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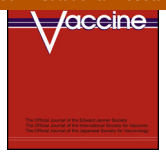

Protection against avian necrotic enteritis after immunisation with NetB genetic or formaldehyde toxoids☆

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ABSTRACT

NetB (necrotic enteritis toxin B) is a recently identified β-pore-forming toxin produced by *Clostridium perfringens*. This toxin has been shown to play a major role in avian necrotic enteritis. In recent years, a dramatic increase in necrotic enteritis has been observed, especially in countries where the use of antimicrobial growth promoters in animal feedstuffs has been banned. The aim of this work was to determine whether immunisation with a NetB toxoid would provide protection against necrotic enteritis. The immunisation of poultry with a formaldehyde NetB toxoid or with a NetB genetic toxoid (W262A) resulted in the induction of antibody responses against NetB and provided partial protection against disease.

1. Introduction

Clostridium perfringens (*C. perfringens*) is a ubiquitous bacterium that is able to colonise a variety of different biotopes and it is not unusual to find *C. perfringens* as a commensal in the normal gut microbiota in domesticated animals. However, under particular circumstances the bacterium is responsible for severe diseases. These diseases are largely a consequence of the actions of toxins on the host [1]. In addition to the four toxins used for typing *C. perfringens* strains (alpha-, beta-, epsilon-, and iota-toxin), the bacterium is able to produce a number of other toxins, including enterotoxin and perfringolysin O [2,3].

C. perfringens has been shown to cause avian necrotic enteritis (NE), a severe gastro-intestinal disease of farmed poultry [4–6]. Until recently, NE has been controlled by the addition of antimicrobial growth promoters to feedstuffs. However, in many countries national and supranational regulations now limit the addition of antimicrobials to animal feeds. Consequently, in these countries, NE is emerging as a disease which is of significant economic consequence to the poultry industry [7,8]. The disease can occur in at least two forms. The acute form of NE typically results in mortality during the last weeks of rear of broilers (week 5–6). However, many cases of NE are associated with relatively mild clinical signs [9–11]. This subclinical form of NE results in decreased digestion and absorption of feedstuffs and consequently reduced weight gain [12,13]. At least in Europe it is now believed that the subclinical NE is the most frequent form of the disease and causes the greatest economic losses to the poultry production industry [14].

Although it is clear that *C. perfringens* is the etiologic agent of NE, a wide range of host and pathogen factors can influence the severity of the disease. These factors include the nature of the feedstuff, co-infection with various *Eimeria* species and the molecular makeup of *C. perfringens* in the gut [15]. Often these factors interact with each other, and this has made the development of reliable infection models difficult [15]. The molecular basis of virulence of *C. perfringens* associated with NE is still being investigated. However, almost all *C. perfringens* isolates from cases of NE possess the *netB* gene [4,16,17] which encodes necrotic enteritis toxin B (NetB), a β-pore-forming toxin [6,18]. Pore formation by NetB can lead to cell lysis by disruption of membrane integrity and a *netB* mutant of *C. perfringens* is reported to be incapable of causing NE [6]. There

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is also accumulating evidence that other virulence factors, such as the TpeL toxin, play a role in disease [19]. The production of these virulence factors might explain the reported ability of some *netB*-negative strains of *C. perfringens* to cause NE [4,6].

Immunisation with either crude toxoids [20] or culture supernatants [21] can provide significant but incomplete protection against experimental NE. Although these vaccines are simple to prepare they suffer from the limitation that it is difficult to configure them for non-invasive dosing, for example by oral delivery. Other workers have explored the possibility of a sub-unit which is able to protect against NE, with a view towards both improving vaccine efficacy and opening the possibility of oral delivery. To date, a range of proteins derived from *C. perfringens* have been evaluated as sub-unit vaccines including alpha-toxin, glyceraldehyde-3-phosphate dehydrogenase, pyruvate-ferredoxin oxidoreductase, fructose 1,6-biphosphate-aldolase, or a hypothetical protein [22]. Immunisation with any of these sub-units provided partial protection against experimental NE. Partial protection against NE has also been reported after immunisation with *C. perfringens* large cytotoxin TpeL, endo-beta-*N*-acetylglucosaminidase or phosphoglyceromutase [23]. A more recent study in which alpha-toxin, NetB, pyruvate-ferredoxin oxidoreductase and elongation factor-Tu were compared as protective antigens concluded that NetB and pyruvate-ferredoxin oxidoreductase given with ISA71 adjuvant provided enhanced protective immunity [24]. However, it is unlikely that a licensed vaccine for widespread use could contain active toxins. Therefore, there is a need to identify non-toxic variants of these toxins.

