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Clostridium perfringens as a pathogenic organism in poultry

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Abstract

Clostridium perfringens causes necrotic enteritis (NE) disease in poultry. Necrotic enteritis has re-emerged as an important disease of poultry in recent years. The use of antimicrobials in poultry feeds has been attributed as one of the main contributing factors for the increasing incidence of necrotic enteritis in commercial poultry. Mortality due to NE is1% which results in great economic losses. Economic losses due to NE are not only associated with mortality but also associated with decreases in bird performance particularly in subclinical cases of NE. Birds that survive usually have a reduced ability to digest and absorb nutrients due to extensive damage to the mucosal lining, which ultimately results in reduced profitability. The poultry industry has been trying to reduce or eliminate the inclusion of sub therapeutic doses of antimicrobials into feed. Formulating diets not only meet bird's nutrient requirements for growth but is also important for gastrointestinal health parameters. Maintenance and enhancement of intestinal integrity is essential for bird performance when antimicrobials are not included in feed, as commercial poultry face numerous enteric pathogen challenges. The most cost-effective control will probably be achieved by balancing the composition of the feed.

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Introduction

Basic characteristics of Clostridium perfringens

Clostridium perfringens is a rectangular gram-positive, rod shape (0.6–2.4 x 1.3–9.0 μm), spore (oval subterminal spores) forming bacterium (Hassan *et al*., 2015). It differs from other clostridia in which the rods are encapsulated, non-motile (Cato *et al*., 1986) and the colonies are smooth and round. *Clostridium perfringens* is classified as an anaerobe but can grow under microaerophilic condition as oxygen is not actively toxic and cultures do not die on exposure to air (Quinn *et al*., 1994).

Growth conditions for Clostridium perfringens

Clostridium perfringens can grow within the temperature range of 12–50°C, though very slowly below 20°C (Adams *et al*., 1995). Under optimal conditions, 43–47°C, *Clostridium perfringens* grows extremely rapidly, with a generation time of 8–10 min, and growth is accompanied by abundant gas production (Bryant *et al*., 1997). Genome analysis has revealed that *Clostridium perfringens* is not able to produce 13 essential amino acids (Myers *et al*., 2006), therefore, *Clostridium perfringens* cannot grow in an environment where amino acids are limiting and it can obtain these via the action of exotoxins, some of which areenzymes.*Clostridium perfringens* grow in the pH range 5–8 but can survive under extreme conditions. Bacterial endospores are the most resistant biological cell type that can survive under extreme conditions, resistant to heat, desiccation, acids and many chemical disinfectants (Novak *et al*., 2002).

Clostridium perfringens habitat

Clostridium perfringens is related with diverse environments including soils, food, and sewage and as gastrointestinal tract microbiota of both diseased and non-diseased humans and animals (Ispolatovskaya, 1971). *Clostridium perfringens* has been constantly associated with various significant systemic and enteric diseases in both humans and animals including gas gangrene, food poisoning, nonfoodborne diarrhoea and enterocolitis (Heida*et al*., 2016). *Clostridium perfringens* is a normal inhabitant of the intestinal tract of chickens as well as a potential pathogen causing necrotic enteritis (Elwinger*et al*., 1998).

Clostridium perfringens strains

Differenttyping methods are used to differentiate between strains that may be associated with serious infection. *Clostridium perfringens* strains are clinically well-known for toxin production having seven toxinotypes: A, B, C, D, E and F. According to the combination of typing toxins, they produce α-toxin, β-toxin, ε-toxin and ι-toxin, enterotoxin (CPE) and NetB (Petit, 1999). Certain toxins are associated with certain hosts and diseases e.g., type B (particularly the β-toxin) is related to dysentery in sheep (Nagahama*et al*., 2015). Food-poisoning associated CPE is genotyped typically in type F strains although CPE can also be produced by certain other types such as C, D and E strains, whereas β2-toxin and θ-toxin could be found in any toxinotypes (Freedman, JC., 2016) but no single strain is known to produce the entire toxins (Kiu, 2017). Strains of *Clostridium perfringens* may also produce several other toxins including sialidiase, hyaluronidase, collagenase (McClane*et al*., 2006), neuraminidase and enterotoxin (Songer, 1996).

