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NetB Toxin and Immunization Against Necrotic Enteritis in Poultry: A Comprehensive Review

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Abstract

Necrotic enteritis, caused by avian-specific NetB toxin-producing strains of *Clostridium perfringens* type A, has gained worldwide concerns due to increased production losses and mortality in broilers, increased veterinarian costs, and the chance of getting contamination in products for human consumption. Prevention strategies include supplementing diet or drinking water with conventional therapeutic antibiotic growth promoters and anti-coccidial drugs.. There are various strategies to prevent the disease, including antibiotic growth promoters. Antibiotic growth promoters are antimicrobial agent that used for control of diseases such as necrotic enteritis. Other factor for prevention, anti-coccidial drugs that are taken orally. However, vaccination against toxin-producing *C. perfringens* type A via nano, toxoid, genetically modified, or other clostridial vaccines is a effective preventive against necrotic enteritis. This comprehensive review describes the recent advances in the development of NetB vaccines, other strategies to enhance immunization, their delivery systems in poultry against necrotic enteritis, and their pathogenesis. This review also exlains future immunization strategies like breeder hen vaccination, *in ovo* vaccination, and live (attenuated) vectors to be used in feed additives and other predisposing factors applicable in the field. All the vaccines discussed in the manuscript have shown their effectiveness against necrotic enteritis in poultry.

Introduction

With the increasing demand due to the increasing number of humans, the poultry industry has undergone many changes over time (Adams, 2004). One of the concerns in this regard is poultry diseases, especially gastrointestinal diseases, which can have an impact on overall productivity in terms of reduced body weight gain and increased mortality rate (Cooper *et al*., 2013). One of the most important poultry diseases is necrotic enteritis (NE), caused by anaerobic toxin-producing gram-positive *Clostridium perfringens* type A.

A high incidence of necrotic enteritis based on a protein-rich diet results in the proliferation of *C. perfringens* in the bird intestine (Riddell & Kong, 1992). *C. perfringens* is widespread in the environment and forms spores that produce a variety of toxins, like necrotic enteritis beta (NetB) toxin (Keyburn *et al*., 2010; Li *et al*., 2013). The role of NetB was subsequently identified by genetic studies, biophysical-biochemical analysis, strain typing, and immunization studies and therefore has been demonstrated to be an important virulence factor for the pathogenicity of necrotic enteritis. However, mutation of the *NetB* gene results in an avirulent strain. In contrast, the *NetB* gene that gets returned correctly into the mutant strain will no longer be able to cause necrotic enteritis in the chicken models (Keyburn *et al*., 2008). Recent studies and initial analysis of NetB toxin describes its potential role in pathogenesis and the next generation of necrotic enteritis vaccines.

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Structural analysis of the NetB toxin

NetB toxin was first identified in an Australian strain of *C. perfringens* isolated from necrotic enteritis-infected chicken (Keyburn *et al*., 2008). Due to its similarity with the β-toxin of *C. perfringens*, it was known as necrotic enteritis toxin, B-like (NetB), a pore-forming toxin secreted in the late exponential growth phase and is encoded on a large plasmid in the *C. perfringens* type A strains. Bioinformatic analysis revealed that NetB encoded 323 amino acids composed of a signal peptide of 26 amino acids. Then, a 30-amino-acid sequence cleaved, resulting in an active toxin (mature β-toxin protein) with a molecular weight of 33 kDa (Keyburn *et al*., 2008). The molecular structure of the toxin monomer is composed of 16 β-strands and a single α-helix which form the β-sandwich, latch, rim, and pre-stem segments. The β-sandwich region consists of five-stranded and six anti-parallel β-sheet. The rim domain consists of four antiparallel β-sheet strands and a loop compromises residue from 205 to 242. The rim domain consists of aromatic groups attached to the lipid membrane. The pre-stem domain forms an antiparallel beta-sheet with three strands, including residue from 140-186, which orients against a five antiparallel stranded beta-sheet (Yan *et al*., 2013). The schematic structure of the netB toxin is shown in Figure 1.

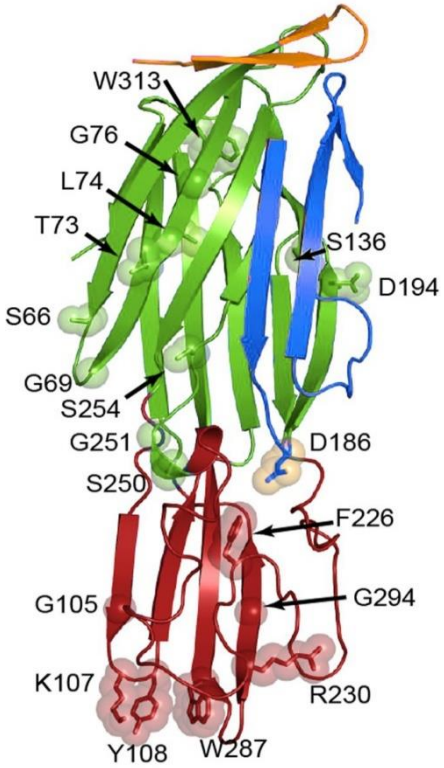

Figure 1. Pictorial representation of the overall fold of NetB: The β-sandwich, latch, rim, and pre-stem domains are shown in green, yellow, red, and blue, respectively (Yan *et al*., 2013).

