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Replacements of Antibiotics in the Control of Necrotic Enteritis: A Review

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Abstract

Necrotic enteritis (NE) is an emerging economic problem of the broiler industry, which is caused by the bacterium *Clostridium perfringens (C. perfringens*). Under the normal physiological conditions, *C. perfringens* may be present in the gut asymptomatically but drastic changes of the gut environment result in damage to the intestinal mucosa, which can lead to a rapid proliferation of *C. perfringens* and the development of NE. Factors associated with the development of NE include parasitism (coccidiosis), high fiber diets and poor hygienic housing conditions. Moreover, excessive use of antibiotic growth promoters (AGP) enhances the capability of C. *perfringens* to induce the disease. The key virulence factors in the pathogenesis of NE of *C. perfringens* are the novel toxins, such as NetB and alpha toxins. Therefore, a toxoid vaccine using the alpha toxin, capable of generating an antibody response can be transferred to the progeny thus achieving partial protection from NE. However, these toxoid vaccines are still under experimentation and an insight of the mechanism which involves the role of alpha toxin in the development of immunity and pathogenesis is desired. This review has three aims: firstly, it intends to summarize the current available information about NE in chickens; secondly, it aims to elaborate the pathogenesis of NE at the molecular level and finally, future prospects of vaccination against NE and other possible novel methods for the control of disease are suggested.

Introduction

Poultry meat is becoming the first-choice source of animal protein worldwide. It contributes approximately 34% of the total share of meat produced in the world. The estimated production of poultry meat during 2012 was about 104.5 million tons [1]. However, a limiting factor in the growth and advancement of the poultry industry is the uncontrolled spreading of deadly diseases [2-4]. One of such diseases is necrotic enteritis (NE), which is causing enormous economic losses in the poultry industry, especially in broilers. The intestinal proliferation of *Clostridium perfringens* results in a sharp decrease in egg production, and eventually the mortality of birds which ultimately leads to higher economic losses in the industry. Financial losses associated with NE have been documented to be about 2 billion dollars annually [5]. Thus, an effective multi-dimensional strategy is required to protect the poultry industry from this devastating disease (NE).

The etiological agent of NE is a gram-positive bacterium, *C. perfringens* (type A and C) [6, 7]. These bacteria grow at wide ranges of temperature varying from 15°C to 50°C. *C. perfringens* is also one of the normal intestinal microflora of clinically healthy broilers. In the range of 33°C to 49°C, bacterial duplication is less than 20 minutes [7]. The ubiquitous presence of *C. perfringens*, its spore forming ability, and resistance to unfavorable growth conditions enhances its potential to cause the disease in animals [6]. The ability of the virulent strains to inhibit the growth of normal intestinal *Clostridium* strains, resulting in a selective proliferation of virulent strains leads to the development of NE and may be considered a virulent trait [8]. This review encompasses various aspects of the molecular basis of pathogenesis, clinical signs and non-conventional control of NE.

Predisposing factors

Nutrition

The environment of the intestine is the most important factor in the occurrence of necrotic enteritis. The type and quality of food is a major non-bacterial factor that plays a vital role in the occurrence of NE. Diets rich in indigestible, water soluble carbohydrates such as wheat, rye, oat, and barley are a major predisposing factor for birds developing necrotic enteritis. In contrast, diets rich in maize prevent/ reduce incidences of NE [9]. It is also reported that poultry with high dietary concentration of the fish meal also shows the increased occurrence of NE [10]. Moreover, fat derived from animals increases the bacterial count of *C. perfringens* compared to oil derived from plants [11]. The particle size of feed also affects the occurrence of NE [12]. A large number of research papers are published with reference to health and nutritional status of different plants [13, 14] and generally, all diets, rich in the concentration of hard to digest feed stuff and require a prolonged intestinal transient time, are associated with an increase in development of NE [15].

Stress

Stress changes the GIT environment in a negative way by enhancing the chance of the occurrence of NE [16]. Sudden stress, such as alteration in feeding pattern or switching from one food type to another is also associated with the development of NE. In addition to this, infectious diseases like viruses of chick anemia, Marek's disease, Gumboro or chemical agents that are immunosuppressive enhance the chance of NE by minimizing resistance against disease. The increase in the number of birds per unit area also induces stress on the birds and ultimately leads to NE [16]. To reduce stress on the birds, the European Union has implemented legislation defining the stocking density limits as 33 kg/m^2, and which can only be increased up to 42 kg/m^2 under some specific conditions (Directive 2007/43/EG).

