Progress and pitfalls in vaccination against necrotic enteritis in broiler chickens

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ABSTRACT

Necrotic enteritis in broilers is caused by *Clostridium perfringens* type A strains that produce the NetB toxin. It is one of the diseases that gained worldwide importance the last decade. Prevention strategies include avoiding predisposing factors, such as coccidiosis, and in-feed supplementation of a variety of feed additives. For protection against a toxin-producing bacterium, vaccination with modified toxin or other secreted immunogenic proteins seems a logical preventive tool. Formalin inactivated crude supernatant has been used for vaccination initially. Recently, several studies have been carried out to identify the most important immunogenic and protective proteins that can be used for vaccination. These include the NetB toxin, but also multiple other proteins. There is evidence that immunization with single proteins is not protective against severe challenge and that combinations of different antigens are needed. Most published studies used multiple dosage vaccination regimens that are not relevant for practical use in the broiler industry. Single vaccination regimens at day-old seem to be non-protective. This review describes the history of vaccination strategies against necrotic enteritis in broilers and gives an update on future vaccination strategies that are applicable in the field. These may include breeder hen vaccination, *in ovo* vaccination and live attenuated vectors to be used in feed or in drinking water.

Keywords: Clostridium perfringens, Necrotic enteritis, Vaccination, Broilers

1. Introduction

Gastrointestinal diseases in broilers have become increasingly important worldwide for multiple reasons. First, high-density floor-housing ensures easy spread of excreted gut pathogens (Guardia *et al*., 2011). Secondly, due to improvements in genetics, broilers have become amazingly capable to convert feed energy in body weight and the gastro-intestinal tract of these animals is highly efficient in absorption of nutrients. Gut micro-organisms play an essential role in degradation of feed components, and there is a complex interplay between gut bacteria and the gastro-intestinal mucosa, either or not beneficial or harmful for the host, depending on the microbial composition. Nutritionists are constantly looking at improving the limits of digestibility and this has caused gut health problems related to bacterial overgrowth, or in other words, an excess of feed nutrients in the gut that are used by harmful micro-organisms, such as *Clostridium perfringens* (*C. perfringens*). Thirdly, there is public and governmental pressure to reduce the use of antibiotics in broilers. The traditional antimicrobial growth promotors (AGPs), used to improve feed conversion ratios and body weight gain, have been banned in the European Union. Also in other countries, consumers put pressure on the poultry industry to rear animals without AGPs. Therapeutic antibiotics are also widely used for preventive and curative interventions against gastro-intestinal pathologies and especially the preventive use is heavily disputed. For all these reasons, some microbial gut pathogens have emerged in broilers, and *C. perfringens* is one of these. This pathogen clearly benefits from high energy diets supporting its fast growth. It is also clear that the use of AGPs in

feed protected broilers from disease caused by *C. perfringens* (Johansson *et al.*, 2004; Martel *et al.*, 2004; Lanckriet *et al*., 2010a).

C. perfringens is a gram-positive spore-forming bacterium causing necrotic enteritis, of which the typical hallmark is small intestinal necrosis. While the acute clinical form is associated with a sudden increase in flock mortality at an average age of 3 to 4 weeks, the subclinical form leads to damage to the intestinal mucosa resulting in decreased digestion and absorption, reduced weight gain and increased feed conversion ratio (Ficken & Wages, 1997; Kaldhusdal, *et al*., 2001). Estimates of the prevalence of necrotic enteritis vary widely because of the unnoticeable subclinical form, but percentages as high as 40% have been reported (Kaldhusdal *et al*., 2001). The economic impact is thus high. The disease is triggered by a variety of predisposing factors. Damage to the intestinal mucosa is an important predisposing factor, and especially coccidiosis co-infection is known to have a high impact (Elwinger *et al*., 1992; Williams, 2005). Also the feed composition is of importance, and high-protein and high-non-starch polysaccharide containing diets are predisposing (Branton *et al.*, 1987; Riddell & Kong, 1992; Branton *et al*., 1997; Gholamiandehkordi *et al*., 2007; Van Immerseel *et al.*, 2009).

