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Animal models to study the pathogenesis of human and animal *Clostridium perfringens* infections

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Abstract

The most common animal models used to study *Clostridium perfringens* infections in humans and animals are reviewed here. The classical *C. perfringens*-mediated histotoxic disease of humans is clostridial myonecrosis or gas gangrene and the use of a mouse myonecrosis model coupled with genetic studies has contributed greatly to our understanding of disease pathogenesis. Similarly, the use of a chicken model has enhanced our understanding of type A-mediated necrotic enteritis in poultry and has led to the identification of NetB as the primary toxin involved in disease. *C. perfringens* type A food poisoning is a highly prevalent bacterial illness in the USA and elsewhere. Rabbits and mice are the species most commonly used to study the action of enterotoxin, the causative toxin. Other animal models used to study the effect of this toxin are rats, non-human primates, sheep and cattle. In rabbits and mice, CPE produces severe necrosis of the small intestinal epithelium along with fluid accumulation. *C. perfringens* type D infection has been studied by inoculating epsilon toxin (ETX) intravenously into mice, rats, sheep, goats and cattle, and by intraduodenal inoculation of whole cultures of this microorganism in mice, sheep, goats and cattle. Molecular Koch's postulates have been fulfilled for enterotoxigenic *C. perfringens* type A in rabbits and mice, for *C. perfringens* type A necrotic enteritis and gas gangrene in chickens

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and mice, respectively, for *C. perfringens* type C in mice, rabbits and goats, and for *C. perfringens* type D in mice, sheep and goats.

Keywords

animal models; *Clostridium perfringens*; enterotoxemia; food poisoning; gas gangrene; necrotic enteritis; alpha toxin; beta toxin; epsilon toxin; enterotoxin; NetB

INTRODUCTION

Clostridium perfringens, an anaerobic, spore-forming Gram-positive rod, can produce ~ 17 toxins (McClane et al., 2006; Li et al., 2013). Four of these toxins, alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) are used to classify this microorganism into five toxinotypes A, B, C, D and E. Most *C. perfringens* isolates produce, in addition to at least one of the typing toxins, other toxins including, but not limited to, enterotoxin (CPE), beta2 toxin (CPB2), NetB and TpeL (McClane et al., 2006; Li et al., 2013).

The different toxinotypes of *C. perfringens* produce a wide variety of diseases in both humans and animals, ranging from *C. perfringens* type A gas gangrene to several enterotoxemias and enteritis syndromes. All of these diseases are mediated by one or more toxins of *C. perfringens* (Uzal et al., 2014).

Several animal models have been used to study the role of the different toxins of *C. perfringens* in the pathogenesis of the infections produced by this microorganism (McDonel 1980; Sayeed et al., 2008; Garcia et al., 2013; Li et al., 2013; Uzal et al., 2014). In particular, over the past few years, some of these animal models have been used to fulfill molecular Koch's postulates for various diseases (Awad et al., 1995; Sarker et al., 1999; McClane et al., 2006; Keyburn et al., 2008; Sayeed et al., 2008; Garcia et al 2013). We review here the information published on the main animal models used to study the pathogenesis of *C. perfringens* infections, with special emphasis on those used to fulfill molecular Koch's postulates.

C. perfringens type A

Gas gangrene—Gas gangrene, or clostridial myonecrosis, is an invasive, anaerobic infection of muscle and is characterized by extensive tissue necrosis and the production of gas (MacLennan, 1962). In humans, this infection can be divided into two types: spontaneous gangrene and traumatic gangrene. The former type is commonly caused by *Clostridium septicum*, while approximately 80% of the latter is caused by *C. perfringens*. However, other clostridia have also been associated with clostridial myonecrosis (Bryant and Stevens, 2010). The focus of this review is the gas gangrene caused by *C. perfringens*, as it is represents the majority of human cases and is by far the best studied.

Cases of *C. perfringens* gas gangrene have been reported in medical literature as far back as the Middle Ages (MacLennan, 1962). Although it was initially thought of as predominantly a disease of war (MacLennan, 1962; Stevens et al., 2012), the incidence of the infection has also been found to increase during times of natural disasters (Stevens et al., 2012), such as

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the 2008 Wenchuan earthquake in China (Chen et al., 2011). The infection begins with the entry of *C. perfringens*, mostly type A (Bryant and Stevens, 1997), from contaminated soil or clothing (Stevens et al., 2012), into a traumatic wound, followed by the multiplication and spread of the organism.