NetB Toxin and Necrotic Enteritis Pathogenicity

NetB gene is encoded on plasmids approximately 82 kilobases a within pathogenicity locus (NELoc-1), which is closely linked to two plasmids that carry 70 kb β2-toxin and 49 kb tetracycline resistance plasmid (Lepp *et al*., 2010; Rood *et al.*, 2016). The Vir-SR two-component signal transduction system regulates NetB gene expression and includes several subunits. The VirS/VirR is compromised by both the *vir*R response regulator gene and the *vir*S histidine kinase sensor gene. The VirS domain is composed of a hydrophobic N-terminal with 6 trans-membrane regions, and autophosphorylation occurs between 4 to 5 N-terminal trans-membrane regions. The VirR N-terminal region contains a preserved domain, which requires a phosphate group from the histidine kinase sensor located in the C-terminal domain (Ohtani & Shimizu, 2016). Seven residues of Net B monomers lead to the formation of the active form of heptavalent toxin, which results in channels formation within the phospholipid membrane which leads to cation input, cell rounding, and lysis (Timbermont *et al*., 2011; Yan *et al*., 2013). Normal and healthy intestinal tissue prevents bacterial adherence to cells (Paap & Additives, 2015). Factors such as intestinal damage caused by *Eimeria* (ECMMs), NetB toxin, and collagenolytic enzymes result in the binding of *C. perfringens* strains to the intestinal tissue. By destroying the epithelial cells, *Eimeria* parasites induce leakage of plasma proteins

and enhance the production of mucus in the intestinal cell. Above two factors provide an increased nutrient availability, allowing the growth of *C. perfringens* in an appropriate environment. Other Clostridia strains are also inhibited by the production of bacteriocins by *C. perfringens* strain. Finally, the NetB toxin leads to channel formation which results in cell rounding and lysis (Timbermont *et al*., 2011; Yan *et al*., 2013). A diagram of intestinal pathogenicity in necrotic enteritis is shown in Figure 2.

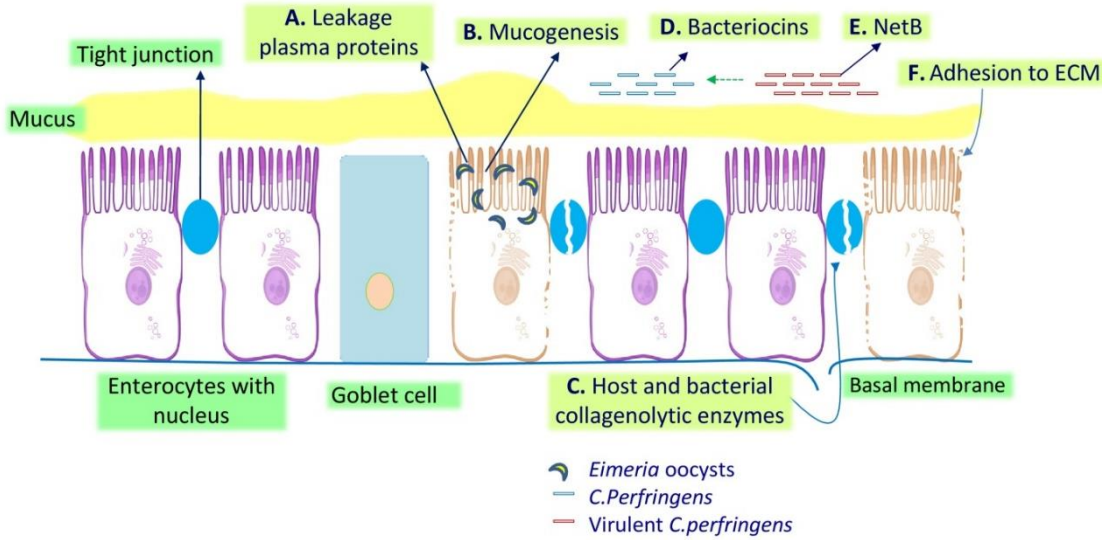

Figure 2. Pictorial representation of intestinal pathogenicity in necrotic enteritis (Paap & Additives, 2015). A: Leakage of plasma proteins, B: Mucogenesis, C: Production of host and bacteria collagenolytic enzymes, D: Production of bacteriocins, E: Release of NetB toxin, F: NetB attachment to ECMM

Clinical features of Necrotic enteritis

Several predisposing factors for necrotic enteritis in poultry, such as coccidiosis co-infection, nutritional stress and protein-rich diet are pre-dominant (Elwinger *et al*., 1992; Paap & Additives, 2015; Riddell & Kong, 1992; Williams, 2005). Coccidiosis in broilers often develops before or concurrently with outbreaks of necrotic enteritis. Although both diseases have different pathology, they act synergistically, and good husbandry practices will help to reduce their transmission risk.