Therapeutic antibiotics, such as amoxicillin and tylosin, are often used to (prevent and) control necrotic enteritis (Hermans & Morgan, 2007). The use of antibiotics is no longer considered as an optimal strategy for keeping gut health problems under control because of issues related to antibiotic resistance. Therefore, better farm management, including biosecurity measures and optimization of feed quality have gained interest. Additionally, feed additives, including organic acids, essential oils and prebiotics, have been tested in animal models and shown to be, at least partially, able to control necrotic enteritis (Lensing *et*

74 *al.*, 2010a; Timbermont *et al.*, 2010; Jerzsele *et al*., 2012). For a disease caused by a toxin producing
75 bacterium, it seems logical however to explore whether vaccines can be developed, either or not based on
76 the causative toxins. Much work has been done in recent years in this area and proteins and toxins have
77 been tested as vaccine candidates. In addition, the use of live vectors is under investigation and studies are
78 being carried out on practical strategies for vaccination in the field. A major question is how birds can be
79 protected by vaccination in the limited time span of 3 to 4 weeks before the lesions mostly develop. The
80 disease thus mostly develops at an age when maternal antibodies have declined. In addition, vaccination of
81 young broilers is hampered by the immature immune system and problems related to mass vaccination (ie.
82 the inability to boost immunity). Solutions are under way to solve these issues. In the current paper an
83 overview is given on the information available of the use of potential vaccine preparations, and a critical
84 view is presented on the practical implementation of vaccination to protect broilers against necrotic
85 enteritis.

86

87 **2. Antibody responses to *C. perfringens* antigens**

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89 The immune response to *C. perfringens* infection, including immune recognition of the pathogen and its
90 secreted proteins and toxins, is still poorly understood. In addition, there are uncertainties about the type
91 of antibodies (IgA, IgY) and the specificity of the antibodies (antigen to which the antibodies are directed
92 to) that are associated with protection. Infection takes place in the small intestines where the pathogen
93 makes contact with the mucosal surface. The enteric immune system of neonatal broilers is poorly

94 developed and matures rapidly up to 4-6 weeks post hatch (Mast & Goddeeris, 1999). Generally, adaptive
95 immune defense at the mucosal surface is mediated by initiation of lymphocyte activation and local
96 secretion of IgA (Muir *et al.*, 2000; Sharma, 1999). Mucosal IgY may be important in protection against
97 necrotic enteritis, since it is the major transferred maternal antibody and it plays an essential role in
98 protection of young chickens against other pathogens. Maternal antibody declines by about 3 weeks of
99 age, which may explain why broiler chickens mostly develop necrotic enteritis around that time point
100 (Ulmer-Franco *et al.*, 2012).

101 It was shown that the level of specific maternal antibodies against alpha toxin was higher in day-old
102 chickens from older hens than in the progenies from younger hens. Broilers with high titers of specific
103 maternal antibodies (IgY) against alpha toxin were shown to have lower mortality (Heier, Lovland,
104 Soleim, Kaldhusdal, & Jarp, 2001). When naturally infected chickens are able to develop an antibody
105 response, this response may have a value for protection against the disease (Lovland *et al.*, 2003). Levels
106 of antibodies (IgY) against NetB and alpha toxin were significantly higher in apparently healthy chickens
107 compared to chickens with clinical necrotic enteritis. This suggests that these antibodies may play a role in
108 the protection against necrotic enteritis (Lee *et al*., 2012).

109 In several vaccination studies a mucosal IgA response against alpha toxin, NetB and other immunogenic
110 proteins was reported in chickens (partially) protected against necrotic enteritis (Kulkarni *et al.*, 2007;
111 Kulkarni *et al.*, 2010; Jang *et al*., 2012). However, in intestinal washings from experimentally infected
112 birds only weak reactivity of mucosal IgA against proteins of *C. perfringens* was found. This might
113 indicate that a serum IgY response plays a more important role in immunity against necrotic enteritis than

mucosal IgA. After systemic immunization with recombinant immunogenic proteins, serum IgY still reaches the mucosal surface under inflammatory conditions caused by *C. perfringens* (Williams, 2005; Kulkarni *et al*., 2007; Kulkarni *et al*., 2010).

3. An overview of vaccination studies against necrotic enteritis

There are various ways to deliver antigens to chickens for immunization purposes. Potential bacterial vaccines can be based on live (attenuated) organisms or killed (inactive) organisms. Live (attenuated) vaccine strains may be superior because they often have the ability to induce a stronger and longer immune response and can be administered orally, but there are some safety concerns (Witter & Hunt, 1994; Plotkin & Plotkin, 2011; Rappuoli *et al*., 2011). For a toxin producing bacterium, however, it seems logical that culture supernatants or toxin-based formulations are used, ideally in inactivated form while preserving antigenicity. Formalin inactivation and genetically engineered inactive toxin variants are an option, as well as the delivery of immunogenic non-toxin proteins. Also DNA vaccines that express *Clostridium* toxins have been tested as vaccine candidates (Saikh *et al*., 1998; Gardiner *et al*., 2009; Li *et al.* 2011; Jin *et al*., 2013).