Function of different toxins

Clostridium perfringens strains are known to secrete more than 20 identified toxins or enzymes that could potentially be the principal virulence factors involved in pathophysiology (Revitt*et al*., 2015). *Clostridium perfringens* can generate a complement of extracellular toxins and hydrolytic enzymes, can survive in aerobic environments and can also produce toxic gases. Therefore, it possesses the capacity to be histotoxic, produce gas gangrene in contaminated wounds, gastroenteritis in human and necrotic enteritis in animals (McClane*et al*., 2006).

The major toxins are alpha, beta, epsilon and iota toxin all are potentially lethal depending on the host. The bacterium is classified into 5 types (A, B, C, D and E) according to different combinations of production of the four major toxins (Songer, 1996).

The alpha toxin gene of the *Clostridium perfringens* is present on chromosome close to the origin of replication and therefore all *Clostridium perfringens* strains carry this gene and produce this toxin in varying amount (Canard *et al.*, 1989). *Clostridium perfringens* alpha toxin (phospholipase C) is a Zn^{+2} metalloenzyme that degrades both lecithin and sphingomyelin. It promotes membrane disorganization resulting in lysis or other forms of cytotoxicity. Alpha toxin causes platelet aggregating, hemolytic, necrotic, and vascular permeabilization activities (Flores-Díaz *et al.*, 2004). It is the main virulence determinant in gas gangrene, which is a serious infection with fever, pain, edema, myonecrosis and gas production. It is shown that mutated strains that are unable to produce alpha toxin failed to cause this disease (Flores-Díaz *et al.*, 2003).

Beta toxin is a protease-sensitive pore-forming toxin. It forms pores by the formation of toxin multimers in the cell membrane, resulting in Ca_2+, Na+, and Cl^- influx and K^+ efflux from the cells (Nagahama*et al.*, 2003).

Epsilon toxin acts by forming large membrane pores by oligomerization into a heptamer resulting in potassium and fluid leakage of cells, which leads to the loss of cell viability (Petit *et al.*, 2003). The beta and epsilon toxins seem to have key roles in enterotoxaemia in calves, lambs, piglets and goats and most of the domesticated livestock in developed countries are immunized with toxoid vaccines.

Iota toxin is a binary toxin. It consists of two independent components, the enzymatic component (Ia) and the binding component (Ib). The Ia is an ADP-ribosyltransferase that modifies actin. The iota toxin is the only *Clostridium perfringens* toxin that acts intracellularly. All other toxins interact with the cell membrane leading to membrane disruption or pore formation (Marvaud*et al.*, 2001).

Enterotoxin is the cause of human food poisoning. Unlike the other toxins, enterotoxin is not secreted but is produced during sporulation (Lukinmaa*et al.*, 2002). It interacts with epithelial tight junction proteins and induces leakage of water and ions by forming pores or channels in plasma membranes of host cells (Smedley *et al.*, 2004).

All other toxins belong to the group of minor toxins. Theta toxin, also known as theta hemolysin, perfringolysin O or the thiol-activated cytolysin are located on the chromosome and are produced by all five toxin types of *Clostridium perfringens* (Rood *et al.*, 1991). Theta toxin is a member of the cholesterol-binding toxin family and causes complete hemolysis of red blood cells by forming oligomers, which subsequently form pores through the cell membrane (Awad*et al.*, 2001). A more recently discovered toxin is Beta2 toxin, a pore forming toxin that is associated with enteritis in neonatal pigs (Jost*et al.*, 2005). Other known toxins produced by Clostridium perfringens are: delta toxin, a hemolysin; kappa toxin, a collagenase; lambda toxin, a caseinase; mu toxin, a hyaluronidase; nu toxin, a nuclease; neuraminidase or sialidase, a N-acetylneuraminic acid glycohydrolase; and the gamma and eta toxins, whose functions are unclear (Hatheway, 1990).