Coccidiosis

Coccidiosis is one of the most important parasitic diseases of poultry [17]. It is an understood factor that results in damage to the membrane of the intestinal tract leading to the colonization of *C. perfringens* [7]. A few studies have demonstrated that coccidia-induced mucogenesis advances the onset of NE by supporting *C. perfringens* growth [18]. The development of colonies of *Eimeria* has also been shown to destroy the epithelium of the intestine [7].

As a result of the *Eimeria* life cycle holes in the epithelial coating of the intestinal lumen develop, allowing plasma proteins to spill into the gut, where they can be utilized as a development substrate by *C. perfringens* [19]. Moreover, the coccidial disease affects a T-cell-interceded provocative reaction that upgrades intestinal mucogenesis. Because of the capacity of C. *perfringens* to utilize bodily fluid as a growth substrate, this improved mucin creation and gives a development favorable position to C. *perfringens* [20]. Experimental studies have confirmed that infection of *Eimeria* and C. *perfringens* can act synergistically to promote the development of NE. Furthermore, *Emeria* and *C. perfringens*co-infections result in a higher mortality rate compared to the single infection of either *Eimeria* or *C. perfringens* [21].

Bacteriocins

Bacteriocins have a key role in the origination and the development of NE. Birds which survive from the NE by natural recovery or by the treatment will carry more than one genotype [22]. Several studies showed the single strain dominance is the key step in the occurrence and propagation of the NE. Isolates of *C. perfringens* from a flock with NE are generally identical within the flock regardless of which bird in the flock or which organ the strain is isolated from [22]. In contrast, amongst healthy flocks of bird's multiple genotypes of *C. perfringens* type A are found between different birds. Furthermore, genetic analysis has revealed that several different genotypes may be isolated from the same intestinal part of an individual healthy bird analysis. Bacteria produce certain chemical compounds, protein in nature that generally retard or inhibit the growth of relatively resembling strains are called bacteriocin [23]. Experimentally, it has been observed that the secretion of growth inhibiting factor by *C. perfringens* is more prevalent in epidemic strains than the normal microbiome strains [24, 9]. Moreover, it has been shown that a single dominant virulent strain could result in the development of NE disease in a study using inoculation of a combination of different strains or a single virulent *C. perfringens* strain [24]. These results suggest that bacteriocins have an effect on virulence. Though, there is a lack of information regarding the bacteriocins that *C. perfringens* produces *in vivo.* It is believed that bacteriocins production enables the virulent strain of *C. perfringens* to displace the intestinal microflora. Treatment of birds with zinc bacitracin at 200 and 400 mg/gallon or adding it to the feed at 100 mg/gallon reduces or prevents NE [9].

Molecular basis of necrotic enteritis pathogenesis

At the molecular level, the pathogenesis of NE is governed by various exotoxins produced by *C. perfringens*. These toxins include the alpha toxin, NetB toxins, and some unknown excreted products of the bacteria. The hydrolytic and proteolytic enzymes are also reported to be involved. The following part of the review provides a glimpse of the structural and functional activities of these toxins.

Alpha toxin

The alpha toxin is considered to be the most important factor involved in the virulence of *C. perfringens* [25, 26]. Earlier studies on *C. perfringens* have shown that the alpha toxin is chemically a zinc-Metallo phospholipase [27]. The alpha toxin of *C. perfringens* is 370 amino acid residues in length and has the ability to bind to membranes in the presence of calcium [28]. A α-helical N terminal domain (residue 1-246), contains the active site of the enzyme while the beta c-terminal domain (residues 256-370) is involved in membrane binding [29]. A linker (residues 247-255) connects the two domains [28]. The alpha toxin hydrolyzes the two important constituents of the eukaryotic cell membrane, i.e., phosphatidylcholine and sphingomyelin [30], thus causing membrane disorganization. Sub lytic concentrations of alpha toxins result in limited membrane damage and the accumulation of diacylglycerol into the cell membrane. This activates the arachidonic acid pathway [27]; a potent inflammatory mediator. Other metabolites of the arachidonic acid pathway are the leukotrienes C4 and D4. They play a pivotal role in increasing the membrane permeability and thromboxane A2, which induces accumulation of platelets [27]. Consequences

of alpha toxin expression include membrane disruption, vascular occlusion, and necrosis of the tissues, in the form of gas gangrene and myo-necrotic lesion.