Avian necrotic enteritis is identified by necrotic lesions in the small intestinal mucosa and has two manifestations, clinical (acute or severe) and subclinical (sub-acute) (Islam *et al.,* 2009). The clinical signs of acute necrotic enteritis include severe depression, dehydration, apathy or ataxia, diarrhea, ruffled feathers, decreased appetite, and acute death can occur within hours (Cooper *et al*., 2013). However, the sub-acute form of NE leads to reduced digestion, absorption, and a decrease in feed consumption, weight loss, and reluctance to move (Kaldhusdal *et al.,* 2001). In subclinical infections, the *Clostridium* strains can reach the bile duct and blood circulation, and colonization of the liver occurs which results in cholangiopathy and the infected liver expands to become pale in appearance with red or white spots (Sasaki *et al*., 2000). The subclinical manifestation is more important than the clinical one because of the asymptomatic cases (Kaldhusdal *et al*., 2001), but their diagnosis is based on gross necrotic lesions in the small intestinal mucosa (Cooper *et al*., 2013). Understanding the pathology and predisposing factors of necrotic enteritis is very helpful for developing an effective control strategy.

Vaccines against Necrotic Enteritis

Necrotic enteritis has long been controlled by administering antimicrobial drugs to water or diet (Islam *et al*., 2009). However, this conventional therapeutic approach conflicts as a result of disease resistance issues and interest in other novel preventive approaches, including immunization or vaccination is increasing. Various commercial and experimental vaccines are used for poultry immunization which are mentioned below.

Bacterin-toxoid vaccine

Keyburn *et al.* (2013b) developed the bacterin NetB vaccine from bacterial culture which was concentrated by centrifugation and ultrafiltration. Then bacterial cells were resuspended and sonicated followed by formaldehyde treatment and this formaldehyde-treated bacterin consisting of sonicated bacterial cells and culture supernatant (50:50 v/v) injected into chickens. The experimental finding showed that the vaccine had the potential to prevent necrotic enteritis, which varied according to the amount of challenge.

Toxoid vaccine

NetB toxoid vaccine has been prepared from the inactivated toxin of *C. perfringens* using formaldehyde and has been studied as vaccine candidate for the prevention of NE in chickens that were vaccinated subcutaneously. The result findings of *in-vitro* (ELISA) and *in-vivo* studies have shown that the toxoid vaccine significantly induces protective immune responses against NE (da Costa *et al*., 2013).

Mutant vaccine

Following structural and functional analysis of the NetB toxin, mutants with lower cytotoxic activity have been developed by researchers (Yan *et al*., 2013). Single amino acid change in the bonding surface of the protein membrane (in the rim region), will significantly reduce its toxicity and result in complete loss of functional expression of protein, which involves protomer and protomer interactions in this region, which are significant for the structural rearrangements in multimer formation (Song *et al*., 1996). An earlier study reported single amino acid substitutions (including Y191A, R200A, W257A and W262A) reduced cytotoxicity in LMH cells and hemolytic activity in red blood cells (RBC) and therefore was proposed as a potential vaccine candidate (Savva *et al*., 2013).

Another study designed mutants based on conserved sequences (K41, D156, and P155), that are involved in the structural formation of NetB crystal heptamer and play a key role in toxicity. However, some unconserved sequences (K71 and D250) and amino acids adjacent to the residues have important functions in Alpha Helix of *Streptococcus aureus*. K41H mutant was found significantly less toxic to human LMH cells and RBC than the NetB wild type and was not able to form heptameric pores in liposomes. Thus NetB K41H mutant can be developed for protection against NE (da Costa *et al*., 2014). Other studies have also shown the protective immunity of mutants NetB vaccine against Necrotic enteritis when chickens were immunized subcutaneously to evaluate the efficacy using *in-vitro* (ELISA) and *in-vivo* (oral administration of virulent strain) studies (da Costa *et al*., 2013). Furthermore, the NetB mutant is the first rationally attenuated strain that has considerable vaccine potential obtained from NE-causing isolate of *C. perfringens.*

Subunit vaccine

Duff *et al.* (2019) was designed mucinase based vaccine using several conserved peptide sequences of carbohydrate binding and evaluated the vaccine potential via *in-vivo* studies in eight group including non-vaccinated, non-inoculated control, non-vaccinated inoculated control using single peptide and/or in combination of all peptides. The study outcome showed that combination of all mucinase peptides was the most significant subunit vaccine type and has the potential to provide the protection against NE, so that can be further used as novel alternative current vaccine.

Recombinant vaccine

The recombinant NetB protein has been cloned, purified and evaluated *in-vivo* and *in-vitro* using pENTR/SD/D-TOPO and pBAD vectors (Keyburn *et al*., 2008; Savva *et al*., 2013). The result suggested that the recombinant NetB has protective efficacy as an immunogenic component and can induce strong immune response or antibodies against NE (Savva *et al*., 2013). A study conducted by Keyburn *et al*. (2013b) also showed that chicken vaccinated with recombinant NetB was significantly protected against mild oral doses of virulent *C. perfringens* strain. However, the level of protection against high dose of vaccination was inadequate (Keyburn *et al.*, 2013b). Whereas the chicken vaccinated with raw toxoid plus recombinant NetB produced a significant increase specific IgY antibodies in serum samples and egg yolk (Keyburn *et al*., 2013a).

Other *in-vitro* ELISA-based studies on the recombinant vaccine containing NetB, pyruvate, ferredoxin oxidoreductase, PFO, α-toxin, and elongation factor (EF-Tu) showed high antibody titers both in chickens with clinical signs and in a healthy one. These results suggested that PFO and EF-Tu are useful antigens for diagnosis (Lee *et al*., 2011) and produce antibodies against them can play an important role in necrotic enteritis and gangrenous dermatitis (Lee *et al*., 2012).