3.1. Live attenuated vaccines

133 The principle that previous infections with *C. perfringens* strains induce protection against challenge was
134 proven by Thompson *et al*. (2006). These authors orally administered virulent strains to 15 day old broiler
135 chickens during 5 consecutive days, followed by treatment with bacitracin for nine days to clear the
136 virulent strains. An oral challenge with virulent strain *C. perfringens* CP4 resulted in significantly fewer
137 chickens with lesions (mean lesion score 0.13 instead of 2.09 in the non-immunized group). These data
138 show the potential of vaccination with live strains, but a major issue with live vaccines is the trade-off
139 between attenuation and protection. Indeed, live strains should be attenuated without losing the ability to
140 protect against disease. When an avirulent strain was used for oral immunization using the same
141 immunization-infection protocol, no protection was conferred. In contrast, an alpha toxin mutant of the
142 challenge strain induced partial protection against infection with an isogenic challenge strain, i.e. a
143 significant decrease in number of birds with necrotic lesions was observed (Thompson *et al.*, 2006). It
144 could very well be that residual virulence (under which maybe NetB production) is essential for a live
145 vaccine strain to be protective. Indeed, an avirulent strain is not providing protective antigens to the gut
146 associated lymphoid tissues, and as a consequence not conferring protection. This observation could
147 hamper the development of live vaccines, as residual virulence is clearly not acceptable.

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149 **3.2.Protein-based vaccines**

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151 Protein-based vaccines are used because they are safer and better characterized as compared to live
152 vaccines, while they can still be protective (Unnikrishnan *et al*., 2012). They include toxoids (inactivated

bacterial toxins) and subunit vaccines, often based on virulence factors or secreted toxins (Berzofsky *et al.*, 2001). *C. perfringens* is known to produce many different toxins and proteins. While in some studies crude culture supernatants (either or not inactivated) were used as vaccines, other vaccination trials were carried out using inactivated toxins and highly antigenic proteins.

Both non-inactivated supernatant and formaldehyde-inactivated supernatant (crude toxoid) of *C. perfringens* have thus been studied as potential vaccines for the prevention of clinical and subclinical necrotic enteritis with variable success. In a study by Saleh *et al.* (2011), subcutaneous vaccination of broilers at an age of 7 and 21 days with *C. perfringens* type A, type C and combined type A and C crude toxoids significantly decreased the number of animals developing intestinal lesions. When breeder hens were vaccinated at 14 and 18 weeks of age with type A and type C crude toxoids and their progeny was challenge exposed under both field conditions and in a disease model, type C crude toxoid was shown to better protect than type A crude toxoid (Lovland *et al.*, 2004). The safety and efficacy of a *C. perfringens* type A alpha toxoid (Netvax^{TM}) was investigated by vaccinating breeder hens intramuscularly at 11 and 18 weeks of age. In this field trial, the progeny from vaccinated hens had a reduced mortality compared to the progeny from unvaccinated hens (Crouch *et al.*, 2010). Lanckriet *et al.* (2010b) compared the non-inactivated supernatant of 8 *C. perfringens* strains, with different alpha toxin and NetB content, using subcutaneous vaccination at an age of 3 and 12 days. They showed important variation in the protective capacity depending on the strain used for supernatant preparation. This suggests that protective immunity is probably determined by an effective combination of different bacterial immunogens or that the expression levels of some (one or more) antigens drives protection conferred by vaccination. The strain

used for crude supernatant collection is thus of crucial importance when designing these vaccine types. It is clear that non-inactivated supernatant always contains a risk because of the presence of active toxins, and thus crude toxoids are preferred for safety reasons. Formaldehyde is mostly used for inactivating the activity of proteins in vaccines but can reduce the protective capacity of the vaccine. Mot *et al.* (2013) showed that the efficacy of subcutaneous vaccination at the age of 3 and 12 days against necrotic enteritis using crude supernatant was abolished when the supernatant was formaldehyde inactivated. A logical way for vaccine development against diseases caused by toxin-producing bacteria is the use of inactivated toxin preparations. The alpha toxin is the most investigated *C. perfringens* toxin in terms of vaccine-induced protection, mainly in mouse gangrene models (Stevens *et al*., 2004; Titball, 2009). Before the NetB toxin was identified as the major toxin in necrotic enteritis in broilers, alpha toxin was believed to be crucial and thus multiple studies used alpha toxin derivatives as vaccine antigen. It has been shown that broilers with a history of clinical or sub-clinical necrotic enteritis have a natural serum antibody response to alpha toxin (Heier *et al*., 2001; Lovland *et al*., 2003). Cooper *et al.* (2009) vaccinated broilers subcutaneously with recombinant alpha toxin at 5 and 15 days of age and showed a decrease in the number of animals with necrotic enteritis lesions. Jang *et al*. (2012) vaccinated broilers subcutaneously at day 1 and day 7 with recombinant alpha toxin and could induce protection against challenge. Using double and triple intramuscular vaccination regimens (day 7, 14 and 21), Kulkarni *et al.* (2007) showed that a prior vaccination with alpha toxoid and a boost with active toxin protected against experimental necrotic enteritis. A triple vaccination of either alpha toxoid or active toxin offered no protection. It was suggested that the failure in protection using the active toxin may have resulted from the toxin activity on immune cells and the failure of alpha toxoid may be the consequence of loss of conformation of the protein,

