Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(6): 2155-2164
Received: 19-09-2017
Accepted: 20-10-2017

Sumiyah Rasool
Department of Microbiology, Skuast-k, Jammu and Kashmir, India

Tufail Hussain
Department of Medicine, Skuast-k, Jammu and Kashmir, India

Shafqut Majeed Khan
Department of Microbiology, Skuast-k, Jammu and Kashmir, India

Asima Zehra
Department of Public Health, Gadvasu, Ludhiana, Punjab, India

Syed Tahreem
Department of Microbiology, Skuast-k, Jammu and Kashmir, India

Abha Marium Kakroo
Department of Pathology, Skuast-k, Jammu and Kashmir, India

Correspondence
Sumiyah Rasool
Department of Microbiology, Skuast-k, Jammu and Kashmir, India

Toxins of Clostridium perfringens as virulence factors in animal diseases

Sumiyah Rasool, Tufail Hussain, Shafqut Majeed Khan, Asima Zehra, Syed Tahreem and Abha Marium Kakroo

Abstract
Clostridium perfringens is a Gram-positive, rod shaped, anaerobic, spore forming bacterium and is responsible for various forms of acute enteric diseases called enterotoxemias, in sheep, goats, and other animals. Based on the toxin production, C. perfringens strains are classified into five toxinotypes, namely alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX). However, this microorganism has the ability to produce more than 16 toxins in various combinations, including lethal toxins such as perfringolysin O (PFO), enterotoxin (CPE), and beta2 toxin (CPB2). C. perfringens produce many toxins with diverse characteristics which imparts a virulence flexibility to it in order to cause an array of diseases. Most diseases caused by this microorganism are mediated by one or more of these toxins. Many animal models experiments have been done for C. perfringens infection and the use of toxin gene knock-out mutants have demonstrated the specific pathogenic role of several toxins of C. perfringens in animal diseases.

Keywords: Clostridium perfringens, toxins, animal diseases

Introduction
Clostridium perfringens is a Gram-positive, spore-forming anaerobe under the genus *Clostridium*. It was first isolated by William Welch and George Nuttall at Johns Hopkins Hospital (Baltimore, MD, USA) following autopsy of a cancer/tuberculosis patient, eight hours post-death and was termed as *Bacillus aerogenes capsulatus* (Welch and Nuttall, 1892) [103]. Most of clostridial toxins are pore forming toxins (PFTs) belonging to the PFT class. They are secretory proteins rich in 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 barrel, which form functional pore into the lipid bilayer (Popoff, 2014) [63].
C. perfringens can be subtyped into five toxin types (A, B, C, D and E) based on the production of four major toxins: alpha, beta, epsilon and iota (Songer, 1996) [82]. Molecular typing of the major toxins produced by *C. perfringens* has been done by many researchers (Engstrom *et al.*, 2003; Nauerby *et al.*, 2003) [17, 59]. Several new virulence factors viz; enterotoxin, net B, Lux S, elongation factor Tu (EF-Tu), pyruvate : ferridoxin oxidoreductase (PFO) and A/B toxin have been studied by a number of workers (Lee *et al*., 2011; Silva *et al*., 2013) [39, 79].

Alpha toxin (CPA)
The most important virulence factor produced by clostridial myonecrosis (gas gangrene)-causing isolates of *C. perfringens* is the toxin, the first toxin for which an enzymatic activity, phospholipase C, was demonstrated (MacFarlane and Knight, 1941) [48]. 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; the N domain, consisting of nine tightly packed helices, and the C-domain, consisting of an eight stranded antiparallel 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. 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) [60].

Role of CPA in Animal Diseases
Intestinal Infections—In sheep, *C. perfringens* type A causes yellow lamb disease, a rare form of acute enterotoxaemia in lambs. Information about pathogenesis of this disease is

minimal and often contradictory, but it is generally assumed that most clinical signs and lesions are due to the effects of CPA (McGowan *et al*., 1988). However, no definitive proof of the action of this toxin in the pathogenesis of yellow lamb disease has been provided. The disease is characterized clinically by depression, anemia, icterus, and hemoglobinuria. Necropsy findings include pale and friable liver and spleen and the presence of red urine in the urinary bladder. Histopathological changes include periacinar necrosis of the liver, splenic congestion, nephrosis with hemoglobin casts, and pulmonary congestion and edema (Manteca *et al*., 2002) [44]. *C. perfringens* type A has been and is still frequently blamed for enteritis, abomasitis and/or enterotoxaemia in cattle (Songer and miskimins, 2005) [75-86], horses (Timoney *et al*., 2005; Waters *et al*., 2005) [90, 100], goats (Songer, 1998) [85] and pigs (Saenz *et al*., 2007) [67].

Gangrene/Malignant Edema—Studies with isogenic mutants strongly suggest that CPA is the major *C. perfringens* toxin involved in gas gangrene in humans (Awad *et al.*, 2001) [2], which is characterized by extensive local tissue destruction and necrosis progressing to shock and death (Flores *et al*., 2003) [25]. *C. perfringens* type A is also involved in gas gangrene (malignant edema) of domestic mammals including sheep, cattle, goats, and horses, acting alone or in combination with other clostridial species. Predisposing factors for this disease in animals include trauma associated to injections, shearing, castration, docking and milking. Also, the fact that most cases of malignant edema in food producing animals are associated with a mixed clostridial infection (which may or may not include *C. perfringens* type A) suggests that, even if CPA is responsible for some cases of malignant edema, other clostridial toxins can also produce this disease (Blood *et al*., 1983) [7]. Grossly, malignant edema is characterized by severe subcutaneous and muscular edema, skeletal muscle necrosis and generalized petechiae on serous membranes, the latter being a manifestation of terminal disseminated intravascular coagulation due to toxemia. Histologically, in contrast to other bacterial myonecrosis, *C. perfringens* gas gangrene and malignant edema are characterized by a marked absence of inflammatory cells in affected tissues and the accumulation of leukocytes within vessels at the lesion periphery, most likely due to the inhibitory effect that CPA has over leukocyte migration. The myotoxic effects could result from a direct activity of CPA on muscle cell membrane, from alteration of local blood flow, and/or inflammatory mediators as a consequence of endothelial cells stimulated by CPA (Stevens *et al*., 1988) [84]. The role of CPA in gas gangrene was demonstrated by protection of mice against this disease by immunization with recombinant CPA, and by reduced virulence of CPA knock-out mutants of *C. perfringens* when injected into mice (Awad *et al.*, 2001) [2].

Beta toxin (CPB)

C. perfringens types B and C produce 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. The *cpb* gene encodes a prototoxin of 336 amino acids that includes a 27-amino acid signal sequence removed during secretion, resulting in a mature toxin of ~35 kDa (Sakurai and Duncan, 1978) [69]. Purified CPB is thermolabile, with >90% of its lethal activity being inactivated by incubation for 1 h at 50°C (or 10 min at 100°C). CPB is also highly sensitive to *in vitro* trypsin and pepsin treatment (Shatursky *et al*., 2000) [76]. Low pH does not seem to affect its activity (Uzal *et al*., 2009) [95]. CPB is encoded by the *cpb* gene, which is carried on virulence plasmids (Katayama *et al*., 1996) [34] of different molecular size. The beta-toxin is a highly trypsin-sensitive protein that causes mucosal necrosis and possibly central nervous signs observed in domestic animals. It is responsible for the diseases caused by *C. perfringens* type C which includes struck of sheep and enterotoxemia of lambs, calves and piglets and necrotic enteritis of man and fowls.