In a previous study, mutational analysis of NetB led to the identification of several amino acids important for toxicity [18]. The mutation of tryptophan to alanine at position 262 (W262A) resulted in a significant reduction in cytotoxicity towards LMH cells, binding to LMH cells and haemolytic activity on red blood cells [18]. The W262A mutation is located within the rim domain of NetB, a region mediating binding of the toxin to the cell membrane.

In this study, we investigated whether a formaldehyde NetB toxoid or a NetB mutant (W262A) were able to induce protection against experimental NE in poultry.

2. Materials and methods

2.1. Bacterial strains and plasmids

Plasmid pBAD (Invitrogen, Paisley, UK) was used as expression vector and *E. coli* TOP10 (Invitrogen, Paisley, UK) as expression host. *E. coli* strains were grown either in Terrific Broth (TB) or Luria–Bertani (LB) agar supplemented with ampicillin (100 μg/ml) at 37 °C and shaken at 300 rpm, where appropriate.

2.2. Animals and housing conditions

Ross 308 broiler chickens were obtained as one-day-old chicks from Vervaeke-Belavi Hatchery (Tielt, Belgium, BE3031) and the parent flock had not been vaccinated with the commercial Netvax or any other *C. perfringens* vaccine. All animals were housed in the same room. The birds were reared in pens at a density of 27 animals per 1.5 m^2 on wood shavings. All pens were separated by solid walls to prevent contact between birds from different treatment groups. Before the trial, the rooms were decontaminated with Metatectyl HQ (Clim'oMedic®, Metatecta, Belgium) and a commercial anticoccidial disinfectant (OOCIDE, DuPont Animal Health Solutions, Wilmington, USA). The chickens received *ad libitum* drinking water and feed. A 23 h/1 h light/darkness programme was applied. The animal experiments were carried out according to the recommendations and following approval of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

2.3. Expression and purification of NetB and NetB W262A

The expression of wild type NetB or NetB W262A, without their native signal peptides and with N-terminal His-tags for purification, was carried out in *E. coli* TOP10 cells and purification was carried out as described previously [18]. In summary, the recombinant *E. coli* carrying the pBAD-NetB expression vector was grown in TB to an optical density (OD_{595nm}) of 0.5 and expression of the toxin induced for 6 h by adding arabinose at a final concentration of 0.02% (w/v). Bacterial cells were harvested by centrifugation, lysed enzymatically using BugBuster (Invitrogen, Paisley, UK), and NetB purified by Ni-NTA chromatography columns (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's instructions. The protein was transferred into Tris-buffered saline (TBS; 20 mM Tris pH 7.5, 150 mM NaCl) using PD-10 desalting columns (GE Healthcare Life Sciences, Little Chalfont, UK) and protein concentrations measured with a UV–vis spectrophotometer (Thermo Scientific, Cramlington, UK).

2.4. Preparation of formaldehyde NetB toxoid

Wild type NetB was suspended at 400 μg/ml in TBS and formaldehyde added to a final concentration of 130 mM. After incubation for 5 days at 37 °C, the reaction was stopped by the addition of L-lysine (30 mM final concentration) and residual formaldehyde was removed by dialysis overnight against TBS by using 10 kDa MWCO Spectra/Por 6 dialysis tubing (Spectrumlabs, Rancho Dominguez, USA).

2.5. SDS-PAGE analysis

Protein purity was analysed by SDS-PAGE on precast 4–12% acrylamide-bisacrylamide gels (Invitrogen, Paisley, UK). Therefore, gels were run in MES running buffer (Invitrogen, Paisley, UK) for 45 min at 200 V and stained with SimplyBlue (Invitrogen, Paisley, UK). The Perfect Protein Marker (Merck, Darmstadt, Germany) was used as a protein standard.

2.6. Cytotoxic activity of NetB and NetB toxoids

Wild type NetB or NetB toxoids were evaluated for cytotoxicity towards a chicken hepatocellular carcinoma epithelial cell line (LMH; ATCC: CRL-2117; ATCC-LGC Standards, Teddington, UK) as described previously [18]. Briefly, LMH cells were grown on 96-well plates to approximately 70% confluency in Waymouth's MB 752/1 medium (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum in a 5% CO_2 incubator at 37 °C. Subsequently, cells were incubated with 100 μl (0.4 mg/ml) of either wild type NetB, formaldehyde NetB toxoid, or NetB W262A for 1 h at 37 °C. Effects on cell morphology were observed with the Inverso-TC optical microscope (Medline Scientific, Chalgrove, UK) and images were taken with the PowerShot S5 IS digital camera (Canon, Reigate, UK).