The incubation period of gas gangrene is usually short; the infection can be well established within 6-8 hours (Bryant and Stevens, 1997), but 1- 3 hours has also been recorded (MacLennan, 1962). Once established, the destruction of the healthy tissue adjacent to the site of infection occurs as quickly as several inches per hour (Bryant and Stevens, 2010). The rapid expansion of the infection is due to the fast growth of *C. perfringens*, which is largely attributed to the readily available supply of amino acids and peptides resulting from tissue trauma, compounded by the extraordinary tissue and cell destructive ability of the bacterium (Bryant and Stevens, 1997). However, the most important, if not the essential, prerequisite for this wound infection is the reduction of the local oxidation-reduction potential (MacLennan, 1962), which creates the anaerobic environment that is required for the growth of *C. perfringens*. Under these conditions, the bacteria not only multiply but also secrete extracellular cytolytic toxins into the surrounding tissue (Stevens et al., 2012). Of the many toxins produced by *C. perfringens*, the two most important in gas gangrene are undoubtedly CPA and perfringolysin O (PFO). These toxins are responsible for the characteristic lesions observed in this infection (Awad et al., 1995; Stevens et al., 1997), in particular, the hallmark of gas gangrene, the paucity of leukocytes at the site of infection (Ellemor et al., 1999; Awad et al., 2001). The role these toxins play in the pathogenesis of gas gangrene could not have been elucidated without the use of experimental animal models.

Early animal studies on *C. perfringens*-mediated gas gangrene were performed using guinea pigs (Armstrong and Rae, 1941; Evans, 1943; Altemeier et al., 1947), as these animals are exceptionally susceptible to the infection and their immunological responses closely mimic those of humans (Altemeier et al., 1947). However, in these models, to achieve a typical and constant infection, it was necessary to either mechanically macerate the muscle tissue (Altemeier et al., 1947) or to inject $CaCl_2$ into the muscle (Armstrong and Rae, 1941; Evans, 1943) prior to infection with *C. perfringens*. The first fulminant gas gangrene model that didn't require pre-treatment of the muscle tissue was developed by Stevens and colleagues (Stevens et al., 1987). In this model, *C. perfringens* cells were injected into the right upper thigh muscles of Swiss Webster mice. It was found that at least 10^9 colony forming units (cfu) was required to achieve fulminant disease; injection of 10^5-10^8 cfu did not cause illness or mortality (Stevens et al., 1987). The importance of the inoculum size was later confirmed by O'Brien and colleagues, when mice injected with 10^9 cfu developed gas gangrene, but those injected with 10^6 and 10^7 cfu failed to develop disease (O'Brien et al., 2007). Mice injected with greater than 10^8 cfu developed extensive tissue necrosis and signs of toxemia (Stevens et al., 1987; Lyristis et al., 1994; Awad et al., 1995). Histologic examination of affected muscle showed a paucity of leukocytes in regions of intense necrosis (Lyristis et al., 1994; Stevens et al., 1997). The inflammatory cells are instead found in the border between healthy and necrotic tissue, particularly clustered within the blood vessels (Stevens et al., 1997). This leukostasis has now been shown *in vivo* to be caused by the synergistic actions of CPA and PFO (Ellemor et al., 1999; Awad et al., 2001). In addition to studying the role of the two essential toxins, this model has been also used to assess the

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efficacy of treatment of gas gangrene with various antibiotics (Stevens et al., 1987) and to immunization with the C-terminal domain of CPA (Williamson and Titball, 1993; Stevens et al., 2004).

Since the development of the original mouse myonecrosis model, a variant of this model has been used successfully to show that CPA is essential for disease. In this model, approximately 10^9 cfu of washed *C. perfringens* cells are injected intramuscularly into the right hind thigh of BALB/c mice (Ellemor et al., 1999; Awad et al., 2000; Awad et al., 2001; Chakravorty et al., 2011; Hiscox et al., 2011; Hiscox et al., 2013; Chakravorty et al., 2014). Following injection, the mice are monitored and scored quantitatively every 30 to 60 mins for 12 to 24 hours (Awad et al., 2000; Awad et al., 2001)(Chakravorty et al., 2011; Hiscox et al., 2011; Hiscox et al., 2013; Chakravorty et al., 2014) for the characteristic signs of disease, namely limping, swelling of the thigh and footpad, and blackening of the thigh and footpad. In addition to using cumulative pathology scores to analyze disease progression, Kaplan-Meier curves are generated to follow the survival of the animals over the course of the infection. (Chakravorty et al., 2011; Hiscox et al., 2011; Hiscox et al., 2013; Chakravorty et al., 2014). More recently, the Department of Microbiology of Monash University has also used mean survival time data to compare different treatment groups (Chakravorty et al., 2014).

