Necrotic Enteritis in Poultry

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Abstract: Necrotic enteritis (NE) is an emerging economically significant problem of poultry caused by a bacterium *Clostridium perfringens (C. perfringens)*. due to high mortality rates and poor feed efficiency. Clostridia are considered to be among the most important agents of enteric disease in poultry which typically occursin broiler chickens but has also been diagnosed in various avian species including turkeys, waterfowl, and ostriches. Diagnosis of enteric diseases produced by clostridia is usually challenging, mainly because many clostridial species can be normal inhabitants of the gut, making it difficult to determine their role in virulence. Diagnosis is based on clinical and pathological findings. Negative culture and toxin detection results may be used to rule out this disease, but isolation of *C. perfringens* and/or detection of its alpha toxin are of little value to confirm the disease because both are often found in the intestine of healthy birds. Prevention strategies include avoiding predisposing factors, such as coccidiosis, and in-feed supplementation with a variety of feed additives. Supplementation of poultry diet with pre and probiotics has proven to be efficient to increase broiler chickens performance (health, weight gain, feed conversion) and to prevent or reduce the incidence of diseases caused by pathogenic bacteria. However, vaccination with modified toxin or other secreted immunogenic proteins seems a logical preventive tool for protection against a toxin producing bacterium.
[Nagwa S. Rabie, Zeinab M. S. Amin Girh and Mona S. Zaki. **Necrotic Enteritis in Poultry.** *Stem Cell* 2018;9(3):16-22]. ISSN: 1945-4570 (print); ISSN: 1945-4732 (online). http://www.sciencepub.net/stem. 3. doi:10.7537/marsscj090318.03.

Keywords: Necrotic; Enteritis; Poultry

Introduction

Enteric diseases are an important concern to the poultry industry because of production losses, increased mortality, reduced welfare of birds, and increased risk of contamination of poultry products for human consumption (**Hafez, 2011**). Necrotic enteritis (NE) is a major disease of poultry caused by infection with *C. perfringens* of which the typical hallmark is small intestinal necrosis **(Umar *et al.* 2016)**.

Avian necrotic enteritis (NE) caused by *C. perfringens* type A, rod shaped, gram-positive, anaerobic spore forming bacterium is wide spread in broilers imposing a significant economic burden on the poultry industry worldwide. It is an acute, often fatal, disease of chickens characterized by depression, loss of appetite and sudden death. It occurs both as an acute clinical disease and as a subclinical disease with necrosis in the intestines or as *C. perfringens*-associated hepatitic change (CPH) witiohepatitis or fibrinoid necrosis in the liver. In broilers, outbreaks of clinical NE have been reported from all poultry growing areas of the world **(Dahiya *et al.*, 2006)**. It has also been recorded in wild geese **(Wobeser and Rainnie, 1987)**, wild crows **(Asaoka *et al.*, 2004)**, ostriches **(Kwon *et al.*, 2004)** and in capercaillies reared in captivity **(Hofshagen and Stenwig, 1992)**. NE has increased in occurrence and severity over the years. The re-emergence of NE has been the most significant threat for the poultry industry, which, in clinical form, causes high mortality and in subclinical forms, affects growth and feed conversion. It is one of the most common and economically devastating bacterial diseases in modern broiler flocks in terms of performance, welfare and mortality. According to estimation, NE losses increased to approximately US$6 billion in 2015 as compared to US$2 billion in 2000 **(Umar *et al.*2016)**. NE is a multi-factorial disease process, in which a number of co-factors are usually required to precipitate an outbreak of the disease. Although, *C. perfringens* has been identified as the etiological agent of the disease, the predisposing factors that lead to over-proliferation of *C.perfringens* and the subsequent progression to disease are poorly understood. The onset of NE is associated with a shift in the microbiota present within the gastrointestinal tract (GIT) **(Antonissen *et al.* 2016)**. Any factor that causes stress in broiler chicks could suppress the immune system and disturb the balance of the intestinal ecosystem, in such a way that the risk of a NE outbreak increases **(Tsiouris, 2016)**. It has been clear that the use of antimicrobials and ionophorous coccidiostats have played a major role in keeping the disease under control for many years **(Kaldhusdal *et al.* 2016)**. The disease is regarded as a typical production disease related to predisposing factors associated with the way the birds are raised. Poultry