2.7. Measurement of antibody to NetB using ELISA

Antibody responses to NetB toxoids were determined using an enzyme-linked immunosorbent assay (ELISA). Three groups 10 of one-day-old Ross 308 broiler chickens were fed a wheat/rye-based (43%/7.5%) diet, with soybean meal (24.6% and 25.3% soybean meal in the starter and grower diet respectively) as a protein source [25]. On days 3, 9, and 15, animals were each immunised with 30 μg of either formaldehyde NetB toxoid or NetB W262A. Quil-A (50 μg;

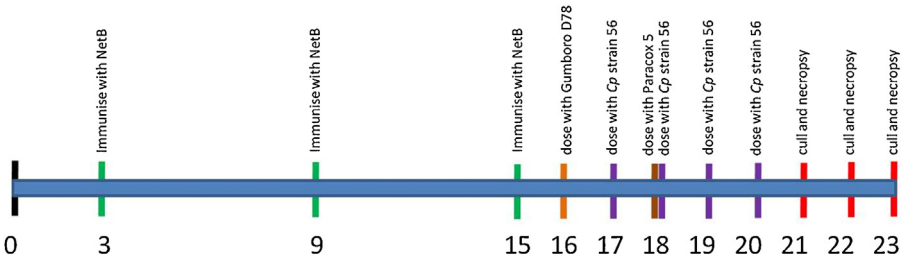

Fig. 1. Experimental design of the *in vivo* NE model. Animals were immunised with formaldehyde NetB toxoid or NetB W262A on days 3, 9, and 15 post-hatching and infected with *C. perfringens* strain 56 on days 17, 18, 19, and 20. Animals were culled, necropsied, and scored for lesions within the small intestines on days 21, 22, or 23.

Brenntag Biosector, Frederikssund, Denmark) was used as an adjuvant. The mixture was diluted in PBS to a total volume of 200 μl, mixed by pipetting and filter-sterilised (0.2 μm pore size). Birds were vaccinated subcutaneously in the neck with a 200 μl dose. Controls consisted of a group receiving only the Quil-A (50 μg) adjuvant. Serum samples were taken on day 15 (prior to third immunisation) and on day 23.

For each ELISA, assays were performed in triplicate in three independent experiments, individual sera in each group were pooled and used at a dilution of 1:20. First, 96-well microtitre plates (Nunc-Immuno Plates–MaxiSorp; Thermo Scientific, Cramlington, UK) were coated with 2.5 μg/well of purified NetB overnight at 4 °C. Plates were washed three times with TBS-T (TBS, Tween 0.05%, v/v) and blocked with Odyssey blocking buffer (LI-COR Biosciences, Cambridge, UK) for 1 h at room temperature. Plates were washed with TBS-T and 100 μl/well of pooled sera was incubated for 2 h at room temperature. Bound antibodies were detected using a HRP-conjugated rabbit anti-chicken IgY (H + L) secondary antibody (1:5000; Thermo Scientific, Cramlington, UK) and the ELISA HRP substrate (680) pack (LI-COR Biosciences, Cambridge, UK). Fluorescent intensity was imaged using the Odyssey CLX infrared imaging system (LI-COR Biosciences, Cambridge, UK). The fluorescence values for each sample were normalised by subtracting the values obtained for using TBS as a sample.

2.8. In vivo NE model

The NE model was based on the subclinical *in vivo* model described previously [25,26]. Groups of 25–28 one-day-old Ross 308 broiler chickens were immunised three times with either formaldehyde NetB toxoid formulated with Quil-A or NetB W262A formulated with Quil-A as described above. Controls consisted of an untreated group and a group receiving only the adjuvant. Nobilis Gumboro D 78 vaccine (Schering-Plough Animal Health, Brussels, Belgium) was given in the drinking water on day 16. From day 17 onwards soy bean meal was replaced by fishmeal (30%) as a protein source. All groups were challenged orally, using a plastic tube inserted in the crop, on days 17, 18, 19 and 20 with a single dose of approximately 4×10^8 cfu of *C. perfringens* strain 56. On day 18, all animals were orally inoculated with a 10× dose of Paracox-5 (Schering-Plough Animal Health, Brussels, Belgium) [26]. On days 21, 22, and 23, one-third of the birds in each group were euthanised and necropsied. A schematic outline of the experimental design is shown in Fig. 1.