Role of CPB in Animal Diseases

CPB is responsible for diseases in several animal species and it is produced by types B and C of *C. perfringens*. Type B isolates cause an often fatal hemorrhagic dysentery in sheep, and possibly in other species, while type C isolates cause enteritis necroticans (also called pigbel) in humans and necrotic enteritis and/or enterotoxaemias in almost all livestock species. Both types B and C animal disease are often accompanied by sudden death or acute neurological signs (McClane *et al*., 2004) [50]. Type B disease is a rare occurrence in farm animals and mostly seen in Middle East countries and the UK. Clinical disease can be characterized by diarrhea, neurological signs or both. Post-mortem gross and histopathological findings are similar to those of type C infections, except for those rare cases of focal symmetrical encephalomalacia, believed to be due to the effect of ETX (Uzal, 2004) [96]. Clinical signs and histopathologic findings in type C infections are very similar in most livestock animal species. The course of disease can be peracute, acute, or chronic, with signs of the acute and peracute condition including intense abdominal pain, depression, and bloody diarrhea. Chronic disease can occur in older animals of some species (e.g. pigs), and it is characterized by persistent diarrhea without blood and dehydration (Songer, 1998) [85]. At necropsy, the predominant lesions are most frequently observed in small intestine, but caecum and spiral colon can sometimes be involved; occasionally, lesions may be confined to large intestine. Lesions are similar in all segments of intestine, and in acute cases consist of intestinal and mesenteric hyperemia, diffuse or segmental, extensive fibrin necrotic enteritis, with emphysema and bloody gut contents. Mesenteric lymph nodes are red, an excess of hemorrhagic peritoneal and pleural fluid is found, there may be fibrin strands on intestinal serosa, and adhesions may develop between intestinal loops. Diffuse edema with variable amounts of protein and inflammatory cell exudate can be seen throughout all intestinal layers, including serosa (Songer and Uzal, 2005) [75-86].

C. perfringens types B and C disease begins in the host intestine with absorption of toxin (s) from the intestines into the circulation that then leads to the death of an infected animal. Different animal models have been developed to help in understanding the mechanism of those diseases involving CPB intoxication (Nagahama *et al*., 2008) [57]. Purified CPB produces abundant dermonecrosis, edema and plasma extravasation when directly injected into the skin of guinea pigs or mice (Nagahama *et al*., 2003) [58]. CPB also provokes the release of TNF-α and IL-1β, as well as activates tachykinin NK1 receptors by a still unknown mechanism (Nagahama *et al*., 2008) [57]. *In vivo*, type B and type C isolates produce intestinal lesions consisting of diffuse or

multifocal hemorrhagic and necrotizing enteritis, mainly in the ileum, with excess of sanguineous serous fluid in the abdominal cavity. Similar intestinal lesions have been observed after inoculating purified CPB into rabbit intestinal loops (Vidal *et al*., 2008) [99]. In recent work, Miclard and his co-workers showed that CPB specifically binds vascular endothelial cells, during peracute or acute cases of type C-induced disease in piglets or humans. The authors reported that binding of CPB to those cells produced acute endothelial degeneration and vascular necrosis (Miclard *et al*., 2009a) [54].

Epsilon toxin (ETX)

ETX is an example of an aerolysin-like, pore-forming toxin. *C. perfringens* ETX and *C. septicum* alpha-toxin are structurally related to aerolysin although ETX shows no significant sequence homology with aerolysin at the amino acid level. ETX and *C. septicum* alpha-toxin form heptameric pores, like aerolysin, and are very potent cytolysins (Sakurai, 1995) [68]. The 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) [51]. The secreted protein (32.9 kDa) is poorly active and is called a prototoxin. The prototoxin is activated by proteases such as trypsin, chymotrypsin, and 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) [55]. ETX is considered the major virulence factor of *C. perfringens* types B and D (Sayeed *et al*., 2007) [73-95]. This toxin causes blood pressure elevation, increased contractility of smooth muscle, vascular permeability increase, as well as brain and lung edema in multiple animal species, while in goats ETX also causes colitis (Tamai *et al*., 2003). ETX is the third most potent clostridial toxin after botulinum toxin and tetanus toxin, with a mouse lethal dose of 100 ng/kg [3]. ETX is secreted as a prototoxin (32,981 Da) (Hunter *et al*., 1992), which is converted into a fully active toxin (~1000 times more toxic than the prototoxin) when activated by proteases such as trypsin, chymotrypsin, and a metalloproteinase named lambda toxin that is produced by *C. perfringens* (Minami *et al*., 1997) [55].

The ETX gene (*etx*) is localized on conjugative plasmids, with most type B isolates possessing the same ~65 kb *etx* plasmid that also carries the gene encoding beta2 toxin (Miyamoto *et al*., 2008) [56]. The *etx* gene in type B, and some type D, isolates is flanked by IS*1151* and a gene related to the transposase (*tnpa*) gene from Tn*3* transposons that lie upstream of *etx.* A region with similarity to transposases from *S. aureus* and *Lactococcus* is located downstream from *etx* (Hughes *et al*., 2007) [32].

As immunity can be conferred by vaccination with a toxoid preparation, the gene for toxin represented a major target for biotechnologists. Among the symptoms produced by 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 sheets. The overall structure of ETX is significantly related to that of the poreforming toxin aerolysin (although having poor sequence identity) produced by *Aeromonas* species (Gurcel *et al.*, 2006) [30], and to the model of alpha toxin from *C. septicum*, an agent of gangrene (Melton *et al.*, 2004) [53]. However, ETX is a much more potent toxin than aerolysin and *C. septicum* alpha toxin having 100 times more lethal activity in mouse (Tweten, 2001) [91].

Role of ETX in Animal Diseases

ETX-producing *C. perfringens* type D strains are the most common cause of clostridial enterotoxemia in sheep and goats. Since the gross and histological changes observed in type D enterotoxemia have been reproduced by intravenous inoculation of ETX in sheep and goats. *C. perfringens* type D produces an acute, subacute, or chronic neurological condition in sheep, characterized by sudden death or neurological and respiratory signs, including blindness, opisthotonos, convulsions, bleating, frothing from the mouth, and recumbency with paddling immediately before death (Uzal and Kelly, 1997) [92-94]. Diarrhoea is occasionally observed, although this is not a common clinical sign in sheep. When present, intestinal gross changes consist of hyperemic small intestine mucosa with slight to marked red fluid contents. Colitis may occur, but is not a consistent finding in sheep enterotoxemia. Several gross findings, such as excess pericardial, pleural, and/or abdominal fluids (with or without fibrin strands), which may clot on exposure to air, serosal petechiation, and lung edema are frequently seen (Uzal, 2004) [96]. Gross changes in sheep are rarely observed in brain, and consist of herniation of the cerebellar vermis (cerebellar coning) in acute or subacute cases and focal symmetrical encephalomalcia (FSE), in chronic cases. FSE is characterized by dark hemorrhagic foci in corpus striatum, thalamus, midbrain, and cerebellar peduncles and white matter. The kidney lesion from which one of the common names of the disease is derived (pulpy kidney disease) is likely to be a postmortem change (Barker *et al*., 1993) [4].