Rostami *et al.* (2016) developed a three folds recombinant CPA- NetB- TpeL vaccine with a high level of antibody titer in vaccinated animals, suggesting its role as a recombinant vaccine that may be a candidate for preventing clostridial infection. Other studies also reported the production of a tetravalent vaccine with four toxins (CPA, CPB, ETX, and NetB toxin) and recombinant vaccine composed of NetB, Alpha-toxin, and metallopeptidase proteins (NAM) and evaluated their *in-vivo* and *in-vitro* efficacy (Katalani *et al*., 2019; Zaragoza *et al.*, 2019).

DNA vaccine

DNA vaccines expressing other Clostridial toxins include *C. botulinum* neurotoxins; *C. difficile* toxin A and toxin B, and tetanus toxoids have also been tested as vaccine candidates (Gardiner *et al.*, 2009; Jin *et al*., 2013; Li *et al.*, 2011; Saikh *et al.*, 1998). But so far, it has not been done for *C. perfringens* toxins.

Nano vaccine

In recent years, a chitosan-nanoparticle vaccine was synthesized using purified native extracellular protein

and cell wall proteins from *C. perfringens* to vaccinate birds. The immunized birds induced an immune response via the oral route (Ramadan *et al*., 2020). Akerele *et al*. (2020) developed a chitosan-nanoparticle with native and toxoids of *C. perfringens* extracellular proteins tagged with *Salmonella* flagella proteins. Their result showed prepared nano vaccine was safe in birds and stimulated the specific immune response (Akerele *et al*., 2020)

Vaccination strategies against Necrotic Enteritis

Several groups have shown that vaccination with a combination of *C. perfringens* toxins and various adjuvants induces a strong immune response against the development of necrotic enteritis. A study conducted by Jang *et al.* (2012) using four vaccines containing NetB, Pyruvate Ferredoxin Oxidoreductase (PFO), α-toxin, and Elongation Factor (EF-Tu) with Montanide™ ISA 71 VG adjuvant tested subcutaneously over broiler chickens. Parameters like intestinal damage, body weight gain, and levels of antibodies against NetB and PFO toxins were measured, and all vaccinated broilers with protein plus ISA 71 VG showed fewer intestinal lesions and more weight gain compared to the ISA 71 VG control group. In addition, higher levels of specific antibodies were observed in the NetB plus ISA 71 LV group compared with the other three groups. So, vaccination with PFO or NetB proteins in combination with ISA 71 VG adjuvant enhances immunity against NE (Jang *et al*., 2012).

In another recent, 18-day chicken embryos were injected with the *Eimeria* profilin (EP) with recombinant NetB and with or without adjuvants (montanide adjuvant IMS 106 and IMS 101). After hatching, they were experimentally challenged with NE, followed by measurement of body weight, intestinal lesion, serum NetB antibodies, and transcriptions of proinflammatory chemokines and cytokines in the intestinal intraepithelial lymphocyte cells. Chickens vaccinated with EP_+ recombinant NetB plus IMS 106 and PE+ recombinant NeB plus IMS 101 showed increased body weight, decrease in intestinal lesions, and strong antibody reaction, respectively, compared to the EP group. Above findings suggests that injecting montanide adjuvants with other antigens into chicken embryos improves chickens' immunity levels against experimental NE (Lillehoj *et al*., 2017).

da Costa *et al.* (2016) vaccinated chickens via the subcutaneous route in two separate experiments involving four vaccine groups, including recombinant NetB+W262A, recombinant CPA, NetB+W262A plus recombinant CPA, and PBS buffer. Quilt A was used as an adjuvant for every group. In the first experiment, all groups were challenged orally, and in the second experiment, all groups were challenged fed. Their results proved that the combination of NetB+W262A and recombinant CPA is the greatest protection against bacterial challenge (da Costa *et al*., 2016).

Mishra and Smyth (2017) studied non-virulent NetB with or without TM 01 Gel adjuvant, cholera toxin (CT), *Escherichia coli* LT toxin, and mutant *E. col*i LT (dmLT) (R192G/ L211A) with multiple ways. All immunization regimens had serum and mucosal antibodies to alpha toxin and secretory proteins. However, in some vaccinated groups, such as the CT adjuvant group, 55% of vaccinated birds were found no lesions up to 6 days after bacterial challenge. The Results of previous studies also indicated that effective immunogens are required to increase immunity (da Costa *et al.,* 2013; Keyburn *et al.,* 2013b; Lanckriet *et al.,* 2010). Several proteins purified from *C. perfringens* have been assessed as appropriate candidates for the NE vaccine. Proteins such as HP, PFO, elongation factor G (EF-G), perfringolysine O, glyceraldehyde-3-phosphate dehydrogenase and fructose-1-6-bisphosphate aldolase (FBA) have been effectively used against virulent *C. perfringens* strain (Kulkarni *et al.,* 2010; Kulkarni *et al.,* 2007). Furthermore, both attenuated and non-virulent bacteria may be considered and used as effective carriers for vaccines (Rappuoli *et al.,* 2011). Attenuated *Salmonella* strains are often considered effective and safe delivery for oral vaccines (Hegazy & Hensel, 2012). Recombinant attenuated *Salmonella* vaccines have advantages including being needle-free, low-cost, stimulating mucosal, systemic, and cellular immune systems (Kong *et al.,* 2013; Wang *et al.,* 2013). Results findings by Zekarias *et al.* (2008), showed that oral immunization by attenuated serotype of *Salmonella enterica* with fructose-1-6-aldolase bisphosphate and *C. perfringens* alpha toxin (CPA) induced a protective reaction against NE in chickens (Zekarias *et al.,* 2008).

