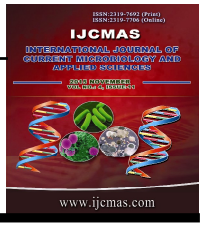
Int.J.Curr.Microbiol.App.Sci (2015) 4(11): 356-371

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 11 (2015) pp. 356-371**
http://www.ijcmas.com



Review Article

Towards an Understanding on Toxins and Infectious Diseases of *Clostridium perfringens* vis-a-vis Prospective Recombinant Vaccines

Pratistha Dwivedi, Shuchi Kaushik* and RS Tomar

Amity Institute of Biotechnology, Amity University, Madhya Pradesh, India
**Corresponding author*

Keywords

Toxin, Vaccine, Gas gangrene, Necrotic enteritis, Protective antigen

A B S T R A C T

Clostridium perfringens is a Gram-positive, rod shaped, anaerobic bacterium that is able to form spores. Various forms of acute enteric diseases, generically called enterotoxemias, in sheep, goats, and other animals have been attributed to *C. perfringens*. In humans, it can cause gangrene and gastrointestinal diseases. *C. perfringens* strains are classified into five toxinotypes, A, B, C, D, and E, based on the production of major toxins α, β, ε, and ι. Interest in development of vaccines which could protect against diseases caused by *C. perfringens* has been increased not only with respect to human but also economically important diseases of domesticated livestock. Following vaccination, there is occurrence of side effects that prompted the use of subunit vaccines. Although it is a fact that high antibody titer does not always correlate with protection, but it can offer clues towards identification of protective antigens of a pathogen in a host surviving infection. Proteomic analysis along with reverse vaccinology approach accelerates the daunting task of vaccine development and leads to the discovery of unique and candidate antigens for further validation and trials.

Introduction

The Clostridia, belong to class of Firmicutes including *Clostridium* and other similar genera. They are lacking aerobic respiration and thus are obligate anaerobes. They are all Gram-positive and have the ability to form spores.

Most of the species of *Clostridium* are saprophytic and found in many places in the environment, most notably the soil. However, the genus does contain some human pathogens. The toxins produced by members of the *Clostridium* genus are among the most dangerous toxins known. The pathogenic members of the genus causes a variety of infections both in humans and animals like botulism, tetanus, gas gangrene, pseudo membranous colitis, and various food poisonings.

Clostridium perfringens was formerly known as *C. welchii*. *C. perfringens* is ever present in nature and can be found as a normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates, insects, and

soil. It is commonly found in the intestines of animals, including humans, where it is pathogenic in certain circumstances. It causes anaerobic cellulitis, gas gangrene, enteritis necroticans, and food poisoning in humans (Rood and Cole, 1991) and gastrointestinal and enterotoxemic diseases in other animals.

Gas gangrene (clostridial myonecrosis), is an infection that originates in that tissues in which the blood supply has been cut off due to trauma or circulatory blockages. From there it soon spreads to healthy tissues and, if left untreated, results in severe shock and cardiac stress due to the release of toxins into the bloodstream (Asmuth *et al.*, 1995). Though the cases of gangrenous infections are known to mankind since Middle Ages, it became frequent during the World Wars of 1914-1918 and 1939-1945. Hundreds of thousands of soldiers died of gas gangrene as a result of battlefield injuries, and further *C. perfringens* was recognized as most important cause of the disease. Moreover, *C. perfringens* and its toxins have been listed as potential biological and toxin warfare (BTW) agents and need attention towards finding approaches for specific detection and protection. Strains of *C. perfringens* are divided into five types, A to E, based on the major lethal extracellular protein toxins that they produce, namely alpha-, beta-, epsilon-, and iota- toxins (Rood and Cole, 1991).