NetB toxin

NetB toxin is a recently described toxin associated with *Clostridium* induced NE. The NetB toxin (33 kDa) shows similarity with different pore forming toxins, for example, 38% uniqueness with beta toxin from *C. perfringens*, 30% distinctiveness with the alpha toxin of *Staphylococcus aureus* and 29% identity with *Bacillus cereus* haemolysin-II toxin [31]. Expression of NetB toxin in male chicken liver carcinoma cells induces cytotoxicity through the formation of pores of 1.6 to 1.8 nm in size in the cell membrane [32, 33]. Deletion of NetB, from the genome of Clostridia, correlates with loss of virulence. Experimentally, virulence of the mutants can be complemented with NetB toxin [32, 34]. Furthermore, the NetB gene is present in the majority of *C. perfringens* isolated from the outbreaks of NE. In contrast, the NetB gene is absent in *Clostridium* isolated from other animal diseases, suggesting a significant involvement of the NetB toxin in NE development [35]. The available evidence suggests the NetB toxin plays a significant role in the development of NE in poultry.

Hydrolytic and proteolytic enzymes

In addition to the alpha and NetB toxins, various secretory products of *C. perfringens* as well as several host factors have a key role in the pathogenesis of NE. These factors include hydrolytic enzymes [36, 37] and proteolytic enzymes that are involved in the destruction of the basal lamina and lateral domains of enterocytes. Proteolytic activities affect the extracellular matrix and junctional complexes or structures. An increase in collagenolytic activity of the matrix metalloproteinase (MMP-2) in the intestinal tissues of birds infected with *C. perfringens*is thought to be associated with morphological changes to the basal and lateral domains of enterocytes [38]. *C. perfringens* preferentially grows in intestine suffering from mucosal damage. Mucosal damaging inducing factors include parasitism (coccidiosis), high fiber diets, poor hygienic and housing conditions in addition to the toxins. Moreover, excessive use of antibiotic growth promoters (AGP) enhances the capability of *C. perfringens* to induce disease because the constant use of AGP eradicates the normal microflora.

Intestinal pathology in poultry

Incidences of necrotic enteritis in broilers usually occur at the age of four weeks [39]. Clinically, the disease is characterized by a sudden increase in mortality of birds during the last weeks of the rearing period, which have exhibited no obvious clinical signs of disease. Pathological findings of these birds show necrosis of the mucosal surface of the Intestine [7]. In per acute cases of the disease, the sick bird may die within 1-2 hours [40] and mortality in broilers may be as high as 50%. In layers, mortality is usually reduced in comparison to broilers, typically around 6.5% only [41]. However, many signs common with other poultry diseases may appear in NE as well. These may include depression, reluctance to move and ruffled feathers [42]. Birds also appear to be sleepy, dehydrated [43], and diarrhea as well [41].

Subclinical NE is characterized by the absence of overt clinical signs and relatively high mortality. The chronicity of the subclinical form of the disease is represented by intestinal mucosal damage leading towards the loss of production, reduction in weight gain and a poor feed conversion ratio [44]. Besides the intestinal lesions, invasion of *C. perfringens* into the liver and bile duct results in hepatic damage, thus, hepatic lesions are taken as an indicator for the presence of NE in broiler birds [45]. Despite the high mortality rate of clinical disease, increased condemnation during post-slaughter meat inspection enhances the importance of subclinical infection due to the persistence of *Clostridium* in broiler flocks without any incidence of overt clinical disease [39]. Macroscopic lesions are most evident in the small intestine, i.e., in jejunum and ileum and to a lesser extent are also present in the duodenum, ceca, liver, and kidneys [46, 42]. Distention of the intestine due to gas accumulation and grey-brown to yellow-green diphtheritic membranes covering the mucosal surface

are the salient features of NE in poultry [47]. Moreover, there is also thinning of the wall of the duodenum, jejunum, ileum and sometimes the ceca. Apart from this, the invasion of *Clostridium* into the bile duct and liver results in cholangiopathies, hepatomegaly, reddish to white foci and an associated pale appearance of the liver [45]. Experimental inoculation of *C. perfringens* into birds resulted in congestion of the blood vessels in the spleen, kidneys, and liver [48].