Microscopic changes in the brain of sheep with type D infection are unique and pathognomonic, and they are present in approximately 90% of cases (Uzal *et al*., 2004) [96]. They consist of perivascular proteinaceous edema (microangiopathy) in the brain In the brain, microvascular endothelial damage by this toxin appears to be the fundamental cause of cerebral parenchymal injury and lesions occur in an apparently dose- and timedependant manner. Large doses of circulating toxin produce a severe, generalized vasogenic cerebral edema and an acute or peracute clinical course to death. With lower doses in partially immune animals, bilaterally symmetrical focal necrosis, occurs in certain selectively vulnerable brain regions (Finnie, 2003) [23].

In goats, type D produces acute, subacute, or chronic disease as well. The acute form occurs more frequently in young, unvaccinated animals and is clinically similar to the acute disease in sheep (Blackwell *et al*., 1992) [6]. The subacute form is more frequently seen in adult goats, vaccinated or not, and is characterized by hemorrhagic diarrhea, abdominal discomfort, severe shock, opisthotonos, and convulsions (Smith and Sherman, 1994) [81]. Gross changes in acute caprine type D enterotoxemia are similar to those in the ovine disease. In the chronic form of the disease in goats, fibrinohemorrhagic colitis with occasional involvement of the distal small intestine seems to be the most consistent lesion (Blackwell *et al*., 1992) [6]. A combination of the findings in acute and chronic forms of disease are frequently seen in subacute forms. The so-called pulpy kidney has not been

reported in caprine enterotoxaemia, nor has cerebellar herniation or FSE. In goats, there are few descriptions of histological changes in type D enterotoxemia, and changes in brain are not considered a consistent feature of caprine enterotoxemia. Nevertheless, perivascular edema and degeneration of white matter, similar to those observed in sheep, can be observed in cases of acute and subacute caprine enterotoxemia (Uzal *et al*., 1997) [92-94].

Iota toxin (ITX)

C. perfringens ITX is a clostridial binary toxin. These toxins have a common structure consisting of two independent protein components that are not covalently linked, one being the binding component (Ib, 100 kDa), and the other the enzymatic component (Ia, 45 kDa). Both components are required for biological activity (Barth *et al*., 2000) [5]. The two proteins that comprise iota toxin were 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) [27]. Ia is an ADPribosyltransferase 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) [65]. 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) [64].

Role of ITX in Animal Diseases

Toxinotype E enteric infection of domestic animals was first reported in the late 1940s, and these infections have generally been considered a rare occurrence. As with type A infections and CPA, infections by *C. perfringens* type E are usually assumed to be mediated by ITX, although no definitive evidence in this regard has been provided. *C. perfringens* type E produces enterotoxemia in rabbits. A very similar disease, thought to be mediated by iota-like toxin, is produced in rabbits by *Clostridium spiroforme.* However, because of cross reactivity between *C. perfringes* type E and *C. spiroforme* toxins with the diagnostic methods currently available, it is thought that some previously reprted outbreaks of entropathies associated with C. perfringensmay have been due to *C. spiroforme*. Both diseases are clinically characterized by diarrhea and loss of condition and characteristic necropsy changes include hemorrhages of the cecal serosa and mucosa, sometimes with involvement of the distal ileum and proximal colon, and the presence of watery mucoid content in these organs. Histologically, there is an acute necrosis of the superficial epithelium with relative sparing of the crypt epithelium, and submucosal hemorrhage and transmural hemorrhage (Perey and Barthold, 2007) [62]. Toxinotype E is an occasional cause of hemorrhagic enteritis and sudden death in beef calves (Songer and Miskimins, 2005) [75-86]. Type E disease has rarely been described in sheep and goats (Songer, 1998) [85].

Beta2 toxin (CPB2)

Over the past decade a variant of the beta toxin known as beta2 (*cpb2*) has been associated with enteric diseases in a wide range of animals including swine, cattle, poultry, sheep, horses, dogs, avian and aquatic species (Boujon *et al*., 2005) [8]. Beta2 toxin has been implicated as an accessory toxin in *C. perfringens* mediated antibiotic associated diarrhea and sporadic diarrhea (Fisher *et al*., 2005) [24]. Schotte *et al.* (2004) [74] observed the clinical signs in CPB2-mediated enteric diseases ranging from pasty to watery diarrhoea with blood in faeces, abdominal pain, and loss of body condition in animals. It was hypothesized that CPB2 toxin may act in synergy with other major toxins of *C. perfringens* in the production of necrotic and hemorrhagic enteritis. The *cpb2* toxin gene is transcribed during vegetative growth, especially during the exponential phase and regulation by the VirS/VirR two-component regulatory system (Ohtani *et al*., 2003) [61]. The *cpb*2 gene is present on *C. perfringens* type D isolates on plasmids 48 kb to 110 kb in size (Sayeed *et al*., 2007) [73-95]. In these isolates, the gene is either present on the same plasmid carrying the *etx* gene or on a different plasmid. In type E isolates, *cpb*2 gene is present on plasmids sizes varying from 70 kb to 90 kb. The gene is always present on different plasmid carrying the iota toxin gene (*itx*) (Li *et al*., 2007) [41].

Role of CPB2 in Animal Disease

Over the past decade CPB2 has been associated with enteric diseases in a wide range of animals including swine (Hendriksen *et al*., 2006) [31], cattle (Ferrarezi *et al*., 2008) [22], horses (Waters *et al*., 2005) [100], sheep and goats (uzal *et al*., 2008) [20-93-99], and wild animals such as deer (Embury *et al*., 2005) [16] and bears (Greco *et al*., 2005) [28]. The clinical signs observed in CPB2-mediated enteric diseases range from pasty to watery diarrhea with blood in faeces, abdominal pain, and loss of body condition. It is hypothesized that CPB2 toxin may act in synergy with other major toxins of *C. perfringens* in the production of necrotic and hemorrhagic enteritis. Based on the reported findings related to the pathology of *C. perfringens* encoding CPB2-related enteritis seen in food producing and wild animals, (Ceci *et al*., 2006) [10], the gross pathology is characterized by hemorrhage and necrosis of the small and large intestines. Other prominent gross pathologic lesions include swollen, hemorrhagic mesenteric lymph nodes, as well as thickened and congested abomasal walls. The characteristic histological lesions include degenerated and necrotic desquamated epithelial cells, cell debris, inflammatory cells, fibrin and variable numbers of bacterial vegetative cells and spores. The pathologic lesions observed suggest that CPB2 toxin is a primary enteric toxin affecting the nutrient absorptive capacity of the intestine, thereby debilitating the affected animal (Embury *et al*., 2005) [16]. A much larger proportion of strains carrying the *cpb2* gene was found in the intestine of pigs with diarrhea than in normal control animals, which strongly suggests that CPB2 is implicated in pig enteritis. However, the same does not seem to be true for other animal species and the role of CPB2 in enteric disease of those animals remains, therefore, undetermined (Uzal *et al*., 2014) [97].