Toxinotyping of necrotic enteritis

It is generally accepted that *Clostridium perfringens* type A is the causative agent of both clinical and sub-clinical necrotic enteritis since strains isolated from birds suffering from necrotic enteritis all belong to toxinotype A (Chalmers *et al.*, 2008). Moreover, clinical and sub-clinical necrotic enteritis are experimentally reproduced using *Clostridium perfringens* type A (Gholamiandehkordi*et al.*, 2007). The major typing toxins, type A strains produce only alpha toxin. Therefore, for a long time it was thought that alpha toxin was the major virulence factor in the pathogenesis of necrotic enteritis in poultry. Several studies have presented evidence for this hypothesis. Bacteria-free crude supernatant from *Clostridium perfringens* type A cultures produce necrotic lesions in broilers (Al Sheikly*et al.*, 1977) or cause mortality in germ-free chickens, after addition of antibodies to C. perfringens alpha toxin to the supernatant no

mortality was seen (Fukata*et al.*, 1988). Lovland*et al*, (2004) showed that maternal vaccination with a crude *Clostridium perfringens* type A and C toxoid induces antibodies against alpha toxin in chicks, which are partially protective against necrotic enteritis. However, care must be taken when interpreting these studies. Hence, crude supernatant was used and the assumption that the observed effects were caused by the dominant protein present in the supernatant (i.e. alpha toxin) did not consider other secreted toxins that the bacteria may have produced. Epidemiological and experimental evidence have supported the proposal that alpha toxin is an important protective antigen. High titers of antibodies to alpha toxin are found in poultry immune to necrotic enteritis. Moreover, immunization of broilers with purified alpha toxoid induces protection against experimentally induced necrotic enteritis (Kulkarni *et al.*, 2007). Thompson *et al.* (2006) showed that spontaneously derived alpha toxin mutants of a virulent strain have an impaired ability to cause NE lesions. However, since it was spontaneously derived mutants, the reduced virulence could be due to the impairment of the production of other toxins than alpha toxin. *C. perfringens* outbreak strains as well as normal broiler microbiota isolates are type A (Nauerby*et al.*, 2003). Moreover, no apparent difference in the levels of alpha toxin was found when the alpha toxin production in vitro was compared between strains associated with necrotic enteritis and isolates derived from the microbiota of normal broilers (Gholamiandehkordi*et al.*, 2006). Yet another study found that the intestinal level of alpha toxin was not correlated with disease lesion scores (Wilkie*et al.*, 2006). More convincing evidence was produced by Keyburn*et al.*, (2006), they showed that an alpha toxin mutant, constructed from a virulent chicken isolate, was equally able to cause necrotic lesion in broiler chickens as compared to the wild-type strain. Another observation that the role of alpha toxin in necrotic enteritis is the heterophil, lymphocyte, and plasma cell infiltration in infected tissues (Gazdzinsky*et al.*, 1992). In gas gangrene, a disease proved to be mediated by alpha toxin, marked leukostasis and lack of inflammatory infiltrate are common in tissues infected by *Clostridium perfringens*(Flores-Díaz *et al.*, 2003). Alpha toxin-negative mutants of *Clostridium perfringens* are not able to cause gas gangrene in mice but do promote profound inflammatory responses (Awad*et al.*, 1995). Thus, the massive immune-cell influx in necrotic enteritis lesions seems to be inconsistent with the known effects of alpha toxin on the innate immune system.

Necrotic enteritis in poultry

Necrotic enteritis (NE) was first described by Parish in 1961 and was first documented in England in 1961 (Parish, 1961). Since then NE has been consistently reported in every continent around the globe. Enteric diseases are an important concern to the poultry industry because of production losses, increased mortality and increased risk of contamination in poultry products for human consumption. It is a widespread disease in broilers and imposing a significant economic burden on the poultry industry worldwide. The total global economic loss because of necrotic enteritis outbreaks in broiler farms is estimated to be over $2 billion annually (Van der Sluis, 2000).