management could significantly affect the pathogenesis of NE. In particular, feed restriction and coccidiosis vaccination can protect against NE, while extreme house temperature, feed mycotoxins and high stocking density predispose to NE **(Moore, 2016)**. Most of the experimental models use a high protein fishmeal supplemented diet and coccidiosis infection as factors **(Wu *et al.* 2014)**. Antibiotics have been commonly used worldwide as growth promoters and for prophylactic treatment of *C. perfringens*-induced NE in poultry. However, with the European ban on antibiotics, alternatives to antibiotics became essential in order to prevent NE occurrence and the consequent economic losses for the poultry industry. However, identification of antibiotic-free, alternative disease control strategies has been hindered by the difficulty of experimentally reproducing NE by *C. perfringens* infection alone **(Umar *et al.* 2016)**. Preventive treatment scan take the form of actions on predisposing factors, such as coccidiosis prevention, diet modifications, or improving overall cleanliness and hygiene. Alternatively they can directly target the causal agent of the disease by controlling the proliferation, colonization and persistence of virulent strains of *C. perfringens* or interfering with virulence and pathogenicity factors *C. perfringens* infections can be reduced or abolished by using natural feed additives, such as probiotics (yeasts or bacteria), plants **(Engberg *et al.* 2012)**. molecules of plant origin for example, essential oils **(Liu *et al.* 2016)** or Annatto extracts **(Galindo-Cuspinera *et al.*2003)**, organic acids **(Geier *et al.* 2010)**, enzymes **(Caly *et al.* 2015)**, lysozyme **(Liu *et al.*2010)**,

Molecular basis of necrotic enteritis pathogenesis:

At molecular level, pathogenesis of NE is governed by various exotoxins produced by *C. perfringens*. *C.perfringens* isolates are toxinotyped by the presence of four major toxins (α, β, ε and ι toxins) various strains can also produce other so-called minor toxins such as CPE, β2 toxin, perfringolysin O (θ-toxin) and collagenase (κ-toxin); the term minor toxin does not refer to the importance or level of production of these toxins, but rather to the fact that they are not part of the toxinotyping scheme **(Rood *et al.* 2016)**. Alpha toxin: For many years, the chromosome-encoded alpha toxin, a membrane active phospholipase, was considered to be the major toxin associated with NE before the discovery of NetB toxins (β-pore-forming toxins) which are now considered the principal virulence factor essential for pathogenesis **(Keyburn *et al.* 2008)**. Almost all *C. perfringens* strains produce α-toxin, a major extracellular toxin that has been shown to be essential for clostridial myonecrosis **(Awad *et al.* 1995)**. Net B toxin: Recent studies have identified a novel toxin linked to NE, designated NetB toxin. It was first identified in an Australian *C.perfringens* type A strain **(Keyburn *et al.* 2008)** and it has been detected in the vast majority of NE-associated strains throughout the world **(Rood *et al.* 2016)**. *Clostridium perfringens* beta toxin, produced by *C. perfringens* type C, has also been implicated in the pathogenesis of NE and has occasionally been detected in the intestinal contents and feces of poultry suffering from NE. **(Hein and Timms,1972)** However, to the authors' knowledge, the role of CPB in pathogenesis of NE has not been investigated. Beta2 toxin (CPB2) was originally purified from a type C isolate from a piglet with hemorrhagic NE, but the toxin also has been suggested to have a role in poultry NE. **(Van et al.,2004)**. However, surveys of isolates from normal chickens and chickens suffering from NE revealed that prevalence of *cpb2* was only 14.3% in NE isolates, which is approximately the same level as in isolates from normal chickens. **(Gholami and ekhordi et al.,2006)** There is therefore little evidence that CPB2 has a role in the pathogenesis of poultry NE. **(Jost et al.,2005)** Enterotoxin (CPE), produced by some isolates of *C. perfringens*, is responsible for the highly prevalent *C. perfringens* food poisoning in human beings. CPE has been detected in the intestines of chickens with NE and has been suggested to have a possible role in the disease 8 by causing minor intestinal damage, allowing for multiplication of *C. perfringens* and eventual development of NE. **(Engström et al.,2003)** However, CPE is not actively secreted by vegetative cells, but is released upon lysis of the mother cell during sporulation. **(Brynestad and Granum,2002)** Fluid accumulation has been elicited in ligated chicken intestinal loops injected with CPE, but histological changes were much milder than those seen in rats and rabbits, animal model species in which CPE causes severe intestinal necrosis. **(Craven et al.,1999)** A role for CPE in NE is therefore still unproved. The rare occurrence of *cpe* in poultry isolates augurs against participation of CPE pathogenesis of NE.