194 resulting in loss of epitopes, as mentioned before. Although alpha toxin has been shown to play no
195 primary role in the induction of necrotic enteritis, the antigen can thus still induce a certain level of
196 protection. It has been shown by Zekarias *et al*. (2008) that anti-alpha toxin antibodies bind to the cell wall
197 of the bacterium and suppress the growth of the bacterium *in vitro*. The binding of the antibodies to the
198 membrane-bound preprotein might block protein transport channels and hereby inhibit proliferation of the
199 bacterium.
200 The discovery of the genetically highly conserved NetB toxin as an essential virulence factor opened new
201 perspectives for the development of vaccines for the control of necrotic enteritis (Keyburn *et al.,* 2006;
202 Keyburn *et al.*, 2010a; Keyburn *et al.*, 2010b). After the structure and function of the NetB toxin protein
203 was analyzed, mutants with reduced cytotoxic activity were designed (Savva *et al*., 2013; Yan *et al*.,
204 2013). The mutation of tryptophan to alanine at position 262 (W262A) resulted in a significant reduction
205 in cytotoxicity to LMH cells and hemolytic activity on red blood cells, and thus showed to be a vaccine
206 candidate (Savva *et al*., 2013). Fernandes da Costa SP *et al*. (2013) vaccinated broilers subcutaneously at
207 day 3, 9 and 15 with a formaldehyde NetB toxoid or the NetB W262A mutant. Both NetB derived
208 vaccines were able to induce significant protection against experimental necrotic enteritis. Keyburn *et al.*
209 (2013b) immunized chickens subcutaneously with purified recombinant NetB (rNetB), formaldehyde
210 treated bacterin (consisting of 50:50 sonicated bacterial cells and culture supernatant) and crude toxoid
211 with or without rNetB supplementation at an age of 7 and 17 days. Chickens vaccinated with rNetB were
212 significantly protected against experimental necrotic enteritis when challenged with a mild oral dose of
213 virulent bacteria, but rNetB was not sufficient to protect against a heavy in-feed challenge. Birds
214 immunized with bacterin and crude toxoid supplemented with rNetB were significantly protected against

moderate and severe in-feed challenge. NetB has thus been shown to have a considerable potential for the development of vaccines against necrotic enteritis. The best protection was observed when birds were vaccinated with the crude toxoid or bacterin supplemented with rNetB (Keyburn *et al.,* 2013b). This study confirmed that NetB alone is not yielding full protection and that supplementation with other antigens increases the protective response. Keyburn *et al.* (2013a) also used a non-toxic NetB variant (S254L) for vaccinating breeder hens (see below).

In addition to toxin-derived protein vaccines, also highly immunodominant proteins can potentially be used to protect animals against necrotic enteritis by vaccination. As mentioned before, neither single NetB nor alpha toxin were capable to induce full protection against the development of lesions after experimental infection. Full protection is probably determined by an effective combination of different bacterial immunogens (Lanckriet *et al*., 2010b; Fernandes da Costa SP *et al.*, 2013; Keyburn *et al.*, 2013b). Several purified *C. perfringens* proteins have been evaluated as potential vaccine candidates. Several authors identified antigens recognized by post infection sera from chickens immune to necrotic enteritis. Hypothetical protein (HP), pyruvate:ferredoxin oxidoreductase (PFOR), elongation factor G (EF-G), perfringolysin O, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a fructose 1,6 biphosphate aldolase (FBA) were identified using post infection serum from chickens immune to virulent *C. perfringens* challenge in infection-immunization experiments (Kulkarni *et al.*, 2006). Jiang *et al.*(2009) identified the *C. perfringens* large cytotoxin (TpeL), endo-beta-N-acetylglucosaminidase (Naglu) and phosphoglyceromutase (Pgm) as dominant antigens using post infection serum from chickens immune to necrotic enteritis. Elongation factor Tu (EF-Tu) and PFO were identified by reaction with immune sera from chickens derived from a clinical outbreak. Kulkarni *et al.* (2007) immunized chickens