Influencing features for necrotic enteritis in poultry

Clostridium perfringens is taken up from the environment including contaminated feed, water or any part of the broiler production plant (Craven *et al.*, 2003). The presence of *Clostridium perfringens* in the intestinal tract of broiler chickens or inoculation of the animals with high doses of *Clostridium perfringens* however does not lead to the development of necrotic enteritis (LaRagione*et al.*, 2003). One or several predisposing factors may be required to elicit the clinical signs and lesions of necrotic enteritis such ascoccidial pathogens (Broussard *et al.*, 1986), antimicrobial growth promotor and immunosuppressors (McDevitt *et al.*, 2006).

Epidemiology of Clostridium perfringens in poultry

The incidence of *Clostridium perfringens* in the

intestinal tract of poultry is high. When the intestinal contents of broiler chickens are analysed for the presence of *Clostridium perfringens*, approximately 75% to 95% were found positive (Craven*et al*., 2000). When poultry meat is analysed for *Clostridium perfringens*, high percentages of positive meat samples are reported, however, in some cases up to 84% (Craven *et al*., 2003). It is suggested that colonization of poultry by *Clostridium perfringens* is a very early eventand can be transmitted within the integrated broiler chicken operation, starting from the hatchery (Craven *et al*., 2003). Ribotyping of isolates from the paper pads, isolates during the grow-out phase and carcass isolates indicate that at least some of the *Clostridium perfringens* contamination found on processed broiler carcasses can originate in the breeder operation and can be transmitted through the hatchery and grow-out operations (Craven *et al*., 2003). It is also shown that intestinal droppings of wild birds contain high numbers of *Clostridium perfringens* and that free-living birds can suffer from necrotic enteritis (Asaoka*et al*., 2003). In environmental samples collected on poultry farms, the highest incidences of *Clostridium perfringens* are detected in wall swabs (53%), fan swabs (46%), fly strips (43%), dirt outside the entrance (43%) and swabs of boots (29%) (Craven *et al*., 2000). All the afore-described studies indicate that *Clostridium perfringens* is a common intestinal inhabitant, but what can be questioned is the significance without further typing of the strains for toxin production.

Prevention and control

Necrotic enteritis (NE) prevention is usually associated with managemental practices that minimize the effects of the predisposing factors that contribute to disease development. Reducing the inclusion of dietary ingredients that may lead to NE, such as fish meal, oats, barley, and rye, has been a noteworthy solution in decreasing NE incidence (Cooper *et al*., 2009). The use of antimicrobial growth promoters (AGP) in feedalso play an important role in the control of NE. The introduction of AGP in the diet assists with coccidiosis management and modifies the intestinal microbial populations, which both result in a reduction in the incidence of NE. Other methods used to control coccidiosis such as vaccination with live Eimeria vaccines may also have an indirect effect on the incidence of NE (Van Immerseel*et al*., 2004). Necrotic enteritis has been treated by administering Lincomycin, Bacitracin, Oxytetracycline, Penicillin and Tylosin in water. Bacitracin, Lincomycin, Virginiamycin, Penicillin, Avoparcin, and Nitrovin can also be used in the feed to treat NE (Opengart, 2008). Necrotic enteritis vaccine studies show varying results for effective methods of NE prevention. Most vaccination efforts have been directed to producing toxoid vaccines by using culture supernatant, of which α-toxin is the major component (Lee *et al*., 2012). Recent findings suggest that netB and not α-toxin are the main virulence factors in the pathogenesis of NE. Strong evidence suggest that netB could be used as a toxoid and offer better protection than α-toxin (Lanckriet*et al*., 2010). Lanckriet*et al*., 2010 reported that vaccination of birds with supernatant from 2 different netB-positive strains of *Clostridium perfringens* significantly protected birds against NE. However, in the same study, no protection against NE was observed when vaccinating birds with the toxoid of the other 3 strains of *C. perfringens* that were also netB positive. These results indicate that immunity to NE induced after vaccination with supernatant of Clostridium perfringens is not entirely determined by netB or α-toxin expression, but probably involves other antigens that have not been identified. Feed additives, such as probiotics, are becoming popular prevention tools for NE. Probiotics are composed of beneficial micro-organism that are administered to birds with the intent of modulating the intestinal microflora. The full mechanism by which probiotics help balance the intestinal microflora is not fully understood and varies depending on the probiotic used. However, it is suggested that beneficial bacteria in probiotic products modulate the intestinal microflora by competing for nutrients and attachment sites with pathogenic bacteria, producing natural antibiotics, and stimulating the immune system (McReynolds *et al*., 2009). *Bacillus licheniformis* has been researched