Theta toxin/ Perfringolysin O (PFO)

PFO is a 54 kDa cytolytic toxin that binds to cholesterol-containing eukaryotic membranes. This toxin forms a large oligomeric prepore complex on the membrane surface prior to insertion into the cell membrane. Structurally, PFO is comprised of 4 domains. The C-terminal domain (domain 4) binds cholesterol and then a conformational change in domain three exposes a β–hairpin that spontaneously inserts into the lipid bilayer (Shepard *et al*., 1998) [77]. PFO pore formation

includes the binding of water-soluble PFO monomers to cholesterol of a lipid bilayer mediated by domain 4. The primary sequence of toxin, deduced from the *pfoA* gene sequence, revealed a 494 residue preprotein from which a 28 residue signal peptide is removed and secreted to the medium. *pfo*A gene is located on the chromosomal DNA near the origin of replication. PFO is synthesized with a 27 amino acid signal peptide, and the mature protein consists of 472 amino acids (53 kDa) (Shimizu *et al*., 2002a) [78].

The *pfo*A gene is directly regulated by the VirS/VirR two-component regulatory system (Lyristis *et al*., 1994) [43]. Once activated by the VirS sensor kinase, the VirR response regulator binds to specific VirR boxes (VirR box 1 and VirR box 2) located upstream of the *pfo*A promoter, to activate its transcription. The signal(s) that activates the VirS/VirR two-component regulatory system is unknown (Cheung *et al*., 2004) [12]. The presence of enterocytes rapidly upregulates PFO production and transcription of the *pfo*A gene, via the same VirS/VirR two-component regulatory system. All five types of *C. perfringens* produce a lethal hemolysin, toxin, which is also known as hemolysin, perfringolysin O, or the thiol activated cytolysin**.** The 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 -toxin cause altered polymorphonuclear leukocyte morphology, metabolism, and migration. Together with toxin, the -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) [88].

Role of PFO in Animal Diseases

PFO participates with CPA in the production of local lesions of gangrene/ malignant edema in humans and animals (Kennedy *et al*., 2009) [35]. When CPA and PFO knock-out mutants of *C. perfringens* type A were used in a mouse model, elimination of both CPA and PFO toxin genes removed most of the histopathological features typical of clostridial myonecrosis. These effects were completely restored when the double mutant was complemented with the alpha-toxin structural gene. However, restoring only PFO activity produced variable results. Reconstitution of both toxins produced histopathology similar to that observed with the alpha-reconstituted strain (Garmory *et al*., 2000) [26]. PFO and CPA stimulate leucocyte adherence, probably by increasing vascular leucostasis and local ischemia. PFO is leukocytotoxic at high doses, and at a sublethal concentration it significantly stimulates the production of ICAM-1 and adherent glycoprotein CD11b/CD18 in endothelial cells, which contributes to leukostasis in vessels adjacent to gangrenous lesions (Bryant *et al*., 1993). The toxin effects on endothelial cells participate in disruption of endothelial integrity resulting in local edema and ultimately in systemic shock and multiorgan failure. Thus, PFO and CPA exhibit synergistic effects (Stevens *et al*., 2002) [87].

PFO might also have a synergistic effect with ETX. The effects of purified CPA and PFO on the lethal activity of purified ETX were studied in a mouse model. Mice were injected intravenously or intragastrically with CPA or PFO with or without ETX. Sublethal doses of CPA or PFO did not affect the lethality of ETX when either was injected together with the latter intravenously. However, sublethal or lethal doses of CPA or PFO reduced survival times of mice injected simultaneously with ETX versus animals given ETX alone. When PFO was inoculated intragastrically with ETX, there was a reduction in survival time. CPA did not alter the survival time when inoculated intragastrically with ETX. These results suggest that both CPA and PFO have the potential to enhance the ETX lethal effects during enterotoxemia in natural hosts such as sheep and goats (Fernandez *et al*., 2008) [20].

Enterotoxin (CPE)

CPE is a 35 kDa polypeptide and the structure that was reported by Kitadokoro *et al.* (2011) [38] 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). Although CPE lacks sequence homology with other toxins, its C-terminal receptor-binding domain structurally resembles that of some *Bacillus thuringiensis* cry toxins (Vanitallie *et al*., 2008) [98]. The gene (*cpe*) encoding CPE can reside on either the chromosome or on plasmids (Cornillot *et al*., 2009). Most human food-poisoning isolates carry *cpe* on the chromosome (McClane *et al*., 2006) [49], and recent multi locus sequence typing (MLST) studies suggest these chromosomal *cpe* isolates are relatively divergent from other *C. perfringens* isolates (Deguchi *et al*., 2009) [15]. In contrast, *cpe*-positive isolates recovered from diseased animals or humans suffering from non-foodborne human gastrointestinal disease typically carry their *cpe* gene on large plasmids (Sparks *et al*., 2001) [83]. In type A isolates, there are two major *cpe* plasmid families : i) a family of ~75 kb plasmids that also carry the beta2 toxin gene (*cpb2*); ii) a family of ~70 kb plasmids lacking the *cpb*2 gene. These two *cpe* plasmid families in type A isolates share a conserved 35 kb region. Some, if not all, *cpe* plasmids of type A isolates can transfer between *C. perfringens* via conjugation, probably because they possess the same *tcp* locus that mediates transfer of *C. perfringens* tetracycline resistance plasmid pCW3 (Bannam *et al*., 2006) [3]. Some type C and D isolates also carry a plasmid-borne *cpe* gene, although those plasmids do not appear to be closely related to the *cpe* plasmids of type A isolates. Interestingly, most type E isolates carry silent *cpe* sequences on their iota toxin-encoding plasmids, which are often related to *cpe* plasmids of type A isolates (Li *et al*., 2007) [41].

CPE action starts when the toxin binds to claudin receptors (claudins are important components of the tight junctions made by epithelial and endothelial cells), forming a small complex (Sayeed *et al*., 2009) [73-95]. Several small complexes then oligomerize on the membrane surface to form a prepore named CH-1; CH-1 minimally contains six copies of CPE, claudin receptor, and some non-receptor claudins that are likely interacting with the claudin receptors (Robertson *et al*., 2007) [66]. After its formation, the CH-1 prepore inserts into the membrane bilayer to generate a cationselective pore (Sayeed *et al*., 2009) [73-95]. The resultant calcium influx activates apoptotic and oncotic cell death pathways that cause morphologic damage to the CPE-treated cells. This morphologic damage exposes the basolateral surface of the cell, allowing CPE to interact with occluding and additional

claudins to form a second CPE-complex named CH-2. Formation of CH-2 triggers internalization of occludin (and probably claudins), which may contribute to paracellular permeability changes and diarrhea (Robertson *et al*., 2007) [66]. All tested mammalian species are sensitive to CPE. This toxin affects all small intestinal regions but is most active in the ileum. CPE has only weak effects on the colon of rabbits or human colonic tissue *ex vivo* (Fernandez *et al*., 2005) [21]. In the ileum, CPE causes villus shortening and epithelial desquamation (Sarker *et al*., 1999) [70], and such damage appears necessary for accumulation of fluids and electrolytes in the lumen (Smedley *et al*., 2008) [80-98].

The Role of CPE in Animal Diseases

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) [47]. Experiments using isogenic mutants have clearly shown that CPE production is important for *C. perfringens* type A food poisoning or non-foodborne gastrointestinal (GI) disease isolates to cause diarrheal symptoms in experimental animals (Sarker *et al*., 1999) [70]. Coupled with extensive epidemiologic evidence, these findings support CPE as an important virulence factor for *C. perfringens* type A food poisoning or CPE-associated nonfoodborne human GI disease (McClane *et al*., 2006) [49].