Histological analysis of muscle tissues from mice infected with the wild-type strain JIR325 showed pervasive myonecrosis, thrombosis and vascular leukostasis, but a scant number of leukocytes in the areas of necrosis (Ellemor et al., 1999; Awad et al., 2001; Chakravorty et al., 2011; Chakravorty et al., 2014). By contrast, the histopathology of the tissues of mice injected with various toxin mutants was markedly different. Tissues of mice infected with an isogenic CPA-toxin mutant showed less myonecrosis, no thrombosis, reduced leukostasis and the presence of many leukocytes, while the tissues of mice injected with an isogenic PFO mutant displayed extensive, albeit delayed, myonecrosis, wild-type levels of thrombosis, reduced vascular leukostasis and the presence of numerous leukocytes in areas of necrosis (Ellemor et al., 1999). Elucidation of the contribution of each toxin to disease was achieved by the use of a double toxin mutant that was complemented with different combinations of toxin genes (Awad et al., 2001). As well as investigating the functional role of CPA and PFO, this model has been used successfully to study the involvement of other potential virulence factors in the disease process (Chiarezza et al., 2009; Chakravorty et al., 2011), the connection between regulatory systems and virulence (Hiscox et al., 2011; Hiscox et al., 2013) and the effect of opioid analgesics on the development of gas gangrene (Chakravorty et al., 2014).

Chicken necrotic enteritis—Necrotic enteritis is a significant threat to the global poultry industries and hence there is an increasing effort to develop treatment methods and management practices to mitigate against the economic impact of the disease. A reliable disease induction model is an important tool in the research effort directed towards understanding and alleviating necrotic enteritis. *C. perfringens* toxinotype A strains expressing the β-pore-forming NetB toxin (Keyburn et al., 2008) are the causative agent of necrotic enteritis, but disease induction is complex, usually requiring more than just bacterial infection. Necrotic enteritis disease models in chickens generally incorporate some

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predisposing stress-inducing factor to assist in the establishment of disease. Feed manipulation is the most widely used factor with changes to feed in the period immediately before *C. perfringens* challenge commonly including a change to a high protein feed, often using high levels of fish meal, and/or an increase in cereals such as wheat or rye that contain high levels of non-starch polysaccharides. Such dietary manipulations may exert their effects in a number of different ways including increasing digesta viscosity and hence transit time in the gut (Annett et al., 2002) and by destabilizing and changing the underlying gut microbiota of the birds, making infection with pathogenic strains of *C. perfringens* more effective (Stanley et al., 2012, 2014). The high animal protein diet change may also provide nutrients such a selected amino acids that *C. perfringens* is unable to synthesize, thus improving bacterial growth. *Eimeria spp.* co-infection is another predisposing factor that has been widely used. The assumption is that *Eimeria spp.* exerts its effect on the disease model by causing damage to the gut epithelium and thus provides an entry point in which *C. perfringens* can colonize and proliferate (Williams et al. 2003; Van Immerseel et al., 2008), although induction of host mucogenesis may also be important (Collier et al., 2008). *Eimeria spp.* may also be the source of additional nutrients, including selected amino acids. A mixture of different *Eimeria* species, which infect different segments of the gastrointestinal tract (GIT), can be effectively used (Collier et al., 2008). An *Eimeria spp.* infection may also exert an immunological stress on the birds, making them less able to fight off a *C. perfringens* infection. Other means of inducing immunological stress, such as use of a bursal disease vaccine, have also been used in necrotic enteritis disease models (McReynolds, et al. 2004).

In our experience, the most reliable disease induction method (Cooper and Songer, 2010), uses large quantities of *C. perfringens* culture, grown through several stages of changing culture conditions, mixed with the feed and delivered over 2 or 3 days. This method has recently been used to test the efficacy of several experimental vaccines (Keyburn et al. 2013a, b). The *Eimeria spp.* co-infection disease induction methods can be problematic for vaccine studies because the lesions induced by this parasite, although distinct from typical necrotic enteritis lesions, can complicate gross disease scoring and there is a concern that the immunological effects of *Eimeria spp.* infection may compromise the response of birds to *C. perfringens* vaccination. Such issues would need to be dealt with for vaccine performance in the field, but they present unnecessary complications in the initial stages of experimental vaccine design and testing. The key to establishing a reliable necrotic enteritis induction model is the strain of *C. perfringens* that is used. A wide variety of pathogenic strains have been used by different authors and the different chicken strains may have different susceptibility to disease induction. The only strains that can reproducibly induce consistent levels of disease are *netB*-positive isolates (Keyburn et al., 2010; Smyth and Martin, 2010). In surveys of *C. perfringens* strains isolated from necrotic enteritis-diseased birds it is generally found that both *netB*-positive and-negative strains are identified. However, in the few studies in which these isolates have then been put back into birds, it has been found that only the *netB*-positive strains can induce disease. The finding that the *netB* gene is carried on a large conjugative plasmid may provide an explanation for the isolation of *netB* negative strains from disease cases – such strains are likely to result from plasmid loss during the initial isolation and culturing of the strains (Bannam et al., 2011). It has become clear that

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strain surveys are of little value unless at least a proportion of the isolates are tested in a reliable disease induction model to identify which strains are actually pathogenic. Strain surveys that have not included pathogenicity testing of isolates in a disease model have led some researchers to erroneous conclusions regarding the basis of pathogenesis in necrotic enteritis-causing strains of *C. perfringens*. Because *C. perfringens* is commonly found in the gastrointestinal tract of healthy birds, it is important to re-isolate and genotype strains from experimentally induced lesions to demonstrate that the observed pathology is due to the deliberately introduced strain rather than any pre-existing strain that may have been present in the birds prior to challenge.