Histopathological changes include intestinal damage starting from the development of edema at the interphase of lamina propria (LP) and enterocytes followed by the separation of the epithelial cells from the basement membrane with only a few cells in the LP showing early signs of necrosis. Subsequent events lead to the extension of the pathological process into the LP and amorphous masses replacing the cellular elements are visible. Hypercellularity of the LP along with a few enterocytes shows signs of necrosis (karyorrhexis and karyolysis). At the later stages, the LP shows complete loss of its normal histological architecture and a large number of enterocytes are necrosed. Preserved integrity of the apical domain of mucosal epithelium showed that mucosal damage is not a prerequisite element of etiology [49].

Control of necrotic enteritis

Rising concerns over the residual effect of antimicrobials on human health and the transfer of antimicrobial resistance to human pathogens [50] have led to the ban of the use of antibiotic growth promoter antibiotics (AGP). The use of AGP was also considered responsible for enhancing the spread of NE disease [51]. Moreover, the use of curative antibiotics and anticoccidials to control NE may also create resistance amongst the *C. perfringens* population [52]. Thus, it is imperative to look for compounds which can effectively replace antibiotics to control NE.

Dietary control of NE provides an excellent opportunity to cope with the losses associated with this devastating disease in a manner compatible with the demands of the post AGP era. Dietary ingredients play an important role in the eruption of NE in situations where the right *Clostridium* species are present. It is known that diets rich in indigestible, water soluble carbohydrates (wheat, rye, oat, and barley) are a major predisposing factor to NE, while a maize based diet prevents the incidence of NE [53]. Cereal grains contain a higher percentage of indigestible non-starch polysaccharides (NSP) [54]. A large amount of NSP's in cereals increases the viscosity of digesta and gut bypass time [31] which in turn leads to intestinal bacterial colonization. A highly viscous intestinal environment also creates favorable conditions for the proliferation of the facultative anaerobes like gram-positive cocci and E*nterobacteriaceae* [55]. Ultimately, though the increased viscosity and intestinal stasis predispose the birds to NE. Therefore, reducing the amount of these cereal grains should be helpful in controlling NE. Moreover, a reduction in NE might also be achieved by improving the digestibility of NSPs of wheat, barley, rye or oats through the use of dietary enzymes like b-glucanase, pentosanase and cellulase [56], or b-glucanase, xylanase, pectinase, amylase and cellulase [57].Wheat and fish meal also contains relatively high concentrations of zinc, which increases production of alpha toxin and protects it from the destruction by trypsin [58]. Therefore, reducing the quantity of fish meal in the diet might also be helpful in controlling NE.

Supplementation of feeds with fatty acids, various salts, oligosaccharides, prebiotics, and probiotics may also help reduce the incidence of NE. Some fatty acids and their derivatives are known to have antibacterial activity [59]. Dietary supplementation of butyric acid along with the combination of medium chain fatty acids, i.e., lauric acid and essential oils which include eucalyptus oil, thymol and cinnamaldehyde can play a significant role in the control of NE [60, 34]. Butyrate, however, does not directly inhibit *C. perfringens* but instead stimulates villi growth [61]. Furthermore, butyrate has anti-inflammatory properties [62] and can act as a source of energy for the epithelial cells which helps maintain villi shape [61]. Thus, butyrate modulates the host pathogen interaction by maintaining the bird intestinal integrity.

Supplementation of feeds with bismuth citrate has also shown encouraging results. Bismuth citrate is shown to reduce colonization of *Campylobacter jejuni*in [63]. A study conducted by Stringfellow et al. [64] to check the effect of bismuth citrate on *C. perfringens* showed that feeding 100 ppm and 200 ppm of bismuth citrate resulted in a significant reduction of colonization of *C. perfringens* in the intestine and the development of lesions when compared with the control group. However, experiments conducted to check the combined effect of lactose and bismuth citrate did not show a significant effect on the *Clostridium* colonization [64]. Lactose, however, reduces the pH of the intestine to a level of 4.4 to 5.5 [65] and growth of *C. perfringens* in the laboratory is inhibited at low pH [66]. Mikkelsen et al. [67] determined that inclusion of potassium diformate into feed at 4.5 g/kg of feed significantly reduced the NE related mortality. However, the effect was not linear as the inclusion level of 6.75 g/kg did not enhance the impact of treatment [67]. It can, therefore, be concluded that under certain circumstances, potassium diformate can be used to alleviate losses associated with NE and can be incorporated in an NE control program.