Diseases caused *by C. perfringens*:

***C. perfringens* type**	**Disease produced**
A	Gas gangrene (clostridial myonecrosis), food poisoning, necrotic enteritis of infants, necrotic enteritis of poultry, necrotizing duodenitis (Hagiya *et al.*, 2012)
B	Lamb dysentery, enterotoxemia of sheep, foals, and goats, haemorrhagic enteritis in neonatal calves and foals
C	Enterotoxemia of sheep (struck), necrotic enteritis in animals, Necrotic enteritis in piglets, lambs, calves and foals, human enteritis necroticans (pigbel, Darmbrand)
D	Enterotoxemia of sheep (pulpy kidney disease), Focal symmetrical encephalomalacia (FSE) (Oliveira *et al.*, 2010)
E	Enteritis of rabbits, bovine and ovine enterotoxaemia

Types of *C. perfringens* with toxins produced[a]

Types	α	β	ε	τ	δ	θ	κ	λ	μ	υ	**En[b]**	**Nm[c]**
A	+	-	-	-	-	+	+	-	+	+	+	+
B	+	+	+	-	+	+	+	+	+	+	+	+
C	+	+	-	-	+	+	+	-	+	+	+	+
D	+	-	+	-	-	+	+	+	+	+	+	+
E	+	-	-	+	-	+	+	+	-	+	+	+

[a]Based on review by Rood and Cole, 1991. [b]En = Enterotoxin.
[c]Nm = Neuraminidase or sialidase.

Toxins and virulence factors

C. perfringens strains are classified into five toxinotypes (A, B, C, D, and E) based on the production of four major toxins (α, β, ε and ι). These toxins are secreted into the medium during the exponential growth phase and can kill mice when the culture supernatant is injected intraperitoneally. In the gangrenous lesions, it also produces virulence factors.

Most of clostridial toxins are pore-forming toxins (PFTs) belonging to the β-PFT class. They are secretory proteins rich in β-strands, recognize a specific receptor on target cells and assemble in oligomers. Then, they undergo a conformational change that leads to the formation of a β-barrel, which form functional pore into the lipid bilayer (Popoff, 2014).

Alpha toxin or phospholipase C

The most important virulence factor produced by clostridial myonecrosis (gas gangrene)-causing isolates of *C. perfringens* is the α-toxin, the first toxin for which an enzymatic activity, phospholipase C, was demonstrated (MacFarlane and Knight, 1941). The toxin is also called sphingomyelinas and it hydrolyzes phospholipids which lead to membrane disorganization. Alpha toxin is lethal and haemolytic having essential role in gas gangrene.

A crystallographic study of alpha-toxin revealed that the structure is divided into two domains (Naylor *et al.*, 1998): the N domain, consisting of nine tightly packed α-helices, and the C-domain, consisting of an eight-stranded antiparallel β sandwich motif. It contains three divalent cations containing zinc ions in the active site, and that amino acid residues involved in zinc-coordination are essential for the enzymatic activities. Nagahama *et al.* (2002) reported that mixing the individual N-domain and C-domain restores the hemolytic activity. The fold of the C-domain is similar to those of the "C2" and "C2-like" domains, present in eukaryotic proteins involved in signal transduction. This finding suggested that C-domain plays a role in membrane interaction and promote binding of to lipid bilayer (Naylor *et al.*, 1998).

Acrylodan-labeled C-domain variants bind to liposomes and exhibit internalization of the domain into the hydrophobic environment in liposomes. As membrane-damaging action of alpha-toxin is dependent on membrane fluidity, invasion of the C-domain into the bilayer may play an important role in its action (Nagahama *et al.*, 2002).

Moreover, diacylglycerol, hydrolytic product of lecithin, activates protein kinase C, leading to stimulation of eukaryotic cell phospholipases C and D and the arachidonic acid cascade, thereby inducing intercellular adhesion molecule 1, interleukin-8, TNF-α, platelet-activating factor, and the endothelial leukocyte adhesion molecule. Cumulatively, these events contribute to blood vessel contraction, increased vascular permeability, platelet aggregation and myocardial dysfunction, all of which results in to clinical manifestations characterized by profound shock and death. The toxin also induces the formation of platelet/platelet aggregates. Further, cells exposed to the toxin undergo morphological changes similar to those induced by exposure to TNF-α or IFN-γ (Bryant *et al.*, 2003).

Beta toxin

C. perfringens types B and C produce β-toxin, which is both lethal and necrotic. Although type B strains cause concern in veterinary medicine, type C isolates can be important in human disease and are

responsible for necrotic enteritis, which has decimated poorly nourished individuals in post war Germany and New Guinea, where the disease is known as darmbrand and pigbel, respectively.