Intestinal pathology and diagnosis:

The gross lesions of NE due to *C. perfringens* vary in both extent and severity and may be focal, multifocal to coalescing, or in severe cases may be diffuse, affecting the entire small intestine. While lesions are most commonly found in the proximal part of the jejunum, any part of the small intestine as well as the caecum may be affected. Necrotizing and fibrinonecrotizing lesions can be recognized as tan discolouration of the mucosa **(Shojadoost *et al.* 2012)**. In some cases, the mucosa becomes thickened due to buildup of adherent fibrin and necrotic debris, and has a marked coarsely granular texture and may be moderately firm and adherent or soft and moist. Ulcers may be recognized as depressed foci with a roughened

red exposed surface and there may be some limited haemorrhages at the margins or in the lumen. In birds with NE, all crater-like lesions are commonly classified as ulcers, when in fact many of these represent areas of mucosal thinning due to sloughing of necrotic mucosa. Such areas are characterized by having a smooth, often glistening, surface which is depressed compared to the surrounding mucosa. Often the rim of mucosa surrounding these reepithelialized craters and ulcers is necrotic, again evidenced by tan discolouration. Ulcers and reepithelialized craters are often visible from the serosal surface. Sloughing of large areas of mucosa together with loss of smooth muscle tone result in a thin flaccid intestinal wall. Foul-smelling gas and sloughed necrotic material may accumulate in the intestinal lumen **(Smyth,2016)**. Frequently, abnormally thick dark-green coloured bile discolours the mucosal surface or luminal content in the duodenum and proximal jejunum. Histologically, lesions of NE have a very characteristic appearance, but this appearance is not pathognomonic for *C. perfringens*; other clostridia, for example, *Clostridium colinum* **(Swayne *et al.* 2013)** and *Clostridium sordellii* **(Rimoldi *et al.* 2015)** Microscopic lesions in field cases of NE are typically characterized by extensive mucosal necrosis, **(Frame and Bickford, 1986)** although in some cases necrotic changes reach the submucosa, **(Ficken and Wages,1997)** and in the most severe cases the necrosis extends into the muscularis mucosa.83,101 Lesions typically develop at the villus tips, with coagulation necrosis and sloughing of the epithelial cells **(Olkowski et al.,2006)** As lesions progress, there is a sharp line of demarcation between necrotic and viable tissue **(Long et al., 1974)** due to accumulation of inflammatory cells in outer areas of the viable tissue. **(Broussard et al., 1986)** Heterophils are the dominant inflammatory cells infiltrating the mucosa in the initial stages of the disease, **(Ficken and Wages, 1997)** but mononuclear cells are also present in more chronic lesions.88 In the lumen, there is fibrinonecrotic material, which frequently forms a pseudomembrane composed of cell debris, bacilli, and inflammatory cells trapped in fibrin **(Long et al., 1974)** Gram-positive rods with square ends are commonly associated with areas of necrosis. **(Ficken and Wages, 1997)** Immunohistochemistry has revealed *C. perfringens* Occasionally, multifocal coagulative necrosis of the liver **(Sasaki et al., 2003)** mand bile ducts **(Kaldhusdal et al., 2001**) is observed. A fibrinous exudates and Gram-positive bacilli are present in the necrotic areas. **(Sasaki et al., 2003)** In such cases, the gall bladder and extrahepatic bile ducts are thickened and distended with yellow inspissated material, **(Sasaki et al., 2000)** and there is hyperplasia in the bile ducts and occasional granulomatous inflammation. **(Van et al.,2004)**