as a direct-fed microbial with the potential to prevent enteric disease and reduce the severity of ongoing enteritis. Knap *et al.*, (2010) reported that the addition of 8 × 10^7cfu/g of feed of *B. licheniformis* was able to reduce NE mortality and lesion score to the same level as virginiamycin-treated birds. *Lactobacillus* spp. are also promising candidates to be used as probiotics in commercial poultry. In a study conducted to evaluate the effectiveness of a direct-fed microbial containing *Lactobacillus acidophilus* and *Lactobacillus casei*, cecal counts of *Clostridium perfringens* were significantly lower when poultry were fed the probiotic in comparison to control birds (Rahimi *et al.*, 2011). In another study using *Lactobacillus fermentum* as a probiotic, NE lesion severity of birds fed probiotic was significantly reduced when compared with challenged birds (not fed probiotics). Probiotic supplementation significantly downregulates the levels of Toll-like receptor 2, IFN-γ and upregulate the expression of IL-10 (Cao *et al.*, 2012). Another class of feed additive being researched with the objective of improving intestinal health is phytogenics and plant-derived compounds. Phytogenics include a broad range of plant materials, and essential oils represent a subcategory of phytogenics. The addition of phytogenic products to bird feeds has shown antimicrobial action, which is attributed to the ability of phytogenics to disintegrate bacterial cell membrane and penetrate bacterial cells. These antimicrobial properties are associated with the lipophilic character of phytogenics (Applegate *et al.*, 2010). Birds fed diets containing phytogenic blends presented significantly reduced severity of NE-associated lesions and mortality due to NE when compared with birds that were fed diets without the phytogenic blends. In the same study, when birds were fed a combination of the phytogenic blends with a multispecies probiotic, the combination of feed additives significantly reduced NE lesion severity when compared with birds fed control diets. However, no improvements in lesion severity were observed when birds were fed the combination of the feed additives in comparison to feeding birds probiotics or phytogenic blend separately ((McReynolds *et al.*, 2009). Research targeting the use of alternative feed additives to help treat and prevent NE have reported inconsistent results.

Conclusion

Necrotic enteritis is a complex disease that is very important to the commercial poultry industry because of the economic cost associated with infected flocks. The complexity of NE pathogenesis makesstreating and preventing this disease a real challenge. The challenge certainly increases with the reduction of the use of antimicrobial growth promoters (AGP) in poultry diets. Efforts in identifying the synergic effects of different virulence factors, such as netB and α-toxin and their mechanisms of action will aid in solving the NE. Additionally, it is important to research the synergic effects among virulence factors with predisposing factors, such as nutrient levels and dietary ingredients, so nutritionists can effectively formulate diets for its effects in gastrointestinal health. In conclusion, NE remains a challenge to the poultry industry and this challenge is becoming greater each day, with more strict regulations and consumers pushing for a product produced with lower levels of AGP. Although advances in NE research have contributed to identifying predisposing factors and preventative resources for NE, research projects focused on identifying the complete pathogenesis of NE and the mode of action of alternative feed ingredients are necessary to effectively prevent and treat NE without the aid of antibiotics.

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