2.9. Assessment of protection

NE severity was assessed by scoring lesions within the small intestine of each animal (duodenum to ileum) as described by Keyburn et al. [27] as follows: 0 = no gross lesions; 1 = congested intestinal mucosa; 2 = focal necrosis or ulceration (1–5 foci); 3 = focal necrosis or ulceration (6–15 foci); 4 = focal necrosis or ulceration (≥16 foci); 5 = patches of necrosis 2–3 cm long; 6 = diffuse necrosis typical of field cases. Animals showing lesion scores of 2 or higher were classified as NE positive.

3. Statistical analysis

For the *in vivo* NE model, differences within the occurrence of NE-positive animals between the controls and the NetB toxoid vaccinated groups were evaluated by a binary logistic regression analysis with the SPSS Statistics software 21.0 (SPSS Inc., Chicago, USA). A 2-way ANOVA analysis with the GraphPad Prism software 5.01 (GraphPad Software, La Jolla, USA) was used to compare the means of ELISA data. In both analysis, a p value of less than 0.01 was considered as significant ($^{***}p < 0.001$; $^{**}p < 0.01$).

4. Results

4.1. SDS-PAGE analyses of NetB W262A and formaldehyde NetB toxoid

Purified NetB W262A was analysed by SDS-PAGE (supplemental Fig. 1A). The predicted molecular size of monomeric NetB, without its native signal peptide but with an N-terminal His-tag, is 37.6 kDa and we observed a major band with an apparent molecular size of approximately 38 kDa. A lower abundance protein migrated with a molecular weight of greater than 200 kDa and this is likely to be the SDS-resistant heptameric form of the toxin [18]. We also prepared a formaldehyde NetB toxoid. The toxoid migrated as a high molecular complex on SDS-PAGE (supplemental Fig. 1B), consistent with the chemical cross-linking of individual proteins.

Supplementary data related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.05.063.

4.2. Cytotoxic activity of NetB and NetB toxoids on LMH cells

LMH cells were incubated with 0.4 mg/ml wild type NetB or NetB toxoids for 1 h at 37 °C and any morphological changes observed by optical microscopy (supplemental Fig. 2). Untreated cells showed epithelial and dendritic-like growth (supplemental Fig. 2A), whereas treatment of cells with purified wild type NetB caused cell blebbing and cell swelling (supplemental Fig. 2B). Incubation with formaldehyde NetB toxoid or NetB W262A did not result in any morphological changes indicative of toxicity to LMH cells (supplemental Fig. 2C and D, respectively).

Supplementary data related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.05.063.

4.3. Measurement of antibody to NetB using ELISA

We used an ELISA to measure the antibody responses to NetB in immunised chicken (Fig. 2). Animals immunised with either formaldehyde NetB toxoid or NetB W262A showed an increasing

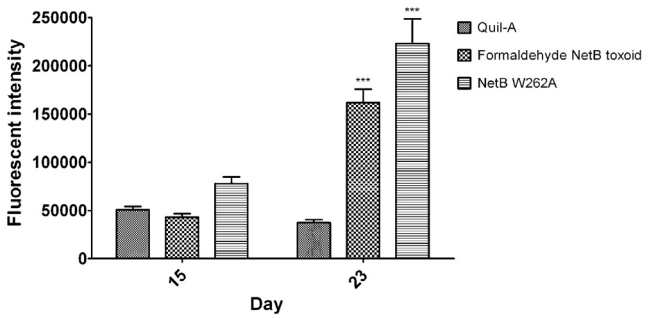

Fig. 2. Measurement of antibody to NetB using ELISA. Chicken were immunised with either formaldehyde NetB toxoid, NetB W262A or the Quil-A adjuvant alone, on days 3, 9, and 15. Sera were taken on day 15 (prior to third immunisation) and on day 23. The graph represents data from three replicates in three independent experiments (data are the means ± SEM; $n = 3$). Asterisks indicate a statistically significant difference (***$p < 0.001$; 2-way ANOVA analysis) relative to the Quil-A immunised control.

antibody response to NetB over the course of the immunisation schedule. By day 23 poultry immunised with the toxoids showed significantly higher levels ($p < 0.001$) of circulating antibody than control animals immunised with Quil-A alone. In addition, on day 23 animals immunised with the NetB W262A showed significantly increased ($p < 0.01$) NetB antibody responses than the group vaccinated with the formaldehyde NetB toxoid.