The identification of NetB toxin as the major virulence factor in necrotic enteritis-causing strains of *C. perfringens* and the fulfilment of molecular Koch's postulates used a disease induction model in which small doses of *C. perfringens* strains were delivered to the challenged birds by direct oral gavage (Keyburn et al., 2008). The disease model also incorporated a feed withdrawal period and a change to high protein feed before the bacterial challenge.

Following *C. perfringens* challenge in small scale trials the scoring of disease severity generally relies on the observation and enumeration of macroscopic lesions in the small intestine. A number of slightly different scoring systems have been used but the 0 to 6 scale, first introduced by Keyburn et al. (2006) and recommended as a standard, with slight modification, by Shojadoost et al. (2012) gives the most complete indication of variation apparent in the manifestation of disease. In larger scale trials, involving hundreds to thousands of birds, productivity measures, for example feed conversion ratios, have been used as measure of the effects of disease induction (Wu et al., 2010). Of course, out in the field it is such effects on productivity and mortality that are the concern of farmers rather than the presence of lesions within the gastrointestinal tract. However, in small trials, involving groups of 10 to 20 birds, which are used to test strain pathogenicity and experimental vaccines, it is not possible to use productivity measures as a reliable indicator.

Historically, it has been difficult to reproducibly induce necrotic enteritis in experimental systems. However, with our increasing understanding of the pathogenesis of disease, it has in recent years been possible to establish refined models that can deliver consistent results. The biggest variable, which still exists and makes comparisons between studies conducted in different laboratories somewhat difficult, is the wide variety of pathogenic strains used – each of which varies in the level of disease induced. There would be value in the research community standardizing research efforts involving disease models around a few well characterized strains.

Type A Enterotoxigenic infections—*C. perfringens* CPE is a non-typing toxin that may be produced by *C. perfringens* type A, C, D and E isolates but not type B (McClane et al., 2006). This toxin is responsible for *C. perfringens* type A food poisoning, currently considered the second most common bacterial disease in the USA (Scallan et al., 2011). Enterotoxigenic type A strains are also responsible for 3 to 15% of nonfood borne gastrointestinal diseases in humans (Robertson et al., 2007).

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CPE is an ~35 kDa single 319 amino acid polypeptide (Czeczulin et al., 1993). This toxin is released in the alimentary canal during sporulation and it binds to specific claudins in the intestine (Katahira et al., 1997a and b; Fujita et al., 2000). CPE receptor-claudins may be found on many epithelial cells, including enterocytes (Smedley et al., 2008). After binding to the claudin receptor, CPE becomes part of a ~90 kDa small complex (Wieckowski et al., 1994; Robertson et al., 2007). Once in these small complexes, CPE oligomerizes to form an ~450 kDa SDS-resistant complex called CH-1, which contains 6 copies of CPE and receptor and non-receptor claudins (Robertson et al., 2007). The CH-1 complex then inserts into cell membranes to form an active pore (Smedley et al., 2007). This pore increases cell membrane permeability and induces influx of Ca^{2+} into cells thereby activating apoptotic (low CPE doses) or oncotic (high CPE doses) death pathways (Chakrabarti et al., 2003; Chakrabarti and McClane, 2005). The morphologic damage resulting from this pore formation also exposes the basolateral cell surface, resulting in the formation of an ~600 kDa CPE complex (CH-2) (Singh et al., 2000, 2001; Robertson et al., 2007). CPE-induced cell death then promotes intestinal damage that initiates fluid and transport changes (McClane et al., 2006).

Rabbit models: For many years rabbit intestinal loops have been used, and are still used today, to study the effects of CPE *in vivo* (Duncan and Strong, 1969; McDonel and Duncan, 1975; Smedley 3rd et al., 2008). Most of these rabbit models have been employed to study the effect of CPE in the small intestine, where it causes fluid and electrolyte secretion and produces significant damage to the mucosa of the jejunum and ileum, but less damage in the duodenum (McDonel and Duncan, 1977; McDonel et al., 1978; McDonel and Demers, 1982,). Molecular Koch's postulate have been fulfilled using rabbit intestinal loops, confirming that CPE is necessary for enterotoxigenic *C. perfringens* type A to produce enteric disease in this animal model (Sarker et al., 1999).