intramuscularly two (or three) times at an age of 7, 14 (and 21) days with recombinant proteins alpha-toxin/alpha toxoid, GAPDH, HP, FBA, and PFOR. All the proteins were able to decrease the mean intestinal lesion score. The degree of protection depended on the severity of the challenge. Alpha toxin, HP, and PFOR protected significantly against heavy challenge. GAPDH and FBA protected only against mild challenge. More recently, double subcutaneous vaccination regimens using alpha toxin, NetB toxin, PFOR and EF-Tu gave similar protection levels after experimental infection (Jang *et al.*, 2012). Immunization with Naglu and Pgm yielded partial protection after challenge with two different strains. Again, the protection level decreased when the challenge severity increased (Jiang *et al.*, 2009). All the above described data thus show that multiple proteins, including derivatives from alpha and NetB toxin, have potential, and that defined mixtures of these proteins need investigation.

3.3. Attenuated live vectors expressing *C. perfringens* proteins

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 several poultry diseases by expressing heterologous antigens (Hegazy & Hensel, 2012). Because the attenuation is usually induced by a deletion mutation in a gene that is essential for the metabolism of the bacterium, the vaccine carrier strains can not overgrow the immune system of the animal host (Spreng *et al.*, 2006). Zekarias *et al.*(2008) evaluated the efficacy of a live recombinant attenuated *S. enterica* serovar Typhimurium vaccine

strain that delivered the C-terminal domain of the alpha toxin. The vaccine strain was twice administered orally at 3 and 17 days of age. Thereafter the birds were challenged by oral inoculation and repeated infection through contaminated feed with a virulent *C. perfringens* strain. A significant reduction in number of birds with necrotic lesions was observed. Kulkarni *et al*., (2008) showed that the delivery of FBA and HP using an attenuated *S. enterica* serovar Typhimurium vaccine vector by the oral route induced a significant protective immune response. Broilers immunized with the vaccine strain, expressing PFOR, at day 1 and day 14, however were not significantly protected against necrotic enteritis. The authors also tested *Salmonella* strains expressing truncated nontoxic alpha toxoid and truncated HP (tHP). The alpha toxoid consisted of a region of 162 amino acid residues that included two sections of immunodominant epitopes as well as regions of weak reactivity. Broiler chickens immunized orally with a *Salmonella* strain expressing nontoxic alpha toxoid, at day 1 and day 10 of age, were significantly protected against moderate experimental necrotic enteritis but not protected against severe challenge, while chickens immunized with tHP were protected against both moderate and severe challenge (Jiang *et al*., 2010; Kulkarni *et al.*, 2010). While *Salmonella* strains are thus potential vaccine carriers for *C. perfringens* proteins, there are other possibilities that, although not yet explored for protection of poultry against necrotic enteritis, can be of value. The expression of the C-terminal domain of alpha toxin on the surface of *Bacillus subtilis* spores was described and shown to be immunogenic in mice (Hoang *et al*., 2008). Also lactic acid bacteria can be used as vaccine carriers for *Clostridium* antigens (Robinson *et al*., 1997; Robinson *et al*., 2004). *B. subtilis* and lactic acid bacteria have the advantage to possess a GRAS (generally recognized as safe) status. The use of live vectors to express *C. perfringens* proteins in the gut

of broilers thus is a promising approach and deserves further attention, mainly in relation to the optimal vector to be used and the proteins to be expressed.

3. The future of vaccine delivery and immunization methods for necrotic enteritis

In recent years, multiple studies have been carried out on the development of vaccines against necrotic enteritis. As reviewed in detail above, non-inactivated supernatants, formalin inactivated crude toxoids, immunogenic proteins and modified toxins have been used in vaccination studies. These have been administered intramuscularly and subcutaneously, either or not in multiple dosage regimens, or have been orally delivered by live attenuated vaccine carrier strains. These studies show clearly that multiple vaccination dosages are necessary for a good immune response and that one parenteral single vaccination, at day of hatch, offers no protection. Ease of administration of a vaccine is important for making vaccines acceptable for the poultry industry. Because large populations of animals must be vaccinated, the most beneficial vaccines are those that can be delivered simultaneously to large numbers of birds with minimum amount of labor (Sharma, 1999). Broilers are mostly slaughtered around 5 to 7 weeks of age, and for practical reasons, vaccines are mostly given in the hatchery. Parenteral vaccination of broiler chickens is theoretically possible at day-of-hatch, but vaccination using live vaccines by spray methods or drinking water application is easier to apply. Parenteral booster vaccinations are practically impossible for broilers. It has been shown that booster vaccinations are essential when non-inactivated supernatant and

crude toxoid are used to provide protection, while single immunization seems to have little benefit (Mot *et al*., 2013). For protection of broilers against necrotic enteritis, there are thus only few options left. These are breeder hen vaccination and the use of live bacterial or viral vectors that can deliver antigens *in ovo* or during rearing (eg. as feed or drinking water additive, thus oral vaccination), and thus presenting the antigens for a longer period as compared to parenteral administration of antigens at day-of-hatch.