Beta toxin was purified and partially characterized in the late 1970s (Sakurai and Duncane, 1977) and shown to correspond to a heat-sensitive 28-kDa protein which is highly sensitive to trypsin. Shatursky *et al.* (2000) later found that the toxin formed pores in phospholipid bilayer of sensitive cells, which provides evidence that the toxin could function as a neurotoxin and produce arterial constriction. It was discovered that sweet potatoes, a vegetable staple often served as an accompaniment to pork (the usual source of contamination), contain a potent trypsin inhibitor, which may contribute to the incidence of Pigbel (Granum, 1990). Although there are unconfirmed reports that, β-toxin production may be associated with a plasmid, very little genetic information is available.

Epsilon toxin

The ε-toxin is the most potent toxin that causes oedema in various organs and is cytotoxic to Madin–Darby Canine Kidney (MDCK) cell cultures. It is produced by type B and D strains and causes a rapidly fatal enterotoxemia which is commonly referred to as pulpy kidney or overeating disease (McDonel, 1986). The secreted protein (32.9 kDa) is poorly active and is called a prototoxin. The prototoxin is activated by proteases such as trypsin, α-chymotrypsin, and λ-protease by proteolytic cleavage. It results in a reduction in size (28.6 kDa) and an important decrease in the p*I* value, probably accompanied by a conformational change (Minami *et al.*, 1997).

Epsilon toxin gene has been cloned and sequenced to produce a second-generation veterinary vaccine and preliminary mapping studies indicate a plasmid location. The ETX gene is located on large plasmids in *C. perfringens*, like the other main toxin genes (beta and iota), which are used for *C. perfringens* typing. The ETX gene harboured by diverse plasmids; at least five (48 – 110 kb) in *C. perfringens* type D (Sayeed and McClane, 2007) and a 65 kb plasmid in *C. perfringens* type B; have been described (Sayeed and McClane, 2010). Plasmids carrying the ETX gene in *C. perfringens* types B and D have probably evolved from a common ancestor by insertion of mobile genetic elements (Miyamoto *et al.,* 2008). As immunity can be conferred by vaccination with a toxoid preparation, the gene for ε-toxin represented a major target for biotechnologists.

Among the symptoms produced by ε-toxin are increased intestinal permeability, lung edema, and excess pericardial fluid accumulation. Its most striking effect is on the kidneys, which become swollen and hyperemic or, in sheep, pulpy a few hours before death. ETX is able to cross the blood–brain barrier and stimulate the release of glutamate, which is the root cause of nervous excitation in animal enterotoxemia. ETX causes rapid swelling in cells followed by cell death involving necrosis.

ETX retains an elongated form and contains three domains that are mainly composed of β sheets. The overall structure of ETX is significantly related to that of the pore-forming toxin aerolysin (although having poor sequence identity) produced by *Aeromonas* species (Gurcel *et al.,* 2006), and to the model of alpha toxin from *C. septicum*, an agent of gangrene (Melton *et al.,* 2004). However, ETX is a much more potent toxin than aerolysin and *C. septicum* alpha toxin having 100 times more lethal activity in mouse (Minami *et al.,* 1997; Tweten, 2001). Domain 1 of ETX consists

of a large α helix followed by a loop and three short α helices, which interacts with the glucosyl phosphatidylinositol anchors of proteins. A cluster of aromatic residues (Tyr49, Tyr43, Tyr42, Tyr209 and Phe212) in domain 1 is involved in receptor binding. Domain 2 is a β-sandwich structurally related to domain 3 of aerolysin. This domain contains a two stranded sheet with a sequence predicted to be the channel-forming domain (Knapp *et al.,* 2009). Domain 3 is also a β-sandwich analogous to domain 4 of aerolysin and contains the cleavage site for toxin activation. Domain 3 is likely involved in the monomer–monomer interaction step of the oligomerization process (Knapp *et al.,* 2010).

Iota toxin

The iota toxin is exclusively produced by type E strains and implicated in sporadic diarrheic outbreaks among calves and lambs and linked to a highly conserved enterotoxin gene localized on the same plasmid. Although *C. perfringens* iota toxin was initially described by Bosworth (1940), its binary nature was elucidated 45 years later by exploiting cross-reacting antiserum against *C. spiroforme* (Stiles and Wilkins, 1986).