In contrast to the well-established role of this toxin in human GI disease, the data implicating CPE in animal disease remains more ambiguous. Animals with diarrhea are rarely tested for the presence of CPE in their feces and diagnostic criteria for establishing CPE-mediated animals disease are lacking (Songer and Uzal, 2005) [75-86]. Nevertheless, there are case reports suggesting CPE may cause GI disease in both domestic animals (including dogs, pigs, horses, and goats) and possibly wild animals (such as penguins, leopards and tortoises) (Fernandez *et al*., 2007) [19]. For example, a relatively recent study definitively showed the presence of CPE-positive, type A isolates and CPE in the small intestines of a goat kid suffering from necrotic enteritis (Deguchi *et al*., 2009) [15]. Additionally, faecal CPE and CPE-positive faecal isolates have been associated with canine diarrhoea and it has been suggested that CPEpositive strains can cause recurrent diarrhea in dogs (Weese *et al*., 2001a) [101]. Finally, in horses, faecal CPE was detected in ~20% of adults with diarrhea and ~30% of foals with diarrhoea ; thus supporting a role for this faecal toxin contributing to disease in those animals, while no faecal CPE was detected in healthy adult horses or foals (Weese *et al*., 2001b) [102].

NetB Toxin

The *C. perfringens* necrotic enteritis B-like toxin (NetB) is a recently discovered member (Keyburn *et al.*, 2008) [36] of the 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) [42]. 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- B) signalling pathway (Guo *et al.*, 2015) [29]. Fernandes *et al.* (2014) [18] identified amino acids that play a role in NetB oligomerisation and poreformation using site-directed mutagenesis.

A novel pore-forming toxin of *C. perfringens* named NetB was identified in the NE strains of *C. perfringens*. It has a similar molecular size to β-toxin, hence the name NetB (necrotic enteritis toxin B-like). Its amino acid sequence has limited similarity with other pore forming toxins similar to the β-toxin of *C. perfringens* (38% identity) and the α-toxin of *Staphlococcus aureus* (31% identity). It has cytotoxic activity against chicken leghorn male hepatoma cell line (LMH) including karyorehexis and karyolysis (Keyburn *et al.*, 2008) [36].

The structure of the NetB monomer has been determined to a resolution of 1.8 Å, which revealed that it had the β-sandwich, latch, rim, and pre-stem domains that are typical of proteins that belong to the α-hemolysin family of β-pore-forming toxins. It has an almost identical structure to δ-toxin from C. perfringens despite the fact that these toxins have very different amino acid sequences. NetB has a four amino acid deletion in the rim domain, in a region that in other β-pore-forming toxins is responsible for lipid binding, suggesting that NetB has a different cell surface target. Finally, analysis of the ability of NetB to form pores in planar phospholipid bilayers revealed that the NetB pore channels have a preference for cations over anions (Yan, *et al*., 2013) [104]. The structure of the NetB pore, without the first 20 N-terminal amino acids, has been solved to a resolution of 3.9 Å after solubilisation and purification of the complex from lipid vesicles (Savva *et al*., 2013) [71]. Like the monomer, the pore structure shows similarity to staphylococcal α-hemolysin. It comprises seven Net B monomers in a ring structure, with the predicted hydrophobic transmembrane domain spanning residues I121 to V146 (numbering from the N-terminal amino acid of the secreted toxin). Liposome studies showed that oligomerisation of Net B was enhanced by cholesterol, which was postulated to play an important role in pore formation. Both site-directed mutagenesis and random mutagenesis have been used to isolate single amino acid substitution derivatives of Net B that have reduced biological activity (Fernandes *et al*., 2014) [18].

The Role of Net B in Animal Diseases

Net B toxin is crucial for the induction of avian necrotic enteritis. It is only found in chicken *C. perfringens* isolates (Martin and Smyth, 2009) [45]. A 100% correlation has been found between Net B production and the ability to produce disease in a necrotic enteritis induction model (Smyth and Martin, 2010) [46]. Net B forms pores in a chicken epithelial cell line *in vitro*. Its activity *in vivo* has not yet been shown, but it may induce necrotic lesions by forming pores in enterocytes leading to cell death (Keyburn *et al*., 2010) [13-37]. The toxin causes cell rounding and lysis in a chicken Leghorn male hepatoma cell line, and it was shown that NetB forms plasma membrane pores with an estimated pore diameter of 1.6 to 1.8 nm (Keyburn *et al*., 2008) [36]. Lepp *et al*. (2010) [40] found that the netB gene is located on an approximately 85 kb plasmid. The VirSR two-component signal transduction system regulates the expression of netB. The NetB toxin production is upregulated when the VirSR system is activated as a result of a high *C. perfringens* population density. This is an efficient environmental adaptation since NetB would only be produced when the *C. perfringens* concentration is high and there is an increased probability of subsequent nutrient limitation. Damage of the host cells caused by NetB might provide enough nutrients for *C. perfringens* (Cheung *et al*., 2010) [13]. Confirmation of the role of NetB in disease came from the finding that most necrotic enteritis outbreak strains

carry the netB gene, whereas non-necrotic enteritis-derived *C. perfringens* isolates lack this gene (Keyburn *et al*., 2010) [13-37]. This gene is only found in poultry *C. perfringens* strains, except for one isolate that was recovered from a cow (Martin and Smyth, 2009) [45]. Moreover, several groups screened *C. perfringens* isolates derived from different countries for the occurrence of netB. In a Canadian study, 95% -of *C. perfringens* isolates from broilers with necrotic enteritis were positive for netB while only 35% isolates from healthy boilers were netB-positive (Chalmers *et al*., 2008) [11].

