with the WT or complemented strains show proteinaceous perivascular edema (arrow) due to increased vascular permeability. Significant histological lesions were absent from the brains of sheep treated intraduodenally with the *etx* KO or control animals.

etx: *C. perfringens* epsilon; KO: Knockout; WT: Wild-type.

Figures and portions of the legend reproduced with permission from [83].

A Histotoxic infection

1. Trauma
2. Wound entry and tissue invasion
4. Localized necrosis
CPA
CPA
PFO
5. Vascular leukostasis
3. Secretion of PFO and CPA

B Intestinal infection

1. Colonization of intestine, toxin release and local toxin action on intestines

CPE
CPB
- Trypsin +
NetB
ETX

Liver Kidney Brain

2. Entry of toxins into circulation, action in distal nonintestinal organs

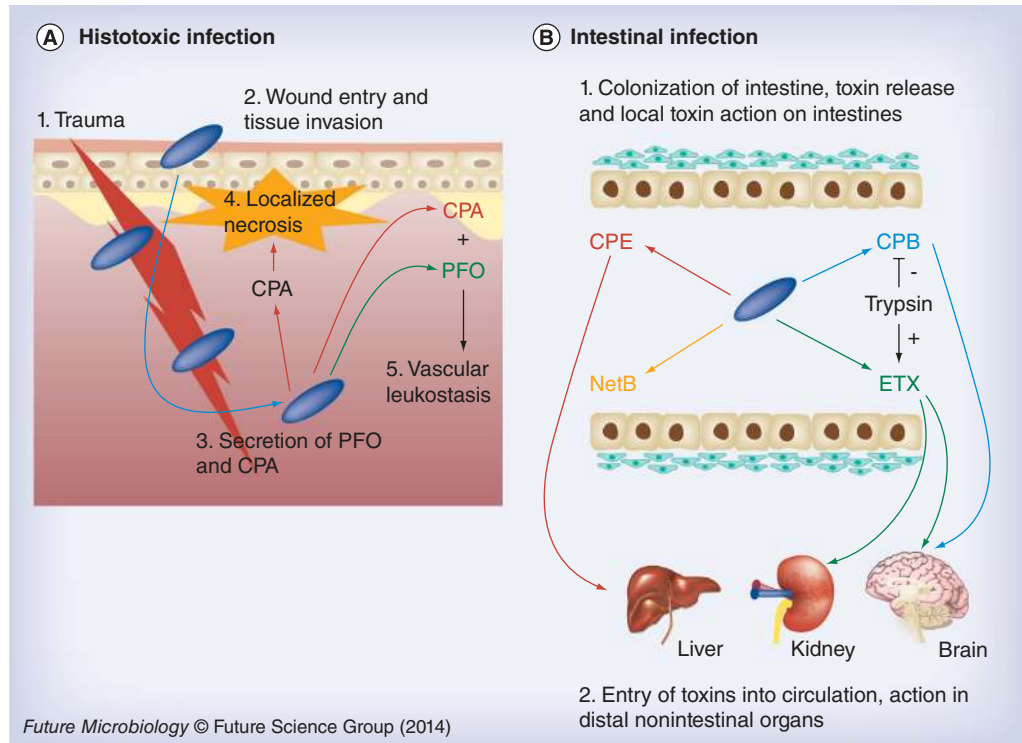*Future Microbiology* © Future Science Group (2014)

Figure 9. Summary of toxin involvement in *Clostridium perfringens* disease. (A) During histotoxic infections CPA plays a predominant role after *C. perfringens* (blue rods) are introduced by trauma into muscle tissue. This toxin induces localized necrosis, as well as toxemic effects in other organs when toxins enter the circulation. Both CPA and PFO are important in vascular leukostasis. **(B)** During intestinal infections, *C. perfringens* (blue rods) can produce several pore-forming toxins, including CPE during sporulation or (during vegetative growth) NetB, ETX or CPB. Host intestinal protease levels can either destroy (e.g., CPB) or activate (e.g., ETX) these toxins. When present in an active form, these toxins can cause local damage (necrosis) or be absorbed into the circulation to damage internal organs such as the brain, kidney or liver.
CPA: *C. perfringens* alpha toxin; CPB: *C. perfringens* beta toxin; CPE: *C. perfringens* enterotoxin; PFO: Perfringolysin O.
For color images please see www.futuremedicine.com/doi/full/10.2217/fmb.13.168

ETX [8]. Notably, those toxins involved in most intestinal infections are associated with mobile genetic elements, typically a large conjugative plasmid [32]. The major exception is the chromosomal *cpe* gene that is present in most type A food poisoning strains; however, even this toxin gene is associated with a putative transposon that has integrated onto the chromosome [1].

The progress in dissecting the contributions of individual toxins to *C. perfringens* virulence is also illuminating why this bacterium produces so many pore-forming toxins. PFO clearly plays a supportive role during gas gangrene [25,26]; however, two lines of evidence suggest this toxin is not a major contributor to most *C. perfringens* intestinal infections. First,

inactivation of the *pfoA* gene of type C animal disease strain CN3685 did not significantly attenuate that strain for causing necrotizing enteritis or lethal enterotoxemias in animal models [76]. Second, PFO cannot be a major contributor to several important *C. perfringens* human intestinal infections since the *pfoA* gene is absent from the chromosomal *cpe* type A strains causing most cases of *C. perfringens* type A food poisoning [33] and from the type C strains causing Darmbrand [84].