Recently, it has also been demonstrated that the rabbit colon is also sensitive to the action of purified CPE, with both fluid secretion and mucosal damage observed (Garcia et al., 2014). The histological changes caused by CPE in both small intestinal and colonic loops of rabbits consist mainly of mucosal necrosis and hemorrhage. These changes are both time- and dose-dependent (Duncan et al., 1968; McDonel and Duncan, 1975, Smedley 3rd et al., 2008; Garcia et al., 2014) and begin at villus tips in small intestinal loops (McDonel and Duncan, 1975, Sherman et al., 1994), where there is a greater density of claudin 4 (Smedley 3rd et al., 2008). Rabbits have also been used to study the binding of CPE to extraintestinal tissues, which led to the demonstration that this toxin binds to liver and kidney (McDonel, 1980), a finding that might explain systemic changes in patients with enterotoxigenic *C. perfringens* type A-associated disease.

Mouse models: Mice have also been used to study the intestinal and systemic effects of CPE (Yamamoto et al., 1979; Caserta et al., 2011). When injected intravenously (i.v.), ~ 2 μg of CPE was found to be lethal for mice (Hauschild and Hilcheimer, 1971; Sakaguchi et al., 1973). Lethality was associated with a rapid fall of blood pressure, respiratory difficulty and changes in the electro cardiogram (ECG). Because mice intoxicated with CPE (administered either i.v. or *via* intestinal loops) show hyperkalemia, it has been suggested that alterations in ECG and death are the direct consequence of elevated potassium in the blood (Hauschild

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and Hilcheimer, 1971; Sakaguchi et al., 1973; Caserta et al., 2011). In small intestinal loops of mice, as in rabbits, CPE causes dose- and time-dependent mucosal necrosis; however, toxin administrated in this manner does not cause fluid accumulation in mice (Caserta et al., 2011). Results of experiments inoculating CPE into intestinal loops of mice, suggest that death observed in constipated human patients with CPE-positive *C. perfringens* type A infection (Bos et al., 2005) could have been a consequence of absorption of CPE from the intestine. As in the rabbit, mice receiving CPE inoculated into intestinal loops were found to bind and form CH-1-like complexes in the liver and kidney.

Rat Models: The *in vivo* effects of CPE have also been studied in rats (Sugimoto et al., 1991), although much less extensively than in mice and rabbits. In rats, as in mice, this toxin produces lethality when injected i.v. and death in rats is preceded by respiratory difficulty, ECG alterations and hyperkalemia. Because these effects were accompanied by an increase of liver enzymes (GPT, GOT and LDH), Sugimoto et al (1991) suggested that CPE-induced hyperkalemia was the consequence of the cytotoxic action of CPE on hepatocytes.

Other animal models: Non-human primates have rarely been used to study the pathogenesis of CPE intoxication and/or enterotoxigenic *C. perfringens* type A-associated disease (Uemura et al., 1975). In the only published study, cynomolgus monkeys fed purified CPE developed vomiting and diarrhea, while monkeys given CPE-positive *C. perfringens* type A orally developed only diarrhea. These effects were only observed when either CPE or CPE-positive *C. perfringens* type A were given together with sodium bicarbonate to neutralize the low gastric pH. Lethality was not observed in these nonhuman primates (Uemura et al., 1975). No information on gross or microscopic changes of these monkeys was published and molecular Koch's postulates have not been fulfilled on non-human primates.

The effects of i.v. administration of extracts of sporulating cultures of CPE-positive *C. perfringens* type A, and of CPE into ligated loops, also have been studied in calves and lambs, respectively (Hauschild et al., 1967; Niilo, 1970, 1973;). The results of these experiments were variable and included diarrhea in calves and mild mucosal changes in the intestinal loops of lambs.

C. perfringens type C

C. perfringens type C isolates must produce, at the minimum, CPA and CPB (McClane et al., 2006). These strains are responsible for highly lethal enteric diseases and enterotoxemias in humans (enteritis necroticans) and in many other mammalian species. Type C disease is mainly characterized by necrotizing enteritis or enterocolitis and systemic disease. It is currently accepted that most clinical manifestations and lesions observed in patients with type C disease are a direct consequence of the action of CPB. This toxin is an ~ 35 kDa pore forming protein whose main cellular effect is cell death and lysis (Uzal and McClane, 2011) and which is lethal for mice (Sakurai and Duncan, 1978; Shatursky et al., 2000; Fisher et al., 2006) and dermonecrotic for mice and guinea pigs (Sakurai and Duncan, 1977; Nagahama et al., 2003). CPB is exquisitely sensitive to the action of proteolytic enzymes such as trypsin, explaining why the neonate is especially predisposed to its action; i.e., the trypsin inhibitors

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in colostrum, which prevent proteolytic degradation of immunoglobulins during the first days of life, also protect CPB toxin (Diab et al., 2012).