Wilde *et al.* (2019) also found that oral vaccination by attenuated strains of *Salmonella* containing α-toxoid, NetB, and Fba alone or in combination can induce strong immune responses against *C. perfringens* antigens. All three vaccines provide protection against *C. perfringens* alone, but along with Fba can provide the best protection against NE (Wilde *et al.,* 2019). In addition, vaccination with the mixture of alpha toxin NetB fusion protein using an attenuated *Salmonella* strain was protective against mild and severe challenges of *C. perfringens*, but vaccination with a single protein was not protective against NE (Jiang *et al.,* 2015). Lactic acid bacteria may also be vaccine carriers for *Clostridium* infections (Robinson *et al.,* 2004; Gangaiah *et al*., 2022).

Future immunization and vaccine delivery systems against Necrotic Enteritis in poultry

Vaccination in Breeder hens

Breeder hens vaccination is preferred because of low labor costs and time-saving in the poultry industry (Schijns *et al.,* 2014). Although immunization of breeder hen has limitations for necrotic enteritis. For example, epidemics usually occur at 3 to 4 weeks when chicken's immune system is still incomplete and passive protection has declined by maternal antibodies (Lovland *et al.,* 2004). Several studies have been developed on maternal vaccination against necrotic enteritis using first and new-generation vaccines (Keyburn *et al.,* 2013a; Lovland *et al.,* 2004).

Chicken vaccination

Immunization of chickens can be done through vector vaccines in ovo or post-hatch vaccination. The advantages of *in-ovo* over post-hatching vaccination include early immunity, reduced stress, precise injection, and lower labor costs (Ricks *et al.,* 1999; Schijns *et al.,* 2014). So, chicken immunization can be preferred in *in-vivo* during the embryonation stage (Muir *et al.,* 2000). Recombinant viruses such as fowl pox virus (FPV) and herpesvirus of turkey (HVT) can be used as vaccine carriers (Schijns *et al.,* 2014) and bacterium like *Salmonella enterica* can be administered orally as a live vaccine. Chicken immunization can be done by feeding, drinking water, or spraying the vaccine (Sharma, 1999). Of course, spraying on chickens can be preferred to oral vaccination because chickens do not drink water regularly during the first days of birth. While spraying will increase its protection against NE (Atterbury *et al.,* 2010). During vaccine production, the choice of adjuvant is also important as some of the adjuvants have embryotoxic side effects (Asif *et al.,* 2004). The protection depends on the degree of colonization and persistence of the strain used in the vaccine (Kulkarni *et al.,* 2008). Other vaccine delivery agents like heat-stable endospores can also be a hopeful opinion because they are easily incorporated into the feed, but it has not been evaluated yet. All the vaccines discussed above have shown some effectiveness against NE in poultry. Thus, it can be an effective alternative for the prevention of Clostridial diseases in the poultry industry, although further studies in this field are needed.

Conclusion

NetB toxin is a major virulence factor of *Clostridium perfringens*, and this toxin increases mortality and morbidity in poultry flocks. NE has shown a clear link between pathogenesis and vaccine production. Before the exploration of NetB, the killed vaccine was evaluated as an experimental NE vaccine. Later, vaccination studies were carried out with genetically-engineered, mutant, and nano vaccines. Also, studies revealed that multiple doses of vaccination are quite effective than a single dose.

Conflict of Interest

No potential conflict of interest to declare.

References

Adams CA. 2004. Nutricines in poultry production: focus on bioactive feed ingredients. Paper presented at the Nutrition Abstracts and Reviews. Series B, Livestock Feeds and Feeding. CAB International. Wallingford. UK. Pages, 1N-12N. DOI: 10.1079/cabireviews20043109361

Akerele G, Ramadan N, Renu S, Renukaradhya GJ, Shanmugasundaram R & Selvaraj RK. 2020. *In vitro* characterization and immunogenicity of chitosan nanoparticles loaded with native and inactivated extracellular proteins from a field strain of *Clostridium perfringens* associated with necrotic enteritis. Veterinary Immunology and Immunopathology, 224: 110059. DOI: 10.1016/j.vetimm.2020.110059

Asif M, Jenkins KA, Hilton LS, Kimpton WG, Bean AG & Lowenthal JW. 2004. Cytokines as adjuvants for avian vaccines. Immunology and Cell Biology, 82(6): 638-643. DOI: 10.1111/j.1440-1711.2004.01295.x

Atterbury RJ, Morris V, Harrison D, Tucker V, Allen V, Davies R & Carrique-Mas J. 2010. Effect of delivery method on the efficacy of Salmonella vaccination in chickens. Veterinary Record, 167(5): 161-164. DOI: 10.1136/vr.b4884