The two proteins that comprise iota toxin were then designated as Iota a or Ia (slow moving) and Iota b or Ib (fast moving), based upon electrophoretic mobility in crossed immuno-electrophoresis. The Ia or Ib are separately non-toxic however, mixture of Ia–Ib is a potent cytotoxin that is lethal to mice and dermonecrotic in guinea pigs (Gibert *et al.,* 2011). Ia is an ADP-ribosyltransferase specific for actin wheareas Ib, although lacking any discernible enzymatic activity, binds to a cell surface protein and subsequently translocates Ia into the cytosol of a targeted cell (Richard *et al.,* 2002). This results in inhibition of cell functions by actin filament depolymerization.

Thus far, the role of iota toxin as a virulence factor is unknown. In one report, iota toxin showed positive effects on adherence and colonization of *C. perfringens* type E by altering the enterocyte morphology and strongly inhibit intra-specific growth of other strains (Redondo *et al.,* 2015).

Theta toxin

All five types of *C. perfringens* produce a lethal hemolysin, θ-toxin, which is also known as θ-hemolysin, perfringolysin O, or the thiol-activated cytolysin**.** The primary sequence of θ-toxin, deduced from the *pfoA* gene sequence, revealed a 494-residue pre-protein from which a 28-residue signal peptide is removed and secreted to the medium. Mature θ-toxin has a predicted molecular weight of 52,469 Da and, when purified from *C. perfringens* or *E. coli*, appears as a 54 kDa species (Tweten, 1988). Theta toxin is placed in cholesterol-binding toxin family as they use cholesterol as a receptor and form large pores in target cell membranes. Diverse group of gram-positive bacteria including the *Bacillus, Streptococcus, Clostridium*, and *Listeria* genera also belong to the same family (Iwamoto *et al.,* 1993).

The θ-toxin plays a role in the tissue necrosis associated with *C. perfringens* gas gangrene and is responsible for the depletion of PML (polymorphonuclear leukocytes) in the affected area.

Low concentrations of θ-toxin cause altered polymorphonuclear leukocyte morphology, metabolism, and migration. Together with α-toxin, the θ-toxin impairs neutrophil migration into the site of infection and to the dysregulation of endothelial cells. This damage causes oedema and ischemia,

leading to reduced oxygen delivery and thus favours the growth of *C. perfringens* (Stevens *et al.,* 1997)

Carbohydrate-active enzymes (μ-Toxin and Sialidases)

Among exotoxins of *C. perfringens,* are a considerable battery of large extracellular carbohydrate-active enzymes, including hyaluronidase μ-toxin which destroys the polysaccharide hyaluronan, and the large sialidases, which remove terminal sialic acid sugars and enhance the lethal cytolytic phospholipase activity of the α-toxin (Boraston *et al.,* 2007). It is a common contaminant in commercial preparations of neuraminidase. The individual modules of such carbohydrate-active enzymes perform a variety of functions, which are most commonly catalysis, protein-carbohydrate interactions, or protein-protein interactions, and these contribute to the overall function and efficiency of the protein.

The *nagH* gene was cloned and sequenced (Canard *et al.,* 1994) and shown to encode a P-N-acetylglucosaminidase which could correspond to μ-toxin. The gene is present in all *C. perfringens* isolates examined, and its product is a 97 kDa secreted protein. Truncated derivatives of *nagH*, containing as little as 421 of the 943 codons, encode enzymatically active products; suggesting multidomain structure of the enzyme. Although definitive identification is still required, it is conceivable that the primary role of the NagH protein / μ-toxin is in cell wall biosynthesis or autolysis and that its action as a virulence factor is secondary. Recently, Ficko-Blean (2009) described the complete structure of NagJ, a 1001-amino acid multi-modular homolog of the *C. perfringens* μ-toxin, which was determined using a combination of small angle x-ray scattering and x-ray crystallography. The structure revealed unprecedented insight into catalytic activity, carbohydrate-specific adherence, formation of molecular complexes with other enzymes, and its involvement in host-pathogen interaction.