The mechanism of action of CPB toxin is not fully understood. The toxin forms pores in cells and in bilayer lipid membranes, creating channels that induce K^+ efflux and Ca^{2+}, Na^+ and Cl^- influxes, which are responsible for cell swelling and lysis (Nagahama et al., 2003). It has been shown to bind to intestinal endothelial cells, a fact that has been hypothesized to be an early step in endothelial cell necrosis, leading to thrombosis and ultimately intestinal necrosis (Miclard et al., 2009a and b; Schumacher et at, 2013). It has been recently demonstrated that CPB induces necrotic cell death in porcine endothelial cells *in vitro,* which is associated with an increase in intracellular calcium and is inhibited by necrostatin-1, suggesting a programmed cell necrosis (necroptosis) mechanism (Autheman et al., 2013).

C. perfringens type C disease has been studied using several animal models including pigs, sheep, goats, rabbits, guinea pigs, and mice (Field and Goodwin, 1959; Kohler et al., 1979; Lawrence and Cooke, 1980; Johansen et al., 1986a,b,c; Sayeed et al., 2008; Uzal et al., 2009; Garcia et al., 2013; Schumacher et al., 2013).

Rabbit and mouse models—A mouse intravenous lethality model was used to demonstrate that CPB is the main factor responsible for systemic lethality in type C culture supernatants (Fisher et al., 2006). In that study, lethality was abolished when culture supernatants were pre-incubated with a CPB monoclonal antibody, but not when the cultures were incubated with a CPA monoclonal antibody, which confirmed the role of CPB in mouse lethality (Fisher et al., 2006).

Conclusive evidence that CPB is required for type C strains to produce disease, was recently provided when molecular Koch's postulates were fulfilled using a series of *C. perfringens* type C strain CN3685 toxin mutants in rabbit intestinal loop (Sayeed et al., 2008) and mouse intragastric and intraduodenal models (Uzal et al, 2009) models. While the wild-type strain produced severe mucosal necrosis and fluid accumulation in rabbit intestinal loops, isogenic *cpb* mutants showed no virulence in the same model. Severe mucosal necrosis and fluid accumulation was still observed in rabbit intestinal loops inoculated with either *cpa* or *pfoA* mutants. Reversal of the *cpb* mutation restored full virulence to this mutant (Sayeed et al., 2008). The same set of CN3685 type C mutants were also used to study *C. perfringens* type C lethality and systemic changes in mice (Uzal et al., 2009). Intragastric and intraduodenal inoculation of the type C wild type in mice produced lethality, which was preceded by swollen abdomen, depression, respiratory and/or neurological signs. These mice, however, did not show diarrhea or intestinal lesions, which are characteristic of spontaneous type C disease in most animal species. This result was hypothesized to be a consequence of lack of receptors in mouse enteric epithelial and/or endothelial cells (Uzal et al., 2009). As in the rabbit intestinal loop model, when the isogenic CN3685 *cpb* mutant was tested in the intragastric and intraduodenal mouse model neither systemic effects nor lethality was observed. Inactivation of the genes encoding CPA or PFO did not have a significant effect in the lethality of CN3685 (Uzal et al., 2009). Altogether, the results of these intragastric and intraduodenal experiments in mice were interpreted as confirmation that CPB is the main

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virulence factor of *C. perfringens* type C and is responsible for the systemic effects observed during infection with this microorganism.

A rabbit intestinal loop model was also used to determine the spatial distribution of the effects of CPB along the alimentary canal (Vidal et al., 2008). In that study, fluid accumulation and necrotizing enteritis was observed only in the small intestine, with the jejunum and ileum being most severely affected. This result is in agreement with natural type C disease in animals and humans, in which the jejunum and ileum are primarily affected (Niilo, 1988; Farrant et al., 1996; Uzal, 2004; Matsuda et al., 2007). Although final evidence in this regard is lacking, an increased trypsin concentration in the duodenum has been blamed for the reduced effect of CPB in this intestinal segment, as this toxin is highly sensitive to trypsin (Zaitlin and Sircus, 1974).

Synergism between CPB and CPE for the virulence of CPE-positive type C strains of *C. perfringens* was recently demonstrated using a rabbit ligated intestinal loop model (Ma et al, 2014). Supernatants of wild-type *C. perfringens* type C,CPE positive, strain CN3758 culture lysates induced significant hemorrhagic lesions and luminal fluid accumulation in these intestinal loops. However, when lysate supernatants of the *cpb* or *cpe* knock out mutants of these strains were inoculated into rabbit ligated intestinal loops, no significant damage or fluid accumulation were observed. Complementing the *cpe* mutant, or reversing the *cpb* mutation, restored the virulent effects of culture lysates. Purified CPB and CPE, inoculated together at concentrations similar to those found in wild type CN3758 culture lysates, also produced lesions and fluid accumulation in the rabbit intestine. However, when either of these toxins was inoculated independently, only higher doses caused damage to the intestine, suggesting that at low concentration, both toxins act synergistically in the intestine (Ma et al, 2014). These experiments provided the first evidence of synergistic toxin activity during intestinal *C. perfringens* infections. It is possible that both CPB and CPE act synergistically at least in some cases of EN.