References

1. Anderson JM, Van Itallie CM. Physiology and function of the tight junction. Cold. Spring. Harb. Perspect. Biol. 2008; 1:a002584.
2. Awad MM, Ellenor DM, Bod RL, Emmins JJ, Rood JI. Synergistic effects of alpha-toxin and perfringolysin O in *Clostridium perfringens*-mediated gas gangrene. Infection and Immunity. 2001; 69:7904-7910.
3. Bannam TL, Teng WL, Bulach D, Lyras D, Rood JI. Functional identification of conjugation and replication regions of the tetracycline resistance plasmid pCW3 from Clostridium perfringens. Journal of Bacteriology. 2006; 188:4942-4951.
4. Barker IK, Van Dreumel AA, Palmer N. The alimentary system, In: Jubb, K.F., Kennedy, P.C., Palmer, N., Pathology of Domestic Animals, 4th ed. Academic Press, San Diego, CA, 1993, 237-245.
5. Barth H, Blocker D, Behlke J, Schutter WB, Brisson AR, Benz R, Aktories K. Cellular uptake of *Clostridium botulinum* C2 toxin requires oligomerization and acidification. Journal of Biology and Chemistry. 2000; 275:18704-18711.
6. Blackwell TE, Butler DG, Bell JA. Enterotoxemia in the goat: the humoral response and local tissue reaction following vaccination with two different bacterin-toxoids. Canadian Journal of Comparative Pathology. 1992; 47:127-132.
7. Blood DC, Radostits OM, Henderson JA. Veterinary medicine: a textbook of the diseases of cattle, sheep, goats and horses. 6th ed. London: Bailliere Tindall, 1983.
8. Boujon P, Henzi M, Penseyres JH, Belloy L. Enterotoxemia involving beta2-toxigenic *Clostridium perfringens* in a white stork (*Ciconia ciconia*). The Veterinary Research. 2005; 156:746-747.
9. Bryant AE, Bayer CR, Hayes-Schroer SM, Stevens DL. Activation of platelet gpIIbIIIa by phospholipase C from *Clostridium perfringens* involves store-operated calcium entry. Journal of Infectious Diseases. 2003; 187:408-417.
10. Ceci L, Paradies P, Sasanelli M, de-Caprariis D, Guarda F, Capucchio MT, *et al*. Haemorrhagic bowel syndrome in dairy cattle: possible role of *Clostridium perfringens* type A in the disease complex. Journal of Veterinary Medicine. 2006; 53:518-523.
11. Chalmers G, Bruce HL, Hunter DB, Parreira VR, Kulkarni RR, Jiang Y-F, *et al*. Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. Journal of Clinical Microbiology. 2008; 46:3957-3964.
12. Cheung JK, Dupuy B, Deveson DS, Rood JI. The spatial organization of the VirR boxes is critical for VirR-mediated expression of the perfringolysin O gene, pfoA, from *Clostridium perfringens*. Journal of Bacteriology. 2004; 186:3321-3330.
13. Cheung JK, Keyburn AL, Carter GP, Lanckri*et al*, Van Immerseel F, Moore RJ, Rood JI. The VirSR twocomponent signal transduction system regulates NetB toxin production in Clostridium perfringens. Infection and Immunity. 2010; 78:3064-3072.
14. Cornillot E, Saint-Joanis B, Daube G, Katayama S, Granum PE, Canard B, Cole ST. The enterotoxin gene (cpe) of *Clostridium perfringens* can be chromosomal or plasmid-borne. Molecular Microbiology. 1995; 15:639-647.
15. Deguchi A, Miyamoto K, Kuwahara T, Miki Y, Kaneko I, Li J, McClane BA, Akimoto S. Genetic characterization of type A enterotoxigenic *Clostridium perfringens* strains. PLoS One. 2009; 4:5598-5601.
16. Embury-Hyatt CK, Wobeser G, Simko E, Woodbury MR. Investigation of a syndrome of sudden death, splenomegaly, and small intestinal hemorrhage in farmed deer. Canadian Veterinary Journal. 2005; 46:702-708.
17. Engstrom BE, C Fermer, A Lindberg, E Saarinen, V Baverud, A Gunnarsson. Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. Veterinary Microbiology. 2003; 94:225-235.
18. Fernandes da Costa SP, Savva CG, Bokori-Brown M, Naylor CE, Moss DS, Basak AK, Titball RW. Identification of a key residue for oligomerisation and pore- formation of *Clostridium perfringens* NetB. Toxins. 2014; 6:1049-1061.
19. Fernandez MME, Fisher DJ, Poon R, Sayeed S, Adams V, Rood JI, *et al*. Both epsilon-toxin and beta-toxin are important for the lethal properties of *Clostridium perfringens* type B isolates in the mouse intravenous injection model. Infection and Immunity. 2007; 75:1443-1452.
20. Fernandez MME, Jost BH, Billington SJ, Uzal FA. Lethal effects of *Clostridium perfringens* epsilon toxin are potentiated by alpha and perfringolysin-O toxins in a mouse model. Veterinary Microbiology. 2008; 127:379-385.
21. Fernandez MME, Pistone CV, Uzal FA, McClane BA, Ibarra C. *Clostridium perfringens* enterotoxin damages the human intestine *in vitro*. Infection and Immunity. 2005; 73:8407-8410.
22. Ferrarezi MC, Cardoso TC, Dutra IS. Genotyping of *Clostridium perfringens* isolated from calves with neonatal diarrhea. Anaerobe. 2008; 4:328-331.
23. Finnie JW. Pathogenesis of brain damage produced in sheep by *Clostridium perfringens* type D epsilon toxin: a review. Australian Veterinary Journal. 2003; 81:219-221.
24. Fisher DJ, Miyamoto K, Harrison B, Akimoto S, Sarker MR, McClane BA. Association of beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. Molecular Microbiology. 2005; 56:747-762.
25. Flores DM, Alape GA. Role of *Clostridium perfringens* phospholipase C in the pathogenesis of gas gangrene. Toxicon. 2003; 42:979-986.
26. Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW. Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiology and Infection. 2000; 124:61-67.
27. Gibert M, Monier MN, Ruez R, Hale ML, Stiles BG, Benmerah A, *et al*. Endocytosis and toxicity of clostridial binary toxins depend on a clathrin-independent