C. perfringens strains typically possess three sialidase-encoding genes, namely *nanH*, *nanI* and *nanJ*, which are located on a conserved region of the chromosome. The *nanH* gene product, which is not secreted, is the ~43 kDa NanH sialidase. The *nanI* and *nanJ* gene products are secreted: NanI sialidase (~77 kDa) and NanJ sialidase (~129 kDa), respectively (Boraston *et al.,* 2007). Sialidase (neuraminidases) production by some pathogenic bacteria has been implicated in their virulence. Sialidases can also apparently contribute to virulence in other ways besides enhancing toxin binding like by providing nutrients for growth, biofilm formation, and enhancing colonization by exposing adhesion sites (King, 2010).

Possible sialidase contributions to *C. perfringens* virulence have received only limited attention. Chiarezza *et al.* (2009) used mutants to evaluate the potential pathogencity contributions of NanI and NanJ sialidase when *C. perfringens* type A strain 13 causes clostridial myonecrosis (gas gangrene).

The study found that sialidases can enhance alpha-toxin-mediated cytotoxic effects *in vitro*, and that sialidase production is not necessary for strain 13 to cause gas gangrene in a mouse model. Possible virulence contribution of sialidases with other *C. perfringens* strains, such as type D strains, has received even lesser attention. Li *et al.* (2011) used both biochemical and isogenic mutant approaches to better evaluate the possible sialidase enhancement of *in vitro* ETX action and its possible role in facilitating *C. perfringens* cell adhesion to host cells.

Enterotoxin

C. perfringens enterotoxin (CPE) causes the symptoms associated with several common gastrointestinal diseases. Most, but not all, *C. perfringens* type A food poisoning strains carry their enterotoxin gene (*cpe*) on the chromosome (McClane, 2007). CPE is a 35 kDa polypeptide and the structure that was reported by Kitadokoro *et al.* (2011) revealed that it consists of three domains; domain I which is C-terminal, responsible for receptor binding, domain II is responsible for oligomerization and membrane insertion, and domain III takes part in physical changes in course of insertion into membranes. Native CPE binds to claudin receptors, a components of the tight junction. The bound toxin then assembles into a hexameric prepore form on the membrane surface, prior to the insertion of this oligomer into membranes to form an active pore (Anderson and Van Itallie, 2009).

NetB Toxin

The *C. perfringens* necrotic enteritis B-like toxin (NetB) is a recently discovered member (Keyburn *et al.,* 2008) of the β-barrel pore-forming toxin family and is produced by a subset of avian *C. perfringens* type A strains. NetB is cytotoxic for avian cells and is associated with avian necrotic enteritis (Lovland and Kaldhusdal, 2001). Its main cell wall component, peptidoglycan (PGN), can be recognized by Toll-like receptor 2 and nucleotide-binding oligomerization domain (NOD). Consequently, the immune response is initiated via activation of nuclear factor kappa B (NF-κB) signalling pathway (Guo *et al.,* 2015). Fernandes da Costa *et al.* (2014) identified amino acids that play a role in NetB oligomerisation and pore-formation using site-directed mutagenesis.

Other toxins and Hydrolytic enzymes

The remaining toxins produced by different *C. perfringens* strains are 5-toxin: α hemolysin, κ-toxin, a collagenase; λ-toxin, a protease; ν-toxin; α nuclease; and eta and gamma toxins, whose existence is dubious.

C. perfringens secretes a variety of hydrolytic enzymes that degrade extracellular substrates and components resulting from cell lysis. These enzymes act synergistically with membrane-damaging toxins during cell disruption. They provide nutrients for *C. perfringens* growth and also contribute to the tissue disorganization observed in the gangrenous lesions. The λ-protease also participates in the activation of ε- and ι- toxins (Minami *et al.,* 1997).

Major infectious diseases caused by *Clostridium perfringens* and their pathogenesis

Enterotoxemia

Enterotoxemia, also called pulpy kidney disease in lambs, is characterized by high levels of toxin production in the intestine. These toxins then pass through the intestinal barrier and disseminate via the circulation (toxemia) to several organs, causing toxic shock and death. Type D strains cause enterotoxemia and high production of ε toxin in the intestine and subsequent disease are conditioned by an overgrowth of ETX-producing *C. perfringens* (Bullen, 1970). A primary target of ETX is the central nervous system, where it produces foci of liquefactive necrosis, perivascular edema, and hemorrhage, especially in the meninges (Buxton *et al.,* 1978).