Cooper KK, Songer JG & Uzal FA. 2013. Diagnosing clostridial enteric disease in poultry. Journal of Veterinary Diagnostic Investigation, 25(3): 314-327. DOI: 10.1177/1040638713483468

da Costa SPF, Mot D, Bokori-Brown M, Savva CG, Basak AK, Van Immerseel F & Titball RW. 2013. Protection against avian necrotic enteritis after immunisation with NetB genetic or formaldehyde toxoids. Vaccine, 31(37): 4003-4008. DOI: 10.1016/j.vaccine.2013.05.063

da Costa SPF, Mot D, Geeraerts S, Bokori-Brown M, Van Immerseel F & Titball RW. 2016. Variable protection against experimental broiler necrotic enteritis after immunization with the C-terminal fragment of *Clostridium perfringens* alpha-toxin and a non-toxic NetB variant. Avian Pathology, 45(3): 381-388. DOI: 10.1080/03079457.2015.1129663

da Costa SPF, Savva CG, Bokori-Brown M, Naylor CE, Moss DS, Basak AK & Titball RW. 2014. Identification of a key residue for oligomerisation and pore-formation of *Clostridium perfringen*s

NetB. Toxins, 6(3): 1049-1061. DOI: 10.3390/toxins6031049

Duff AF, Vuong CN, Searer KL, Briggs WN, Wilson KM, Hargis BM, Berghman LR & Bielke LR. 2019. Preliminary studies on development of a novel subunit vaccine targeting *Clostridium perfringens* mucolytic enzymes for the control of necrotic enteritis in broilers. Poultry Science, 98(12): 6319-6325. DOI: 10.3382/ps/pez448

Elwinger K a, Schneitz C, Berndtson E, Fossum O, Teglöf B & Engstöm B. 1992. Factors affecting the incidence of necrotic enteritis, caecal carriage of *Clostridium perfringens* and bird performance in broiler chicks. Acta Veterinaria Scandinavica, 33(4): 369-378. DOI: 10.1186/BF03547304

Gangaiah D, Ryan V, Van Hoesel D, Mane SP, Mckinley ET, Lakshmanan N, Reddy ND, Dolk E & Kumar A. 2022. Recombinant *Limosilactobacillus* (*Lactobacillus*) delivering nanobodies against *Clostridium perfringens* NetB and alpha toxin confers potential protection from necrotic enteritis. Microbiology Open, 11(2): e1270. DOI: 10.1002/mbo3.1270

Gardiner DF, Rosenberg T, Zaharatos J, Franco D & Ho DD. 2009. A DNA vaccine targeting the receptor-binding domain of *Clostridium difficile* toxin A. Vaccine, 27(27): 3598-3604. DOI: 10.1016/j.vaccine.2009.03.058

Hegazy WAH & Hensel M. 2012. *Salmonella enterica* as a vaccine carrier. Future Microbiology, 7(1): 111-127. DOI: 10.2217/fmb.11.144

Islam M, Rashid S, Juli M, Hoque M & Akter M. 2009. Necrotic enteritis in chickens: Pathological, bacteriological and therapeutical investigation. International Journal of Sustainable Crop Production, 4(3): 1-7.

Jang SI, Lillehoj HS, Lee SH, Lee KW, Lillehoj EP, Hong YH, An DJ, Jeong W, Chun JE, Bertrand F & Dupuis L. 2012. Vaccination with *Clostridium perfringens* recombinant proteins in combination with Montanide™ ISA 71 VG adjuvant increases protection against experimental necrotic enteritis in commercial broiler chickens. Vaccine, 30(36): 5401-5406. DOI: 10.1016/j.vaccine.2012.06.007

Jiang Y, Mo H, Willingham C, Wang S, Park JY, Kong W, Roland KL & Curtiss III R. 2015. Protection against necrotic enteritis in broiler chickens by regulated delayed lysis *Salmonella* vaccines. Avian Diseases, 59(4): 475-485. DOI: 10.1637/11094-041715-Reg

Jin K, Wang S, Zhang C, Xiao Y, Lu S & Huang Z. 2013. Protective antibody responses against *Clostridium difficile* elicited by a DNA vaccine expressing the enzymatic domain of toxin B. Human Vaccines & Immunotherapeutics, 9(1): 63-73. DOI: 10.4161/hv.22434

Kaldhusdal M, Schneitz C, Hofshagen M & Skjerve E. 2001. Reduced incidence of *Clostridium perfringens*-associated lesions and improved performance in broiler chickens treated with normal intestinal bacteria from adult fowl. Avian Diseases, 149-156. DOI: 10.2307/1593022

Katalani C, Nematzadeh G, Ahmadian G, Amani J, Kiani G & Ehsani P. 2019. Design and expression optimization of a chimeric derivative of NetB, Alpha-toxin and metallopeptidase proteins as a subunit vaccine against *Clostridium perfringens*. Vaccine Research, 6: 5-12.

Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Di Rubbo A, Rood JI & Moore RJ. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathogens, 4(2): e26. DOI: 10.1371/journal.ppat.0040026

Keyburn AL, Portela RW, Ford ME, Bannam TL, Yan XX, Rood JI & Moore RJ. 2013a. Maternal immunization with vaccines containing recombinant NetB toxin partially protects progeny chickens from necrotic enteritis. Veterinary Research, 44(1): 108. DOI: 10.1186/1297-9716-44-108

Keyburn AL, Portela RW, Sproat K, Ford ME, Bannam TL, Yan X, Rood JI & Moore RJ. 2013b. Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis. Veterinary Research, 44(1): 54. DOI: 10.1186/1297-9716-44-54

Keyburn AL, Yan XX, Bannam TL, Van Immerseel F, Rood JI & Moore RJ. 2010. Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. Veterinary Research, 41(2): 1-8. DOI: 10.1051/vetres/2009069

Kong W, Clark-Curtiss J & Curtiss III R. 2013. Utilizing *Salmonella* for antigen delivery: the aims and benefits of bacterial delivered vaccination. Expert Review of Vaccines, 12(4): 345-347. DOI: 10.1586/erv.13.7

Kulkarni R, Parreira V, Jiang YF & Prescott J. 2010. A live oral recombinant *Salmonella enterica* serovar Typhimurium vaccine expressing *Clostridium perfringens* antigens confers protection against necrotic enteritis in broiler chickens. Clinical and Vaccine Immunology, 17(2): 205-214. DOI: 10.1128/CVI.00406-09

Kulkarni R, Parreira V, Sharif S & Prescott J. 2007. Immunization of broiler chickens against *Clostridium perfringens*-induced necrotic enteritis. Clinical and Vaccine Immunology, 14(9): 1070-1077. DOI: 10.1128/CVI.00162-07

Kulkarni R, Parreira V, Sharif S & Prescott J. 2008. Oral immunization of broiler chickens against necrotic enteritis with an attenuated *Salmonella*

vaccine vector expressing *Clostridium perfringens* antigens. Vaccine, 26(33): 4194-4203. DOI: 10.1016/j.vaccine.2008.05.079

Lanckriet A, Timbermont L, Eeckhaut V, Haesebrouck F, Ducatelle R & Van Immerseel F. 2010. Variable protection after vaccination of broiler chickens against necrotic enteritis using supernatants of different *Clostridium perfringens* strains. Vaccine, 28(36): 5920-5923. DOI: 10.1016/j.vaccine.2010.06.035

Lee KW, Lillehoj HS, Park MS, Jang SI, Ritter GD, Hong YH, Jeong W, Jeoung HY, An DJ & Lillehoj EP. 2012. *Clostridium perfringens* α-toxin and NetB toxin antibodies and their possible role in protection against necrotic enteritis and gangrenous dermatitis in broiler chickens. Avian Diseases, 56(1): 230-233. DOI: 10.1637/9847-070711-ResNote.1

Lee K, Lillehoj HS, Li G, Park MS, Jang SI, Jeong W, Jeoung HY, An DJ & Lillehoj EP. 2011. Identification and cloning of two immunogenic *Clostridium perfringens* proteins, elongation factor Tu (EF-Tu) and pyruvate: ferredoxin oxidoreductase (PFO) of *C. perfringens*. Research in Veterinary Science, 91(3): e80-e86. DOI: 10.1016/j.rvsc.2011.01.017

Lepp D, Roxas B, Parreira VR, Marri PR, Rosey EL, Gong J, Songer JG, Vedantam G & Prescott JF. 2010. Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. PloS One, 5(5): e10795. DOI: 10.1371/journal.pone.0010795

Li J, Adams V, Bannam TL, Miyamoto K, Garcia JP, Uzal FA, Rood JI & McClane BA. 2013. Toxin plasmids of *Clostridium perfringens*. Microbiology and Molecular Biology Reviews, 77(2): 208-233. DOI: 10.1128/MMBR.00062-12

Li N, Yu YZ, Yu WY & Sun ZW. 2011. Enhancement of the immunogenicity of DNA replicon vaccine of *Clostridium botulinum* neurotoxin serotype A by GM-CSF gene adjuvant. Immunopharmacology and Immunotoxicology, 33(1): 211-219. DOI: 10.3109/08923971003782327

Lillehoj HS, Jang SI, Panebra A, Lillehoj EP, Dupuis L, Arous JB, Lee SK & Oh ST. 2017. In ovo vaccination using Eimeria profilin and *Clostridium perfringens* NetB proteins in Montanide IMS adjuvant increases protective immunity against experimentally-induced necrotic enteritis. Asian-Australasian Journal of Animal Sciences, 30(10): 1478. DOI: 10.5713/ajas.17.0053

Lovland A, Kaldhusdal M, Redhead K, Skjerve E & Lillehaug A. 2004. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Pathology, 33(1): 81-90. DOI: 10.1080/0379450310001636255

Mishra N & Smyth JA. 2017. Oral vaccination of broiler chickens against necrotic enteritis using a non-virulent NetB positive strain of *Clostridium perfringens* type A. Vaccine, 35(49): 6858-6865. DOI: 10.1016/j.vaccine.2017.10.030

Muir W, Bryden W & Husband A. 2000. Immunity, vaccination and the avian intestinal tract. Developmental & Comparative Immunology, 24(2-3): 325-342. DOI: 10.1016/s0145-305x(99)00081-6

Ohtani K & Shimizu T. 2016. Regulation of toxin production in *Clostridium perfringens*. Toxins, 8(7): 207. DOI: 10.3390/toxins8070207

Paap P & Additives O. 2015. Dietary treatments for major poultry diseases.