3.1. Breeder hen vaccination

Vaccination of breeder hens is often preferred in the poultry industry. Due to the generation of large numbers of protected progeny per vaccinated hen, the vaccine cost per chicken is lower as compared to post-hatch vaccination (Schijns *et al*., 2008). Passive protection by maternal antibodies in broiler chickens by breeder hen vaccination could have some limitations with regard to necrotic enteritis. Outbreaks of necrotic enteritis mostly occur at the age of 3-4 weeks. The immune system of broiler chickens is still developing at that age and maternal antibodies already have declined (Lovland *et al*., 2004). Until now, three studies have reported data on maternal vaccination against necrotic enteritis, two of them using crude supernatant toxoids and one using rNetB (S254L) either or not in combination with crude toxoid (Lovland *et al*., 2004, Crouch *et al.,* 2010, Keyburn *et al.,* 2013a). When breeder hens were vaccinated intramuscularly at 14 and 18 weeks of age with *C. perfringens* type A or type C crude toxoid, an increase in antibody response to alpha toxin in serum samples of parent hens was shown. In a field trial under

predisposing conditions a partial protection against necrotic enteritis in their progeny was shown (Lovland *et al*., 2004). The safety and efficacy of a commercial *C. perfringens* type A alpha toxoid (Netvax™) was analyzed by immunizing breeder hens intramuscularly at 11 and 18/19 weeks of age. An increase in specific alpha toxin IgY antibody response was shown in serum from hens, in the egg yolk from eggs collected from those hens and in serum from 7-day-old chickens hatched from those eggs (Crouch *et al*., 2010). In a field trial the progeny (from eggs collected at 27 and 32 weeks) from a group NetVax™-vaccinated hens had a reduced overall mortality as compared to the progeny from an unvaccinated group, especially at those time points at which necrotic lesions were observed in the progeny from the unvaccinated group (Crouch *et al.*, 2010). Recently, a recombinant non-toxic NetB variant (S254L) was tested in breeder hens, single or combined with crude toxoid (Keyburn *et al.*, 2013a). Hens were vaccinated subcutaneously at 22, 24 and 26 weeks of age. A significant IgY antibody response against NetB was detected in serum samples from hens, in the egg yolk of their eggs and in serum from hatched chickens from vaccinated hens. When the progeny (from eggs collected at 30 weeks) of vaccinated hens was infected with in-feed *C. perfringens* at 26 and 27 days of age, only chickens derived from hens vaccinated with rNetB (S254L) combined with crude toxoid had a significantly lower lesion score. When the *C. perfringens* infection was performed at 14 days of age, chickens derived from hens vaccinated with single rNetB (S254L) or single crude toxoid were also protected (Keyburn *et al*., 2013a). The authors hypothesized that a higher level of specific antibodies at the time of challenge is responsible for the protection against challenge at earlier age.

3.2. *In ovo* vaccination and oral immunization using viral or bacterial vector vaccines

Chickens can be vaccinated using vector vaccines *in ovo* or during rearing. Benefits of *in ovo* vaccination compared to post-hatch vaccination include earlier immunity, reduction in bird stress, precise and uniform injection and reduced labor costs (Ricks *et al*., 1999; Schijns *et al*., 2008). The vaccine is injected in eggs during the late embryonation stage, usually at 17-18 days of incubation (Muir *et al*., 2000). Recombinant fowl poxvirus (FPV) and herpesvirus of turkey (HVT) replicating viruses are examples of vector vaccines for *in ovo* application (Schijns *et al*., 2008). If a non-replicative vector for *C. perfringens* antigens would be injected *in ovo*, possibly protective antibodies would already decline at the time the diseases occurs. Also the choice of the adjuvant is important as some adjuvants are known for inducing embryotoxic side effects (Asif *et al.*, 2004). According to our knowledge there are no studies reporting efficacy of *in ovo* vaccination against necrotic enteritis.