In contrast, recent studies have clearly demonstrated a key role for a number of other pore-forming toxins in *C. perfringens* infections originating in the intestine **(Figure 9)**. Why then does this bacterium employ so many different pore-forming toxins to cause intestinal infections?

One answer is that these toxins likely provide the species with tremendous versatility in causing these diseases. For example, CPE is only produced during sporulation, which occurs during food poisoning when *C. perfringens* is ingested in contaminated foods, briefly undergoes a period of intense growth in the anaerobic and nutrient-rich intestinal environment, and then (for still unknown reasons) synchronously sporulates and produces CPE. This pore-forming toxin then induces a strong diarrhea that flushes large numbers of spores back into the environment so they can eventually repeat this cycle [1].

Unlike CPE, the other pore-forming toxins with established roles in intestinal infections are produced by vegetative cells. As for gas gangrene, intestinal infections involving ETX, CPB and NetB often result in rapid death of the host, possibly providing the infecting *C. perfringens* strains with a food source for their multiplication [8]. Why then might *C. perfringens* use so many different pore-forming toxins to induce host death during vegetative growth? There could be at least two answers. First, some of these toxins appear to be exquisitely adapted for particular hosts. For example, NetB and CPB are clearly related with respect to sequence, yet NetB-producing strains overwhelmingly cause disease in poultry while CPB-producing strains affect mammals [45,62]. A second potential explanation for producing so many different pore-forming toxins is that it provides *C. perfringens* with flexibility in causing disease under varying host conditions. For example, ETX is activated by intestinal proteases [80], yet CPB is easily destroyed by those same intestinal proteases [85]. Therefore, ETX-producing strains are adept at causing disease in 'healthy' animals, while CPB-producing strains are well-suited for affecting young or compromised hosts (animals or people) with reduced levels of intestinal proteases. Type B strains may illustrate this point particularly well, that is, presumably these strains can use their ETX to cause disease in healthy animals but employ their CPB to cause disease in animals with reduced intestinal protease levels (see next section) [85].

Finally, while it is clear that toxins play a critical role in *C. perfringens* pathogenicity, it should be appreciated that other factors, such as strain clonality, toxin production regulation, enzyme production and conjugative toxin plasmid transfer [32,33,56,86], are also key contributors to the virulence of this bacterium.

Future perspective

Many gaps still remain in our knowledge of toxin contributions to *C. perfringens* infections. For example, type B strains cause an often fatal hemorrhagic dysentery in sheep, and possibly in other species. Some earlier studies using IV injection of type B culture supernatants into mice suggested that both CPB and ETX are important for virulence of these strains [85], with host factors (i.e., the presence or absence of trypsin) apparently tipping the scale for the relative importance of these two toxins for lethality. However, there is a clear need to demonstrate molecular Koch's postulates using a natural infection model to confirm these findings. Similarly, conclusive evidence for a role of type E strains in animal disease remains unclear, although these strains have been associated with enteric disease in rabbits, lambs and calves. These are the only *C. perfringens* strains producing iota toxin, which is a binary toxin that ADP-ribosylates actin to cause a depolymerization of actin filaments in host cells [8]. There is currently little information available regarding toxin contributions to type E strain virulence. Type E strains are also worthy of study because iota toxin is one of only two toxins (TpeL being the other) made by this bacterium that act intracellularly [8,87]. Therefore, careful virulence dissections of type E strains and TpeL-producing strains now appear warranted to evaluate whether intracellularly active toxins, as well as plasma membrane-active toxins, contribute to virulence.

EXECUTIVE SUMMARY

Background

- *Clostridium perfringens* causes a spectrum of important human and animal diseases ranging from histotoxic to enteric infections.

- These include clostridial myonecrosis (gas gangrene), *C. perfringens* type A food poisoning, *C. perfringens* enterotoxin (CPE)-associated nonfoodborne GI diseases, avian necrotic enteritis, human enteritis necroticans, and animal enteritis or enterotoxemia.

- These diseases are mediated by different toxins, whose production varies among *C. perfringens* strains.

Histotoxic infections

- Alpha toxin and perfringolysin O are important during histotoxic infections such as gas gangrene.

Diseases originating in the intestines

- When produced, enterotoxin (CPE), NetB toxin, epsilon toxin and beta toxin are critical contributors to intestinal infections.

- CPE is necessary for *C. perfringens* type A food poisoning and CPE-associated nonfoodborne human GI diseases.

- NetB is required for avian necrotic enteritis.

- CPB or ETX, respectively, are important for type C and D infections originating in the intestines.

- The toxins contributing to intestinal infections vary in their targets when produced (sporulation vs vegetative growth) and their protease sensitivity, giving *C. perfringens* the ability to cause disease under varying host intestinal conditions.

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