Differential diagnosis

Coccidiosis is a known predisposing factor of NE and can occur prior to or simultaneously with the disease, and thus it must be differentiated from NE. Typically, coccidiosis in chickens will result in blood in the intestinal tract, which is rarely observed in cases of NE. **(Johnson and Reid, 1970)** Histologically, in all avian species, the presence of oocysts at the tips of the intestinal villi or large schizonts in the lamina propria indicates coccidiosis, **(Al-Sheikhly and Al-Saieg,1980)** whereas large Gram-positive rods in the lesions would indicate a clostridial infection such as NE or UE. **(Williams, 2005)** Ulcerative enteritis is another clostridial enteric disease that must be differentially diagnosed from NE and is described in detail in the following. In short, UE will present with ulcers in the small intestine that often perforate the intestinal wall and produce peritonitis and intestinal adhesions. Isolation of *C. colinum* from the intestine acknowledges a diagnosis of UE instead of NE. **(Wages, 1997)** Histomoniasis must also be differentiated from NE. Gros pathology of histomoniasis includes necrotic liver lesions with depressed centers and well-defined edges, as well as cecal lesions composed of necrotic mucosa, with blood and other debris. The observation of living organisms by phase contrast microscopy can assist in diagnosing histomoniasis **(McDougald,2005)**

Control of necrotic enteritis:

Three general strategies have been proposed to control NE in poultry: (1) reduction of environmental exposure **(biosecurity measures)**, (2) an increase in poultry's host resistance to reduce pathogenic *Clostridium* species carriage in the gut (e.g., competitive exclusion, vaccination, and host genetics selection), and (3) the use of antimicrobial alternatives to reduce and even eliminate *Clostridium* species from colonised chickens (e.g., bacteriophage therapy and bacteriocin treatment). The genetics of birds appears to have some influence on susceptibility to NE as different lines of birds have different degrees of susceptibility **(Jang *et al.* 2013)** to NE and this may result from subtle difference in immune responses to *C. perfringens* **(Kim *et al.* 2014)**. Due to legistlative constraints on the use of antibiotics in feed, control of C. perfringens through natural approaches of intervention has become urgent for chicken production **(Liu *et al.* 2016)**.

Dietary control of Necrotic enteritis:

The protective effect of the feed restriction against NE was attributed to the neuroendocrine and immune system influence, as well as to the absence of nutrients in the intestinal tract. Furthermore, feed restriction improves blood circulation to the intestinal mucosa and may protect it from becoming necrotic **(Tsiouris *et al.* 2014)**. Feeding the birds with diets

based on wheat, rye oats and barley increases the incidence of NE in the birds than maize based diets **(Annett *et al.* 2002)**. Maize is considered an excellent ingredient in broiler diet due to its high energy value however it also has privilege over wheat and barley as high inclusion of maize in diet reduces the incidence of NE in birds. Cereal grains have high level of indigestible non-starch polysaccharides (NSP). Large amount of NSPs in cereals increases viscosity of digesta and gut passage time **(Moore, 2016)** which in turn leads to the bacterial colonization in the intestine. The ultimate results of increase viscosity and intestinal stasis predisposes the birds to NE. Thus reducing the amount of these cereal grains would be helpful in controlling the NE. High levels of animal protein in the diet, particularly fishmeal, have been used as a predisposing factor in experimental disease models **(Wu *et al.* 2014)**. Supplementation of the broilers' diet with one or several beneficial bacteria has proven to be efficient to prevent the overgrowth of pathogens and the subsequent diseases. Several bacterial strains have been shown toincrease broiler chickens performance (health, weight gain, feed conversion) and to prevent or reduce the incidence of diseases caused by pathogenic bacteria. Probiotics, or direct-fed microbials, and competitive exclusion (CE) cultures are thus commonly used in broiler farms. There are several commercially available products that have been shown to be efficient against *C. perfringens* and NE in poultry. A probiotic is defined as "a live microbial food supplement that beneficially affects the host by improving the intestinal microbial balance" **(Caly *et al.* 2015)**. Probiotic bacteria have also been shown to produce molecules with antimicrobial activities, such as bacteriocins, that target specific pathogens, or even inhibit the adhesion of pathogens or the production of pathogenic toxins **(Schoster *et al.* 2013)**. Yeasts are also known to have antimicrobial properties, which were recently reviewed **(Hatoum *et al.* 2012)**. In addition the cell wall is, for many types of yeast, rich in beta- glucans, which have immunomodulatory properties **(Alizadeh *et al.* 2016)**. Bacteriophages are highly species-specific viruses that infect and kill bacteria. Upon replication within the bacterial cell, phages produce endolysins, which target peptidoglycans and lyse the bacterial cell wall, freeing the phages and allowing them to spread to other cells **(Nakonieczna *et al.* 2015)**. Many bacteriophages of *C. perfringens* have been described and sequenced **(Caly *et al.* 2015)**, including several that were isolated from strains of poultry origin and that hadnspecific anti-*Clostridium* activity **(Volozhantsev *et al.* 2011)**. The use of bacteriophages to limit *C. perfringens* infection has proven efficient in field trials. The use of bacteriophages to control NE is a promising alternative; however, the application can be problematic. Indeed, it is hard to predict the behavior of the molecule *in vivo* and many factors can interfere. Recently, encapsulated carvacrol (essential oil) has been proved effective in controlling NE **(Liu *et al.* 2016).** Moreover, organic acid supplementation in feed can also inhibit growth of harmful bacteria and maintain intestinal heath by modification of host-pathogen interactions **(Timbermont *et al.* 2010)**.