Ramadan NM, Akerele G, Renu S, Renukaradhya G & Selvaraj R. 2020. Oral immunization of broilers with chitosan nano-encapsulated extracellular and cell wall proteins of necrotic enteritis-induced *Clostridium perfringens*. BioRxiv. DOI: 10.1101/2020.10.23.351817

Rappuoli R, Black S & Lambert PH. 2011. Vaccine discovery and translation of new vaccine technology. The Lancet, 378(9788): 360-368. DOI: 10.1016/S0140-6736(11)60440-6

Ricks CA, Avakian A, Bryan T, Gildersleeve R, Haddad E, Ilich R, King S, Murray L, Phelps P, Poston R & Whitfill C. 1999. In ovo vaccination technology. Advances in Veterinary Medicine, 41: 495.

Riddell C & Kong X-M. 1992. The influence of diet on necrotic enteritis in broiler chickens. Avian Diseases, 36: 499-503. DOI: 10.2307/1591740

Robinson K, Chamberlain L, Lopez M, Rush C, Marcotte H, Le Page R & Wells J. 2004. Mucosal and cellular immune responses elicited by recombinant *Lactococcus lactis* strains expressing tetanus toxin fragment C. Infection and Immunity, 72(5): 2753-2761. DOI: 10.1128/IAI.72.5.2753-2761.2004

Rood JI, Keyburn AL & Moore RJ. 2016. NetB and necrotic enteritis: the hole movable story. Avian Pathology, 45(3): 295-301. DOI: 10.1080/03079457.2016.1158781

Rostami A, Goshadrou F, Langroudi RP, Bathaie SZ, Riazi A, Amani J & Ahmadian G. 2016. Design and expression of a chimeric vaccine candidate for avian necrotic enteritis. Protein Engineering, Design and Selection, 30(1): 39-45. DOI: 10.1093/protein/gzw060

Saikh KU, Sesno J, Brandler P & Ulrich RG. 1998. Are DNA-based vaccines useful for protection against secreted bacterial toxins? Tetanus toxin test case. Vaccine, 16(9-10): 1029-1038. DOI: 10.1016/S0264-410X(97)00280-6

Sasaki J, Goryo M, Honda J, Okoshi N, Okada K & Furukawa H. 2000. Cholangiohepatitis in broiler

chickens in Japan: histopathological, immunohistochemical and microbiological studies of spontaneous disease. Acta Veterinaria Hungarica, 48(1): 59-67. DOI: 10.1556/AVet.48.2000.1.7

Savva CG, da Costa SP, Bokori-Brown M, Naylor CE, Cole AR, Moss DS, Titball RW & Basak AK. 2013. Molecular architecture and functional analysis of NetB, a pore-forming toxin from *Clostridium perfringens*. Journal of Biological Chemistry, 288(5): 3512-3522. DOI: 10.1074/jbc.M112.430223

Schijns VE, van de Zande S, Lupiani B & Reddy SM. 2014. Practical aspects of poultry vaccination. Avian Immunology, 288(5): 345-362. DOI: 10.1016/B978-0-12-396965-1.00020-0

Sharma J. 1999. Introduction to poultry vaccines and immunity. Advances in Veterinary Medicine, 41: 481-494. DOI: 10.1016/s0065-3519(99)80036-6.

Song L, Hobaugh M R, Shustak C, Cheley S, Bayley H & Gouaux J E. 1996. Structure of staphylococcal α-hemolysin, a heptameric transmembrane pore. Science, 274(5294): 1859-1865. DOI: 10.1126/science.274.5294.1859

Timbermont L, Haesebrouck F, Ducatelle R & Van Immerseel F. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathology, 40(4): 341-347. DOI: 10.1080/03079457.2011.590967

Wang S, Kong Q & Curtiss III R. 2013. New technologies in developing recombinant attenuated *Salmonella* vaccine vectors. Microbial Pathogenesis, 58**:** 17-28. DOI: 10.1016/j.micpath.2012.10.006

Wilde S, Jiang Y, Tafoya AM, Horsman J, Yousif M, Vazquez LA & Roland KL. 2019. *Salmonella*-vectored vaccine delivering three *Clostridium perfringens* antigens protects poultry against necrotic enteritis. PloS One, 14(2): e0197721. DOI: 10.1371/journal.pone.0197721

Williams R. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology, 34(3): 159-180. DOI: 10.1080/03079450500112195

Yan XX, Porter CJ, Hardy SP, Steer D, Smith AI, Quinsey NS, Hughes V, Cheung JK, Keyburn AL, Kaldhusdal M & Moore RJ. 2013. Structural and functional analysis of the pore-forming toxin NetB from *Clostridium perfringens*. MBio, 4(1): e00019-00013. DOI: 10.1128/mBio.00019-13

Zaragoza NE, Orellana CA, Moonen GA, Moutafis G & Marcellin E. 2019. Vaccine Production to Protect Animals Against Pathogenic Clostridia. Toxins, 11(9): 525. DOI: 10.3390/toxins11090525

Zekarias B, Mo H & Curtiss, R. 2008. Recombinant attenuated *Salmonella enterica* serovar *typhimurium* expressing the carboxy-terminal domain of alpha toxin from *Clostridium perfringens* induces protective responses against necrotic enteritis in chickens. Clinical and Vaccine Immunology, 15(5): 805-816. DOI: 10.1128/CVI.00457-07