It is important to note that trypsin inhibitor had to be added to all of the type C cultures, supernatants and purified CPB to develop pathology in the intestine of mouse and rabbit models. This need to protect CPB from trypsin digestion is in agreement with the natural disease process in which only hosts with low trypsin levels are susceptible to CPB mediated disease.

Large animal models—Large animal models have also been used to study the pathogenesis of type C disease. Initially piglets were experimentally used for this purpose (Field and Goodwin, 1959; Kohler et al., 1979; Johansen et al., 1986a,b,c). However, those experiments were performed using whole cultures or crude culture supernatants, and although the results confirmed that *C. perfringens* type C is a pathogen for piglets, they did not identify the main virulence factor(s) involved in the pathogenesis of those infections. The mechanism of action of CPB in type C infection was recently studied in intestinal loops of piglets (Schumacher et al., 2013). This study indicated that there is a tropism of CPB toward endothelial cells, suggesting that endothelial damage induced by CPB plays a role in the early stages of *C. perfringens* type C enteritis in pigs. Molecular Koch's postulates for type C disease were fulfilled in goats (Garcia et al., 2012), using the same set of *C.*

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perfringens type C mutants previously used to fulfill those postulates in rabbits and mice (Sayeed et al., 2008; Uzal et al., 2009). The results of the goat experiments confirmed, this time in a natural host of the disease, the key role of CPB in the pathogenesis of natural *C. perfringens* type C disease.

C. perfringens type D

C. perfringens type D is responsible for a highly lethal enterotoxemia in sheep, goats and other ruminants. Type D isolates produce CPA and ETX, but several toxinotype D isolates also produce several other toxins (McClane et al., 2006).

ETX is produced in the form of a relatively inactive prototoxin, which becomes fully activated when a string of 14 amino acids from the C-terminus are proteolytically removed (Minami et al., 1997). Activation of ETX in the host intestine is mediated by serine proteases such as trypsin, chymotrypsin, and other proteases, including carboxypeptidases (Freedman et al., 2014). This activation occurs in a stepwise fashion, resulting in 3 distinct ~27 kDa ETX species and a higher-molecular-mass form (Freedman et al., 2014). When ETX is produced and activated within the intestinal tract of animals, the active toxin is absorbed into the systemic circulation and distributed to the brain, lungs, kidneys and other organs (Uzal and Songer, 2008). In the brain, and probably other organs, ETX binds to endothelial cells, producing degeneration and necrosis of these cells, leading to increased vascular permeability and perivascular edema, which, if the animals survive long enough, causes necrosis of the adjacent parenchyma (Buxton and Morgan, 1976). ETX crosses the blood-brain barrier and accumulates in the brain (Nagahama and Sakurai, 1991; Finnie, 2003; Soler-Jovel et al., 2007; Dorca-Arévalo et al., 2008), where it has been shown to have a direct effect on neurons (Finnie, 2003). In the brain of rats ETX binds to synaptosomes (Nagahama and Sakurai, 1992) *via* an as yet undetermined receptor, which is thought to be a sialoglycoprotein (Nagahama and Sakurai, 1991, 1992). The action of ETX on the hippocampus leads to an excessive release of glutamate, which seems to be at least in part be responsible for the neurological disorders observed in type D disease and/or ETX intoxication of several animal species (Miyamoto et al., 1998; Dorca-Arevalo et al., 2008, 2014).

Multiple animal models have been used to study the intravenous effects of ETX, including sheep (Buxton and Morgan, 1976; Uzal and Kelly, 1997), goats (Uzal and Kelly, 1997), cattle (Niilo et al., 1963; Uzal et al., 2002), mice (Finnie, 1984a and b; Fisher et al., 2006; Dorca-Arevalo et al., 2014) and rats (Finnie et al., 1999). The most significant effects of ETX are edema of the lungs and brain. It has also been demonstrated that rats and sheep intoxicated with ETX, suffer upregulation of aquaporine 4 (a membrane water-channel proteins) in the brain (Finnie et al., 2008; Garcia et al., 2014). A mutant with cysteine substitutions in the membrane insertion domain of ETX has been tested in a mouse model. The mutant was not lethal and the substituted toxin did not cross the blood-brain barrier or affect renal epithelial cells (Dorca-Arevalo et al., 2014). Based on these results it was suggested that there is a direct correlation between the lethal effect of ETX with its ability to cross the blood brain barrier and its effect on renal tubular cells (Dorca-Arevalo et al., 2014).