C. perfringens type D enterotoxemia is very common in lambs, less frequent in sheep and goats, and occasional in other animal

species. Rapidly growing lambs are most susceptible. More recently, the pathology of enterotoxemia by *Clostridium perfringens* type C has been described. The gross changes observed in the gastrointestinal tract of calves consisted of multifocal subserosal haemorrhages of the rumen, diffuse congestion and multifocal haemorrhages of the small intestinal mucosa, dilated cecum with bloody liquid contents and diffuse coagulation necrosis of the intestinal mucosa (Garcia *et al.,* 2013).

Necrotic enteritis

Necrotic enteritis (NE) is being considered among the most important infectious diseases in the current poultry production system globally. A novel pore forming toxin, NetB, has been identified in these virulent avian *C. perfringens* strains (Keyburn *et al.,* 2008). Furthermore, it has now been established that only certain *C. perfringens* strains are capable of inducing necrotic enteritis under specific conditions that predispose to the disease and they constitute only a minority in the intestinal tract of healthy chickens (Pedersen *et al.,* 2008). Recently, isolates from healthy and diseased turkey were characterized for the presence of *cpa*, *cpb*, *iA*, *etx*, *cpb2*, *cpe*, and *netB* genes (Lyhs *et al.,* 2013).

It has been reported that proteolytic enzymes have an important role in the initial stages of necrotic enteritis where the villi and the lateral domain of the enterocytes are affected (Olkowski *et al.,* 2008).

Food poisoning in human

C. perfringens is also one of the major bacterial causes of human food poisoning. The most common cause of *C. perfringens* associated food poisoning is sporulation of *C. perfringens* vegetative cells once it is consumed and then production of enterotoxin in the gut. It has been demonstrated that production of CPE is essential for CPE-positive type A disease isolates to cause gastrointestinal (GI) effects in animal models. The outcome of CPE action during GI disease is desquamation of the intestinal epithelium, intestinal necrosis, and the accumulation of luminal fluid (McClane *et al.,* 2006). These effects account for diarrhoea and abdominal cramps. Typically, people are sickened with *C. perfringens* type A food poisoning for 12–24 hours and then recover. However, this illness can be fatal in the elderly or in people suffering from medication-induced constipation (Bos *et al.,* 2005).

Despite the importance of spore formation in *C. perfringens* pathogenesis, the details of the regulation of sporulation have not yet been defined fully. In a recent study by Ohtani *et al.* (2013), a candidate gene (the RNA regulator virX) was identified for the repression of genes encoding positive regulators (SpoA and sigma factors) of *C. perfringens* sporulation. The virX RNA regulator plays a key role in the drastic shift in lifestyle of the anaerobic flesh eater *C. perfringens* between the vegetative state (for gas gangrene) and the sporulating state (for food poisoning). Since virX homologues were not found in any *Bacillus* species but were present in other *Clostridial* species, their findings identify further differences in the regulation of sporulation between *Bacillus* and certain *Clostridium* species.

Clostridial myonecrosis or gas gangrene

Myonecrosis with gas gangrene is the most fulminant disease that affects humans and is typically caused by *C. perfringens* type A. Rapid destruction of viable, healthy tissue is characteristic of gas gangrene due to *Clostridium perfringens*. Indeed, in victims

of traumatic injury – whether on the battlefield or following accidents and natural disasters such as earthquakes – clostridial myonecrosis can become well established in as little as 6–8 h and the destruction of adjacent healthy muscle can progress several inches per hour despite appropriate antibiotic coverage (Stevens *et al.,* 2005). The rapid progression of infection and tissue necrosis associated with clostridial gas gangrene is related to the absence of an acute tissue inflammatory response, to tissue perfusion deficits resulting from toxin-mediated vascular dysfunction and injury, and to the elaboration of potent cytotoxins and proteases (Bryant *et al.,* 2006). Similarly, the onset of severe pain in gas gangrene is "sometimes so sudden as to suggest a vascular catastrophe".