Oral immunization of broilers can be done through the feed or drinking water or by spraying the vaccine on the chickens (Sharma, 1999). These delivery systems are labor- and time-saving and practically feasible for the broiler industry. Chickens do not always drink regularly in the first days after hatching. In contrast, spray application may increase the vaccine uptake and lead to a more consistent level of protection against the pathogen (Atterbury *et al*., 2010). Orally administered live vaccine strains expressing *C. perfringens* antigens and colonizing the intestinal tract of the broilers have been described (Kulkarni *et al.*, 2008, Zekarias *et al.*, 2008; Kulkarni *et al.*, 2009). The obtained protection depends on the colonization level and persistence of the vaccine strains. Kulkarni *et al.* (2008, 2009) immunized

354 broilers orally at day of hatch and at day 14 with a recombinant *S. enterica* serovar Typhimurium strain
355 expressing truncated proteins of the alpha toxin, FBA, PFOR or HP. They induced a significant protective
356 immune response but the degree of protection was less than observed when these proteins were
357 administered intramuscularly in multiple dosages (Kulkarni *et al*., 2006). Zekarias *et al.* (2008) inoculated
358 chickens orally with a *S. enterica* serovar Typhimurium strain, expressing a nontoxic fragment of alpha
359 toxin, at day 3 and 13. The antibody response was low, but the immunized chickens had a reduced number
360 of necrotic enteritis lesions after challenge. The above mentioned studies however used oral gavage of the
361 vaccine strains. Practical delivery methods, such as in-feed, drinking water or spray application were not
362 tested yet. Recombinant *B. subtilis* endospores that express the C-terminal domain of alpha toxin have
363 been used to vaccinate mice against *C. perfringens* infection (Hoang *et al.*, 2008). The endospores appear
364 to provide an adjuvant effect, boosting the immune response to the antigens. The use of these heat-stable
365 endospores as vaccine delivery agents is a promising idea because they could be incorporated into feed.
366 This type of bacterial vectors has not been evaluated for necrotic enteritis in broilers.

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368 **3.3. Summary and concluding remarks**

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370 The history of research on necrotic enteritis clearly shows a link between pathogenesis studies and vaccine
371 development. Before the identification of the major toxin NetB and before the identification of important
372 immunogenic proteins, formalin-inactivated crude supernatants were tested. The last few years studies
373 have been carried out using single proteins or combinations of proteins, mostly by parenteral

374 immunization. A summary is given in table 1. These studies have been important to identify proteins as
375 vaccine candidates (such as the NetB toxin), and it became clear that combinations of immunogenic
376 proteins are yielding better protection as compared to single protein immunization. Most of these studies
377 used multiple dosage parenteral immunization regimes which suffer from lack of practical value for
378 broilers. Single dosing at day-of-hatch, a possible method that can be used in the field, results in total loss
379 of protection compared to multiple dosage vaccination. Breeder hen vaccination is an option and several
380 studies have shown promising results, but the antibody decline in the progeny will decrease the efficacy at
381 later ages, which may be important for necrotic enteritis which typically occurs at 3 to 4 weeks of age. *In*
382 *ovo* vaccination could be a valuable method, but no data have been reported so far on this strategy. When
383 immunogenic proteins need to be presented to the immune system for a more prolonged period of time
384 using a single dosage, live attenuated bacterial (or viral or parasitic) vectors are a potential strategy for the
385 future. The obtained protection depends on the colonization level and persistence of the live vaccine
386 strains and the combination and levels of the expressed antigens. The ideal strain would be one that, apart
387 from inducing immunity and protection, can be added to the feed or drinking water, or sprayed on the day-
388 old chicks in the hatchery. Considering the progress made in recent years, it can be expected that new
389 protective vaccines will become available in the next few years.

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Table 1: Summary of all studies on vaccination against necrotic enteritis described in the scientific literature. The table shows the route of administration, vaccine regimen, antigen, dose, vector (if used), adjuvant, the result of the vaccination study and the literature reference.

Route of Administration	Vaccination regimen	Vector/Adjuvant	Antigen and dose	Protection	Reference
IM	Double (breeder hens week 14 and 18)	20% Alhydrogel and 0.013% thiomersal	- Type A crude toxoid (0.25ml of 1TCP*) - Type C crude toxoid (0.25ml of 30TCP*)	- Specific antibody response against alpha toxin in breeder hens and their progeny - Less mortality in progeny	(Lovland *et al.*, 2004)
Oral	Infection-immunization for 5 consecutive days	Mixed in feed at ratio 2:1 (feed:broth culture)	- Avirulent strain CP5 - Virulent strain CP1 - Virulent strain CP4 - Alpha toxin deficient mutants (Cpa^{-1},Cpa^{-2},Cpa^{-3} and Cpa^{-4})	- Reduction in chickens with lesions that were infection-immunized with CP1, CP4, Cpa^{-2} and Cpa^{-4}	(Thompson *et al.*, 2006)
IM	Double or triple (day 7, 14 (and 21)	Quil A	- Alpha toxin - Alpha toxoid - HP* - FBA* - GDP* - tPFOR* 20µg in triple vaccination, 40µg in double vaccination	- Serum and intestinal antibody response against immunogens - Reduction in chickens with lesions depending on the severity of challenge	(Kulkarni *et al.*, 2007)
Oral	Double (day 1 and 14)	Attenuated *S. enterica* serovar *Typhimurium X*9241	- FBA* - tPFOR* - tHP* 100µl containing 10^9 CFU	- Serum and intestinal antibody response against immunogens - Reduction in main lesion score and increase in body weight gain (FBA and tHP)	(Kulkarni *et al.*, 2008)
Oral SC	Double (day 3 and 13) Double (day 3 and 17) Triple (day 3, 13 and 35)	Attenuated *S. enterica* serovar *Typhimurium X*8914 Complete Freunds adjuvant (SC)	- C-terminal domain of alpha toxin (rPLC) 50µg (SC) 500µl containing 10^9 CFU (oral)	- Low serum antibody response - Reduction in number of chickens with lesions - Reduction in lesion score	(Zekarias *et al.*, 2008)
SC	Double (day 5 and 15)	Quil A	- Alpha toxin 20µg	- Specific serum antibody response against alpha toxin - Reduction in number of chickens with lesions	(Cooper *et al.*, 2009)