Vaccination: required in the post growth promoter antibiotic era?

The current situation suggests that the use of immunization might be an alternative strategy to the use of antibiotics in controlling NE. Unfortunately; no effective vaccine has yet been developed for use by the poultry industry [68]. Different attempts have been made to develop an effective vaccine against NE. Most of the *Clostridium* strains involved in NE are known to have *plc* gene that encodes for the alpha toxin [22], which is a metalloenzyme having sphingomyelinase and lecithinase activity and is thought to be involved in virulence of NE [69]. A toxoid vaccine using *C. perfringens* type A toxoid, produced an antibody response, resulted in partial protection from the NE, and which was capable of being transferred to the progeny has been developed [70]. However, vaccination using the *C. perfringens* alpha toxoid is still controversial [5] and further research into the mechanisms involving the role of alpha toxin in the development of immunity and pathogenesis needs to be undertaken.

Recombinant histidine tagged type-A toxin has been used to immunize birds and resulted in partial protection from NE. Subcutaneous vaccination of broiler chicks with recombinant alpha toxin and quail A as a vaccine adjuvant (1:1 W/V) at day 5^{th} and 15^{th} of age provided partial protection against NE after experimental *Clostridium* challenge. Amongst the vaccinated birds, 54.9% developed lesions. In contrast, 87.8% of non-vaccinated birds developed lesions associated with NE. Further, IgG titers 15 fold greater were observed in vaccinated birds when compared to non-vaccinated birds [5]. These results explain the possible use of alpha toxin as a vaccine candidate. However, more investigation on the role of alpha toxins in the development of NE is required as *Clostridium* strains, carrying a deletion of the PLC gene, were still capable of producing lesions in the gut of broilers in experimental trials [71]. Use of live attenuated oral vaccines has also resulted in immunization against NE. A combination of infection, subsequent treatment with bacitracin and then re-challenge can be used as a preventive measure to control the disease. Immunization is associated with virulent strains and not with the avirulent strains. Interestingly, the protection against NE after using live alpha toxin deficient isolates has turned attention towards bacterial factors other than an alpha toxin, as candidates for vaccine development [72].

The discovery of a novel toxin in NetB has opened new horizons for exploration of the pathogenesis of the NE and the development of better vaccines. Deletion of the NetB gene is associated with the loss of virulence, which shows the critical importance of NetB toxin as a virulence factor in the pathogenesis of NE [71]. However, NE is also observed in the birds having NetB negative *Clostridium* strains [35]. This highlights the importance of other virulence factors, i.e., hydrolytic enzymes like collagenase which may be involved in the virulence of NE, as they cause necrosis in the enterocytes [50]. Involvement of virulence factors other than the alpha and NetB toxins attracts our

attention to the experiments conducted by Riddell and Kong [53], where the supernatant from 8 strains of *C. perfringens* type A resulted in the variable protection from NE after challenge with strain 56 and 61. Subcutaneous administration of 200 µl of supernatant containing 7µl and 70µl of total protein at day 3 and 12 post hatching, respectively, with 50 µl of quial A as vaccine adjuvant resulted in complete protection from NE by using supernatant from strain 23 (NetB positive and low alpha toxin producer). The supernatant of strain 48 (NetB negative and high alpha toxin producer) provided partial protection from NE. It shows the involvement of immunogen other than NetB or alpha toxin for the prevention of NE [52].

Conclusions

Following the ban on the use of the AGPs, NE has become an increasing problem for the poultry industry. A better understanding of the molecular basis of the disease pathogenesis and discovery of new potential bacterial virulent factors will ultimately contribute towards the development of improved control measures for the disease. By improving hygiene, management, feed, and the construction and climate of stables, the outbreaks of the disease can be reduced. However, it is also necessary to control NE in an antibiotic free condition. Dietary control of the disease and protective immunization are the two main strategies that may be exploited to tackle the NE issue in a post AGP's regimen.

Conflict of interest

The authors declare that they have no conflict of interest.

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