Most of the knowledge about bacterial toxin-mediated vascular dysfunction in these necrotizing infections comes from studies involving phospholipase C (PLC), the principal lethal (alpha) toxin of *C. perfringens*. Many more studies have shown that within minutes, PLC stimulates the formation of large intravascular aggregates of platelets and leukocytes that irreversibly block blood flow and impair leukocyte extravasation into infected tissues (Bryant *et al.,* 2006). The θ toxin of *C. perfringens* (known as perfringolysin O) also contributes to aggregate formation and aggregate-mediated vascular occlusion.

Clostridium perfringens vaccines

Currently available research tools are becoming very important in the field to develop more effective vaccines that will prevent lethal diseases produced by *C. perfringens.* The recombinant vaccines are well-defined and standardized and thus the immunogenicity of these vaccines is more uniform than the conventional vaccines.

In addition, recombinant vaccine is easier and cheaper to produce as it is bypassing the need to grow large volumes of a dangerous pathogen, relying instead on the culture of a laboratory strain of *E. coli* or any other host. Finally, taking advantage of highly purified form, it could be combined in future with other purified vaccine proteins to produce a multivalent vaccine formulation to protect against a range of diseases.

Alpha toxin vaccines

During the middle part of the 20th century several workers explored the possibility that immunisation could be used to prevent gas gangrene, caused by alpha toxin. Work on the generation of α-toxoids commenced in the 1930s and probably reached a peak of activity during World War II. Two approaches to the development of α toxoid vaccine have been proposed: the expression of non-toxic fragments (or domains) of α-toxin or the use of non-toxic forms of the whole toxin as immunogens.

The use of fragments as immunogens is driven largely by the finding that α-toxin is composed from 2 domains (Naylor *et al.,* 1998) which are associated with phospholipase C activity (N-domain) and membrane recognition (C-domain), respectively. These domains can readily be produced using recombinant DNA technology in *E. coli*. However, only the immune response against the C-domain provided protection against a subsequent challenge with α-toxin and experimental gas gangrene in mice (Stevens *et al.,* 2005). Protection appeared to extend to a range of sequence-variant forms of the toxin from different strains of *C. perfringens* and even to homologues of α-toxin found in *C. bifermentans* and *C. absonum* (Neeson *et al.,* 2007).

Immunisation of mice using vaccinia virus, expressing the carboxy-terminal segment of the α-toxin gene fused to glutathione-S-transferase (GST-C-domain), resulted in the induction of an immune response capable of protecting against toxin (Bennett *et al.,* 1999). Zeng *et al.* (2011) developed recombinant α-toxin, β2/β1-fusion toxin and α/β2/β1 trivalent fusion-toxin as vaccine candidates that may be used to vaccinate against *C. perfringens* α, β1 and β2-toxins. Mice immunized with these recombinant toxoids demonstrated a strong protective immunological response when administered a lethal dose of *C. perfringens.* Earlier, recombinant α-β fusion protein was produced and used for immunization and shown to be immunogenic. The antibody induced by immunization with α-β fusion protein could neutralize the toxicity of α-toxin and β-toxin (Bai *et al.,* 2006).

More recently, the recombinant α toxin and PLC were expressed as glutathione S-transferase (GST) fusion proteins. Partial protection from the toxin and *C. perfringens* was elicited by immunization with these fusion proteins suggesting it as a promising candidate for vaccine against clostridial-induced gas gangrene (Nagahama *et al.,* 2013). An alternative approach to the use of domains of α-toxin as vaccines is to exploit naturally occurring or genetically engineered variant forms of α-toxin with markedly reduced toxicity. The prior vaccination of mice with a naturally occurring α-toxin, devoid of enzymatic (PLC), hemolytic and lethal activity has been shown as a useful vaccine candidate (Schoepe *et al.,* 2006).

In one study, a bivalent chimeric protein r-Cpae is synthesized comprising C-terminal binding regions of alpha toxin (αC) and enterotoxin (CPE) using structural vaccinology rationale and demonstrated its efficacy as a potential sub unit vaccine candidate against αC and CPE of *C. perfringens* type A toxaemia (Shreya *et al.,* 2015). While in another study, Uppalapati and co-workers (2014) synthesized a non-toxic chimeric molecule r-αCS encompassing the binding domains of *C. perfringens* and *S. aureus* alpha toxins and assessed its protective efficacy against alpha toxin mediated *C. perfringens* and *S. aureus* soft tissue co-infections.