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Most of the clinical and pathological changes observed in natural type D enterotoxemias have also been reproduced by intraduodenal inoculation of type D whole cultures in sheep, goats and cattle (Buxton and Morgan, 1976; Blackwell et al., 1991; Uzal and Kelly, 1997, 1998). Although this evidence pointed to ETX as the main virulence factor of type D isolates, conclusive evidence for the role of this toxin was only obtained when molecular Koch postulates were recently fulfilled in several animal species (Garcia et al., 2013)

Mouse models—A *C. perfringens* type D wild-type strain CN1020, and its isogenic *etx* mutant were examined in an oral mouse model (Garcia et al., 2013). When mice were inoculated with the wild-type strain, most animals developed neurological signs followed by death, but no major anatomopathological changes were observed. By contrast, mice challenged with the isogenic *etx* mutant, remained clinically healthy and no lesions were observed. Virulence was restored by complementation of the *etx* mutant with the wild-type *etx* gene; these mice developed disease that was clinically and pathologically similar to that observed in the animals infected with the wild-type strain. These results indicate that ETX is necessary for type D isolates to induce disease in mice, supporting a key role for this toxin in type D disease pathogenesis.

Sheep and goat models—The same set of isogenic type D strains used to fulfill Koch's postulates in mice was used to study the contribution of ETX to *C. perfringens* type D virulence in an intraduodenal model in sheep and goats, both natural hosts of type D disease (Garcia et al., 2013). When sheep and goats were inoculated with the wild-type strain, most animals developed full blown disease characterized by respiratory and neurological signs, followed by death. Anatomopathological changes in sheep included edema of the brain, lungs and heart, and hydropericardium, while goats developed necrotizing colitis, pulmonary edema and hydropericardium. When another group of sheep and goats were challenged with the isogenic *etx* mutant, all animals remained clinically healthy and no lesions were observed in any animal. Virulence was restored by complementation of the mutant and most goats and sheep inoculated with the complemented mutant developed disease clinically and pathologically similar to that observed in the animals infected with the wild-type strain. These results indicate that ETX is necessary for type D isolates to induce disease, supporting a key role for this toxin in type D disease pathogenesis.

Other animal models—The action of purified or semi-purified ETX preparations has been studied by i.v. injection in cattle (Uzal et al., 2002), sheep (Buxton and Morgan, 1976), mice (Finnie, 1984a and b), goats (Uzal and Kelly, 1997) and rats (Finnie et al., 1999). With minor variations between animal species, the most significant effect of i.v. administration of ETX was brain and pulmonary edema, which are responsible for the neurological and respiratory signs of the disease. *C. perfringens* type D intraduodenal inoculation models have also been developed and used to study type D disease in natural hosts of the disease, including sheep (Uzal et al., 2004), goats (Uzal and Kelly, 1998) and cattle (Filho et al., 2009). These models allowed for a detail clinical and pathological characterization of type D disease in different animal species.

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Concluding remarks

Although significant progress has been made over the last few decades towards the understanding of *C. perfringens* infections in humans and animals, it was not until relatively recently that the development of reverse genetics, combined with the use of several animal models, allowed researchers to determine the importance of individual toxins in the pathogenesis and virulence of different toxinotypes and strains of this microorganism. In addition, although several animal models have been developed and used to study the pathogenesis of *C. perfringens* infections it is important to stress that different animal species must be used to study different types of *C. perfringens* and/or different effects of the same toxin. For instance, although the large animal i.v. models are useful to study the systemic effects of ETX, the changes produced in the intestine by this toxin could only be studied with intraduodenal inoculation models. Another example is the need to use both rabbit and mouse models to study the intestinal and systemic effects, respectively, of CPE. It is also important to note that although toxins are critical for disease pathogenesis the various diseases caused by *C. perfringens* are all infections. Therefore, a true understanding of the pathogenesis process can only be obtained by using infection models.

One of the difficulties for the understanding of the pathogenesis and diagnosis of *C. perfringens* enteric disease is based on the fact that most types of this microorganism can frequently be found in the intestine of normal animals. Simple isolation of *C. perfringens* is therefore not diagnostic for several enteric *C. perfringens* infections. However, it is possible that certain strains of *C. perfringens* carry unrecognized virulence factors that are not present in commensal strains. Finding those virulence factors would therefore be very helpful in determining the pathogenic role of several *C. perfringens* strains.

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Highlights

* Gas gangrene in humans was mainly elucidated using a mouse model coupled with genetic studies

* A chicken model was used to understand type A-mediated necrotic enteritis in poultry

* Food poisoning and necrotic enteritis was studied using mainly rabbits and mice

* *C. perfringens* type D infection has been studied using models in mice, rats, sheep, goats and cattle

* Molecular Koch's postulates have been fulfilled using animal models for most of these diseases