IM	Double (breeder hens week 11 and 18-19)	Light mineral oil	- Type A crude toxoid (0.5ml of 3 TCP*)	- Specific antibody response against alpha toxin in breeder hens and their progeny - Lower mortality rate in field trial	(Crouch *et al.*, 2010)
Oral IM	Double (day 1 and 10) Triple (day 1, 10 and 17)	Attenuated *S. enterica* serovar *Typhimurium X*9352	- Alpha toxoid (region of 162 amino acid residues) - tHP 100µl containing 10^9 CFU	- Serum and intestinal antibody response against immunogens - Reduction in chickens with lesions depending on the severity of challenge - Increased body weight	(Kulkarni *et al.*, 2010)
SC	Double (day 3 and 12)	Quil A	- Supernatant of 8 type A strains (variable NetB and alpha toxin content) 7 and 70µg	- Reduction in number of chickens with necrotic lesions	(Lanckriet*et al.*, 2010b)
IM	Double or triple (day 7, 14 (and 21)	Quil A	- Naglu* - Pgm*	- Serum and intestinal antibody response against immunogens - Reduction in chickens with lesions depending on the severity of challenge and challenge strain	(Jiang *et al.*, 2009)
SC	Double (day 7 and 21)	Unknown	- Crude toxoid A - Crude toxoid C - Crude toxoid AC	- Serum antibody response against immunogens - Reduction in number of chickens with necrotic lesions	(Saleh *et al.*, 2011)
SC	Double (day1 and 7)	Montanide ISA 71 VG	- Alpha toxin - NetB - EF-Tu* - PFO* 50µg	- Specific serum antibody response against NetB and PFO - Reduction in lesion score	(Jang *et al.*, 2012)
SC	Single (day1 or 3) Double (day 3 and 12)	Quil A	- Supernatant NetB positive toxin type A strain 7 and 70µg	- Reduction in number of chickens with necrotic lesions - Reduction in lesion score	(Mot *et al.*, 2013)
SC	Triple (day 3, 9 and 15)	Quil A	- NetB toxoid - NetB (W262A) 30µg	- Reduction in number of chickens with necrotic lesions - Reduction in mean lesion score	(Fernandes da Costa *et al.*, 2013)
SC	Double (day 7 and 17)	60% Montanide 40% QuilA DEAE-dextran	- NetB - Bacterin (50:50 bacterial cells and culture supernatant) - Bacterin + NetB 50µg	- Specific serum antibody response against NetB - Reduction in average lesion score depending on the severity of challenge	(Keyburn *et al.*, 2013b)
SC	Triple (breeder hens week 22, 24 and 26)	60% Montanide 40% QuilA	- rNetB(S254L) - Crude toxoid (type A,	- Specific antibody response against NetB in breeder hens and progeny	(Keyburn *et al.*, 2013a)

	DEAE-dextran	NetB positive) - Crude toxoid (type A, NetB positive) + rNetB(S254L) 50µg	- Reduction in number of chickens with necrotic lesions in experimental infection trial in progeny

*TCP (total combining power), (t)HP ((truncated)Hypothetical protein), FBA (fructose 1,6-biphosphate aldolase), GDP (glyceraldehyde-3-phosphate dehydrogenase), (t)PFO(R) ((truncated) pyruvate: ferredoxin oxidoreductase), Naglu (endo-beta-N-acetylglucosaminidase), Pgm (phosphoglyceromutase), EF-Tu (elongation factor Tu), CFU (colony forming units)

SC (subcutaneous), IM (intramuscularly)

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