pathway regulated by Rho-GDI. Cell Microbiology. 2011; 13:154-170.
28. Greco G, Madio A, Martella V, Campolo M, Corrente M, Buonavoglia D, Buonavoglia C. Enterotoxemia associated with beta2 toxin-producing *Clostridium perfringens* type A in two Asiatic black bears (Selenarctos thibetanus). Journal of Veterinary Diagnosis and Investigation. 2005; 17:186-189.
29. Guo S, Li C, Liu D, Guo Y. Inflammatory responses to a *Clostridium perfringens* type A strain and toxin in primary intestinal epithelial cells of chicken embryos. Avian Pathology. 2015; 44:81-91.
30. Gurcel L, Iacovache I, Van der Goot FG. Aerolysin and related Aeromonas toxins. In: Alouf, J.E., Popoff, M.R. (Eds.). The source book of bacterial protein toxins. Elsevier, Amsterdam, 2006, 606-620.
31. Hendriksen SW, VanLeengoed LA, Roest HI, vanes A. Neonatal diarrhoea in pigs: alpha- and beta2-toxin produced by *Clostridium perfringens*. Tijdschrift voor Diergeneeskunde. 2006; 131:910-913.
32. Hughes ML, Poon R, Adams V, Sayeed S, Saputo J, Uzal FA, *et al*. Epsilon-Toxin Plasmids of *Clostridium perfringens* Type D Are Conjugative. Journal of Bacteriology. 2007; 189:7531-7538.
33. Hunter SEC, Clarke IN, Kelly DC, Titball RW. Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in Escherichia coli. Infection and Immunity. 1992; 60:102-110.
34. Katayama S, Dupuy B, Daube G, China B, Cole ST. Genome mapping of *Clostridium perfringens* strains with I-CeuI shows many virulence genes to be plasmid-borne. Molecular Genetics and Genomics. 1996; 251:720-726.
35. Kennedy CL, Smith DJ, Lyras D, Chakravorty A, Rood JI. Programmed cellular necrosis mediated by the pore-forming alpha-toxin from *Clostridium septicum*. PLoS Pathogenesis. 2009; 5:516-518.
36. Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, *et al*. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathologyl. 2008; 4:26.
37. Keyburn AL, Yan XX, Bannam TL, Van Immerseel F, Rood JI, Moore RJ. Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin. Veterinary Research. 2010; 41:21.
38. Kitadokoro K, Nishimura K, Kamitani S, Fukui-Miyazaki A, Toshima H, Abe H, *et al*. Crystal structure of *Clostridium perfringens* enterotoxin displays features of beta-pore-forming toxins. Journal of Biological Chemistry. 2011; 286:19549-19555.
39. Lee K, Lillehoj HS, Li G, Park MS, Jang SI, Jeong W, *et al*. Identification and cloning of two immunogenic *Clostridium perfringens* proteins, elongation factor Tu (EF-Tu) and pyruvate:ferredoxin oxidoreductase (PFO) of C. perfringens. Research in Veterinary Science. 2011; 91:80-86.
40. Lepp D, Roxas B, Parreira VR, Marri PR, Rosey EL, Gong J, *et al*. Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. PLoS ONE. 2010; 5:795-810.
41. Li J, Miyamoto K, McClane BA. Comparison of virulence plasmids among *Clostridium perfringens* type E isolates. Infection and Immunity. 2007; 75:1811-1819.
42. Lovland AM. Severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated hepatitis. Avian Pathology. 2001; 30:73-81.
43. Lyristis M, Bryant AE, Sloan J, Awad MM, Nisbet IT, Stevens DL, *et al*. Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. Molecular Microbiology. 1994; 2:761-777.
44. Manteca C, Daube G, Jauniaux T, Linden A, Pirson V, Detilleux J, *et al*. A role for the *Clostridium perfringens* beta2 toxin in bovine enterotoxaemia? Veterinary Microbiology. 2002; 86:191-202.
45. Martin TG, Smyth JA. Prevalence of netB among some clinical isolates of *Clostridium perfringens* from animals in the United States. Veterinary Microbiology. 2009; 136:202-205.
46. Martin TG, Smyth JA. The ability of disease and nondisease producing strains of *Clostridium perfringens* from chickens to adhere to extracellular matrix molecules and Caco-2 cells. Anaerobe. 2010; 16:533-539.
47. McClane BA. *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R. (Eds), Food microbiology: fundamentals and frontiers. ASM Press, Washington, DC, USA, 2007, 423-444.
48. McFarlane MG, Knight BCJG. The biochemistry of bacterial toxins: The lecithinase activity of *Cl. Welchii* toxins. Biochemistry Journal. 1941; 35:884-902.
49. McClane BA, Lyerly DL, Wilkins TD. Enterotoxic clostridia: *Clostridium perfringens* type A and Clostridium difficile. In: Fischetti, RPNVA. Feretti, J.J., Portnoy, D.A., Rood, J.I. (Eds). Gram positive pathogens. Washington DC: ASM Press, 2006, 703-14.
50. McClane BA, Uzal FA, Fernandez-Miyakawa M, Lyerly D, Wilkins TD. The enterotoxigenic clostridia. In: Dworkin, SFM, Rosenburg, E., Schleifer, KF. Stackebrandt, E. (Eds). The Prokaryotes. New York: Springer-Verlag, 2004, 698-752.
51. McDonel JL. Toxins of *Clostridium perfringens* types A, B, C, D and E. In: Dorner, F., Drews, J. (Eds), Pharmacology of bacterial toxins. Permagon Press, Oxford, 1986, 477-517.
52. McGowan G, Moulton JE, Rood SE. Lamb losses associated with *Clostridium perfringens* type. Journal of American Veterinary Medicine Association. 1958; 133:219-221.
53. Melton JA, Parker MW, Rossjohn J, Buckley JT, Tweten RK. The identification and structure of the membrane-spanning domain of the *Clostridium septicum* alpha toxin. Journal of Biology and Chemistry. 2004; 279:14315-14322.
54. Miclard J, Jaggi M, Sutter E. *et al*. *Clostridium perfringens* beta-toxin targets endothelial cells in necrotizing enteritis in piglets. Veterinary Microbiology. 2009a; 137:320-325.
55. Minami J, Katayama S, Matsushita O, Matsushita C, Okabe A. Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. Microbiology and Immunology. 1997; 41:527-535.
56. Miyamoto K, Li J, Sayeed S, Akimoto S, McClane BA. Sequencing and diversity analyses reveal extensive similarities between some epsilon toxin encoding plasmids and the pCPF5603 *Clostridium perfringens* enterotoxin plasmid. Journal of Bacteriology. 2008; 190:7178-7188.
57. Nagahama M, Kihara A, Kintoh H, Oda M, Sakurai J. Involvement of tumour necrosis factor-alpha in