Control of coccidial infections:

Coccidial infection has multiple effects on the GIT that predispose birds to NE. Physical damage to the GIT epithelium compromises gut integrity which opens direct access to the intestinal basal layer, which may be an important site in the early stages of disease; may expose extracellular matrix molecules, such as collagen, that have a role in *C. perfringens* adherence **(Wade & Keyburn, 2015)** causes serum leakage into the gut which acts as a rich nutrient source for *C. perfringens* growth; and induces mucus production, providing another protein-rich nutrient source to support *C. perfringens* proliferation **(Moore,2016)**. Therefore, control of coccidiosis will ultimately help to reduce NE in poultry.

Immune interventions:

Several strategies have been used to vaccinate broilers against *C. perfringens* to include use of live bacteria or inactivated toxins. Vaccines can be delivered by spraying chicks upon hatching, by addition to the feed or the drinking water, or even injected *in ovo* **(Mot *et al.* 2014)**. Vaccination using non- virulent *C. perfringens* strains have proven to be inefficient, and it has been shown that strains used in vaccines need to remain mildly virulent. **Thompson *et al*. (2006)** showed that strains with a mutation in the gene coding for the alpha toxin that were still virulent (but less than the wild-type) were able to protect chickens against NE, where as an avirulent strain of *C. perfringens* did not have any immunizing effects. Several trials have shown that chickens could be protected against *C. perfringen s*induced NE by injection with inactive and active toxins **(Jang *et al.* 2012)** and antigenic proteins **(Jiang *et al.* 2015)**. Protein-based vaccines are used because they are safer and better characterized when compared with live vaccines, while still providing protection **(Unnikrishnan *et al.* 2012)**. They include toxoids (inactivated bacterial toxins) and subunit vaccines, often based on virulence factors or secreted toxins. DNA vaccines that express Clostridium toxins, but not *C. perfringens* toxins, have also been tested as vaccine candidates **(Jin *et al.* 2013)**. Attenuated or avirulent bacteria can be used as vehicles for the effective delivery of vaccine candidates **(Rappuoli *et al.* 2011)**. Attenuated Salmonella strains are often used in poultry for the control of salmonellosis and they can serve as safe and effective oral carrier vaccines to prevent NE

by expressing heterologous antigens **(Jiang *et al.* 2015)**. The belief that alpha-toxin was important in disease misdirected vaccine efforts for many years but the recent advances made in our fundamental understanding of the basis of pathogenesis is now enabling the development of effective vaccines **(Moore, 2015)**. Since the discovery of its role in NE, the NetB toxin has been intensively studied with regards to vaccination, with some promising results **(da Costa *et al.* 2013)**. Several groups have shown that vaccination with NetB induces an immune response that delivers a degree of protection from the development of NE. Protection has been shown both in directly vaccinated birds **(Jiang *et al.* 2015)**.

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