Epsilon toxin vaccines

Toxoid vaccines for use in domesticated sheep and goats are widely available commercially and have been used extensively over the past decades. Although these toxoid vaccines are effective in preventing enterotoxaemia in animals, there are reports of variable immune responses following vaccination.

Recombinant vaccine against ε toxin has received lesser attention than vaccines against α-toxin. As a veterinary vaccine for sheep, lambs and cattle, the non-toxic site-directed mutated toxin has several advantages over the commercial toxoid vaccine. Consequently, the focus was on the evaluation of site-directed mutants with the intention of producing a stable and non-toxic mutated ε-toxin which could be considered as a vaccine candidate. Eventually, of 10 mutants tested, one non-toxic mutated toxin was identified and immunisation of mice with this non toxic ε toxin mutant resulted in the induction of an antibody response against ε-toxin and immunised mice were protected against a subsequent challenge with 1000 MLD doses of wild-type ε-toxin (Oyston *et al.,* 1998). A single (conformational) epitope on the toxin has been previously shown to be sufficient to protect against purified ε-toxin but the location of this epitope is not known (Percival *et al.,* 1990). Use of recombinant combined vaccines also opens a number of avenues for the animal industry. Chandran *et*

al. (2010) reported that the recombinant epsilon toxoid with an adjuvant preparation and freeze dried attenuated combination vaccine protected the sheep against both sheep pox and enterotoxemia.

Formalin-inactivated toxin can successfully induce antibody-mediated protection in animals, but their usefulness in humans is limited due to safety concerns. For this reason, developing recombinant, attenuated vaccines based on a detailed understanding of the molecular mechanisms by which this toxin function has become a research target. Epsilon toxin encoding *etx* gene, was cloned into pET vector and recombinant epsilon toxin (rec-ε) was expressed in inclusion bodies and was used for animal immunization. Serum protection was evaluated and cross-serum neutralization tests were used to characterize the recombinant toxin. Based on the findings, this rec-ε was considered as a good candidate for vaccine production against enterotoxemia caused by epsilon toxin of *C. perfringens* type D (Souza *et al.,* 2010). Site-directed mutant of Etx was exploited as a recombinant vaccine against enterotoxemia in another study. Protection study in rabbits suggested that mutant could form the basis of an improved recombinant vaccine against enterotoxemia (Bokori-Brown *et al.,* 2014).

NetB toxin vaccines

As the recently identified pore-forming toxin NetB (Keyburn *et al.,* 2008; 2013) is a key virulence determinant of necrotic enteritis in chickens, so it is a promising target for vaccine development. Vaccine development for necrotic enteritis in chicken was previously focused on α-toxin, but the experimental vaccines did not produce the level of efficacy.

Several *C. perfringens* recombinant proteins, principally NetB toxin and perfringolysin O (PFO), in combination with the Montanide TM ISA 71 VG adjuvant were identified as novel vaccine candidates for experimental Necrotic Enteritis (NE). Protection against field NE might be associated with augmented humoral and cellular immune responses (Jang *et al.,* 2012). The efficacy of NetB as a vaccine antigen to protect chickens from necrotic enteritis was examined using purified recombinant NetB (rNetB). Birds immunized with rNetB were significantly protected against necrotic enteritis when challenged with a mild oral dose of virulent bacteria (Keyburn *et al.,* 2013). Unfortunately, till date there are no reports in the literature addressing the use of NetB in vaccine formulations.

Clostridium perfringens is the most widely occurring pathogenic bacterium, readily found in soil samples and intestinal contents of animals and humans. *C. perfringens* has been shown to be cause of several human and animal diseases. Isolates of *C. perfringens* is divided into five types from A to E based on the particular toxins which they secrete. Subunit vaccines based on selected antigens recognized as the safest type of antibacterial vaccines. Choice of antigen in the development of effective subunit vaccines is the crucial step because a proper candidate for immunization must possess a wide range of different properties such as extracytoplasmic localization, abundance in the cell, capability of stimulating the immune system and conservation among different pathogens serotypes / genotypes. Moreover, a vaccine candidate should also express *in vivo* during pathogen infection.

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