Clostridium perfringens beta-toxin-induced plasma extravasation in mice. British Journal of Pharmacology. 2008; 153:1296-1302.
58. Nagahama M, Morimitsu S, Kihara A, Akita M, Setsu k, Sakurai J. Involvement of tachykinin receptors in *Clostridium perfringens* beta-toxin-induced plasma extravasation. British Journal of Pharmacology. 2003; 138:23-30.
59. Nauerby B, Pedersen K, Madsen M. Analysis by pulsed-field gel electrophoresis of the genetic diversity among Clostridium perfringens isolates from chickens. Veterinary Microbiology. 2003; 94:257-266.
60. Naylor CE, Eaton JT, Howells A, Justin N, Moss DS, Titball RW, Basak AK. Structure of the key toxin in gas gangrene. Nature Structural Biology. 1998; 5:738-746.
61. Ohtani K, Kawsar HI, Okumura K, Hayashi H, Shimizu T. The VirR/VirS regulatory cascade affects transcription of plasmid-encoded putative virulence genes in *Clostridium perfringens* strain 13. FEMS Microbiology Letters. 2003; 222:137-141.
62. Percy DH, Barthold SW. Pathology of laboratory rodents and rabbits. Victoria, Australia: Blackwell Publishing, 2007.
63. Popoff MR. Clostridial pore-forming toxins: powerful virulence factors. Anaerobe. 2014; 30:220-238.
64. Redondo LM, Carrasco JM, Redondo EA, Delgado F, Miyakawa ME. *Clostridium perfringens* type E virulence traits involved in gut colonization. PLoS One. 2015; 10(3).
65. Richard JF, Mainguy G, Gibert M, Marvaud JC, Stiles BG, Popoff MR. Transcytosis of iota-toxin across polarized CaCo-2 cells. Molecular Microbiology. 2002; 43:907-917.
66. Robertson SL, Smedley JG, Singh U, Chakrabarti G, Vanitallie CM, Anderson JM, McClane BA. Compositional and stoichiometric analysis of *Clostridium perfringens* enterotoxin complexes in Caco-2 cells and claudin 4 fibroblast transfectants. Cell Microbiology. 2007; 9:2734-2755.
67. Saenz MG, Venturini L, Assis RA, Uzal F, Risso MA, Idiart JR, Perfumo CJ. Fibrinonecrotic enteritis of piglets in a commercial farm: a postmortem study of the prevalence and the role of lesion associated agents *Isospora suis* and *Clostridium perfringens*. Pesquisa Veterinaria Brasileira. 2007; 27:297-300.
68. Sakurai J. Toxins of *Clostridium perfringens*. Review in Medical Microbiology. 1995; 6:175-185.
69. Sakurai J, Duncan CL. Some properties of beta-toxin produced by *Clostridium perfringens* type C. Infection and Immunity. 1978; 21:678-680.
70. Sarker MR, Carman RJ, McClane BA. Inactivation of the gene (cpe) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two cpe-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. Molecular Microbiology. 1999; 33:946-958.
71. Savva CG, Fernandes da Costa SP, Bokori-Brown M, Naylor CE, Cole AR, Moss DS, *et al*. Molecular architecture and functional analysis of NetB, a pore-forming toxin from *Clostridium perfringens*. Journal of Biological Chemistry. 2013; 288:3512-3522.
72. Sayeed S, Li J, McClane BA. Virulence plasmid diversity in *Clostridium perfringens* type D isolates. Infection and Immunity. 2007; 75:2391-2398.
73. Sayeed S, Robertson SL, Caserta JA, McClane BA. Improved understanding of the action and genetics of *Clostridium perfringens* enterotoxin suggests potential application for cancer therapy and drug delivery. In: Bruggemann, H.; Gottschalk, G. (Eds). Clostridia: Molecular Biology in the post-genomic era. Norfolk: Caister academic press, 2009, 29-46.
74. Schotte U, Truyen U, Neubauer H. Significance of beta 2-toxigenic *Clostridium perfringens* infections in animals and their predisposing factors--a review. Journal of Veterinary Medicine. 2004; 51:423-426.
75. Songer JG, Miskimins DW. Clostridial abomasitis in calves: case report and review of the literature. Anaerobe 2005; 11:290-294.
76. Shatursky O, Bayles R, Rogers M, Jost BH, Songer JG, Tweten RK. *Clostridium perfringens* beta toxin forms potential-dependent, cation-selective channels in lipid bilayers. Infection and Immunity. 2000; 68:5546-5551.
77. Shepard LA, Heuck AP, Hamman BD, Rossjohn J, Parker MW, Ryan KR, *et al*. Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin *Clostridium perfringens* perfringolysin O: an alpha-helical to beta-sheet transition identified by fluorescence spectroscopy. Biochemistry. 1998; 37:14563-14574.
78. Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, *et al*. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proceedings of the National Academy of Sciences. 2002; 99:996-1001.
79. Silva ROS, Ribeiro MGM, Palhares S, Borges AS, Maranhão RPA, Silva MX, *et al*. Detection of A/B toxin and isolation of *Clostridium difficile* and *Clostridium perfringens* from foals. Equine Veterinary Journal. 2013; 45:671-675.
80. Smedley JG, Saputo J, Parker JC, Miyakawa MEF, Robertson SL, McClane BA, *et al*. Noncytotoxic *Clostridium perfringens* enterotoxin (CPE) variants localize CPE intestinal binding and demonstrate a relationship between CPE induced cytotoxicity and enterotoxicity. Infection and Immunity. 2008; 76:3793-3800.
81. Smith MC, Sherman DM. Goat medicine. Philadelphia: Lea and Febiger, 1994.
82. Songer JG. Clostridial enteric diseases of domestic animals. Clinical Microbiology Review. 1996; 9:216-234.
83. Sparks SG, Carman RJ, Sarker MR, McClane BA. Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. Journal of Clinical Microbiology. 2001; 39:883-888.
84. Stevens DL, Troyer BE, Merrick DT, Mitten JE, Olson RD. Lethal effects and cardiovascular effects of purified a- and Q-toxins from *Clostridium perfringens*. Journal of Infectious Diseases. 1988; 157:272-279.
85. Songer JG. Clostridial diseases of small ruminants. Veterinary Research. 1998; 29:219-232.
86. Songer JG, Uzal FA. Clostridial enteric infections in pigs. Journal of Veterinary Diagnosis and Investigation. 2005; 17:528-536.
87. Stevens DL, Bryant AE. The role of Clostridial toxins in the pathogenesis of gas gangrene. Clinical Infectious Diseases. 2002; 35:93-100.
88. Stevens DL, Tweten RK, Awad MM, Rood JI, Bryant AE. Clostridial gas gangrene: evidence that alpha and theta toxins differentially modulate the immune response and induce acute tissue necrosis. Journal of Infectious

Diseases. 1997; 176:189-195.
89. Tamai E, Ishida T, Miyata S, Matsushita O, Suda H, Kobayashi S, Sonobe H, Okabe A. Accumulation of *Clostridium perfringens* epsilon-toxin in the mouse kidney and its possible biological significance. Infection and Immunity. 2003; 71:5371-5375.
90. Timoney JF, Hartmann M, Fallon L, Fallon E, Walker J. Antibody responses of mares to prepartum vaccination with *Clostridium perfringens* bacterin and beta2 toxin. Veterinary Record. 2005; 157:810-812.
91. Tweten RK. *Clostridium perfringens* beta toxin and *Clostridium septicum*alpha toxin: their mechanisms andpossible role in pathogenesis. Veterinary Microbiology. 2001; 82:1-9.
92. Uzal FA, Kelly WR. The effects of intravenous administration of *Clostridium perfringens* type D epsilon toxin on young goats and lambs. Journal of Comparative Pathology. 1997; 116:63-71.
93. Uzal FA, Fisher DJ, Saputo J, Sayeed S, McClane BA, Songer G, *et al*. Ulcerative enterocolitis in two goats associated with enterotoxin- and beta2 toxin-positive *Clostridium perfringens* type D. Journal of Veterinary Diagnosis and Investigation. 2008; 20:668-672.
94. Uzal FA, Glastonbury JR, Kelly WR, Thomas R. Caprine enterotoxemia associated with cerebral microangiopathy. Veterinary Record. 1997; 141:224-226.
95. Uzal FA, Saputo J, Sayeed S, Vidal JE, Fisher DJ, Poon R, *et al*. Development and application of new mouse models to study the pathogenesis of *Clostridium perfringens* type C enterotoxemias. Infection and Immunity. 2009; 77:5291-5299.
96. Uzal FA, Kelly WR, Morris WE, Bermudez J, Baison M. The pathology of experimental *Clostridium perfringens* type D enterotoxemia in sheep. Journal of Veterinary Diagnosis and Investigation. 2004; 16:403-411.
97. Uzal FA, Freedman JC, Shrestha A, Theoret JR, Garcia J, Awad MM, *et al*. Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. Future Microbiology. 2014; 9:361-377.
98. VanItallie CM, Betts L, Smedley JG, McClane BA, Anderson JM. Structure of the claudin binding domain of *Clostridium perfringens* enterotoxin. Journal of Biology and Chemistry. 2008; 283:268-274.
99. Vidal JE, McClane BA, Saputo J, Parker J, Uzal FA. Effects of *Clostridium perfringens* beta-toxin on the rabbit small intestine and colon. Infection and Immunity 2008; 76:4396-4404.
100. Waters M, Raju D, Garmory HS, Popoff MR, Sarker MR. Regulated expression of the beta2-toxin gene (cpb2) in *Clostridium perfringens* type a isolates from horses with gastrointestinal diseases. Journal of Clinical Microbiology. 2005; 43:4002-4009.
101. Weese JS, Greenwood SJ, Staempfli HR. Recurrent diarrhea associated with enterotoxigenic *Clostridium perfringens* in 2 dogs. Canadian Veterinary Journal. 2001a; 42:292-294.
102. Weese JS, Staempfli HR, Prescott JF. A prospective study of the roles of clostridium difficile and enterotoxigenic *Clostridium perfringens* in equine diarrhoea. Equine Veterinary Journal. 2001b; 33:403-409.
103. Welch WH, Nuttal GHF. A gas producing bacillus, *Bacillus capsulatus* (Nov. spec.) capable of rapid development in the blood vessel after death. Bulletin Johns Hopkins Hospital. 1892; 3:81-91.
104. Yan X, Porter CJ, Hardy SP, Steer D, Smith AI, Quinsey NS, *et al*. Structural and functional analysis of the pore-forming toxin NetB from Clostridium perfringens. mBio. 2013; 4:19-13.