4.4. Protection against experimental NE after immunisation with formaldehyde NetB toxoid or NetB W262A

Immunisation with either the formaldehyde NetB toxoid or with NetB W262A reduced lesion scores relative to the control groups in poultry experimentally infected with *C. perfringens* (Fig. 3A). In the control groups of untreated chickens, or chickens dosed with adjuvant only, the mean lesion scores were 1.04 and 1.07, respectively. In contrast, animals immunised with the formaldehyde NetB toxoid showed a mean lesion score of 0.33 and animals immunised with NetB W262A a mean lesion score of 0.25. There was also a reduced occurrence of NE-positive chicken in vaccinated groups compared with control groups (Fig. 3B). In the non-vaccinated and the adjuvant only immunised groups 48% of the birds were NE-positive, whereas in animals vaccinated with formaldehyde NetB toxoid or NetB W262A only 15% and 11% of animals were NE-positive, respectively.

5. Discussion

In broilers, acute NE is often evidenced as a sudden increase in flock mortality during the last weeks of the rearing period (week 5–6) and at necropsy large necrotic foci and extensive necrosis are found at the mucosal surface of the gut. The infection model we have used in our study reflects the subclinical form of disease. In our model approximately 50% of the control animals develop focal lesions which are typical of the lesions seen in field cases of subclinical NE. We do not observe massive necrosis of the gut which is associated with acute disease. In previous studies we have used this model, in some cases with minor modifications, to evaluate the effect of feed additives, vaccines or antibiotics on disease or to perform studies on the pathogenesis of NE [21,26,28–30].

Recently, we have solved the heptameric pore-forming structure of NetB and determined the roles of selected amino acids mediating binding and toxicity to target cells [18]. The NetB mutant (W262A) showed the greatest reduction in binding and toxicity, relative to wild type NetB. Cytotoxicity of wild type NetB and NetB W262A on LMH cells was tested *via* the release of lactate dehydrogenase (LDH) into the culture medium [18]. Wild type NetB caused LDH release and the median cytotoxic dose (CT_{50}) was 800 nM. In contrast, LDH release could not be detected after incubation of cells with NetB W262A, even at a concentration of 4 μM [18].

This study was conducted to test NetB W262A or a formaldehyde NetB toxoid for their potential to induce an immune response able to protect chicken against the subclinical form of NE. Vaccination with either antigen increased the antibody response to NetB, relative to the control groups. In addition, antibody levels to NetB were significantly higher on day 23 for the NetB W262A immunised group than for the formaldehyde NetB toxoid vaccinated group. As expected, control chickens did not have high levels of antibody to NetB on days 15 and 23 of our study. We recorded an induced protection after immunisation with either of the NetB toxoids in our NE disease model. However, a direct comparison with protection studies carried out by other researchers with different antigens is difficult, due to differences in the immunisation regimens and disease models used.

Although our findings are encouraging, the vaccination schedule we have reported here would not be suitable for widespread use in poultry flocks. The lifespan of broiler poultry is typically 4–8 weeks and using our immunisation schedule we could not detect a significant increase in serum NetB antibody at 15 days post immunisation. Protection of younger animals might be achieved by the vaccination of breeder hens with the subsequent transfer of maternal antibody to eggs [31,32], by *in ovo* immunisation [33,34], or using a vaccine delivery system that results in a rapid development of immunity [35].

We could not demonstrate complete protection of poultry after immunisation with either NetB W262A or the formaldehyde NetB toxoid. It is possible that NetB variants with mutations in other residues might lead to higher levels of protection. However, our findings that immunisation with either NetB W262A or the formaldehyde toxoid induced broadly similar levels of protection suggests that it is more likely that delivery with a different adjuvant or by a different route is required for improved protective immunity. If NetB is directly responsible for the gut lesions seen in diseased animals, then it is possible that immunisation by a route that would induce a mucosal antibody response would provide increased protection against NE. Immunisation with an attenuated strain of *Salmonella enterica* serovar Typhimurium expressing NetB W262A could result in the induction of mucosal antibody which would then protect the gut mucosal surface from the toxin. The use of a *Salmonella*-vectored vaccine might also allow a single dose of vaccine to be given – an important consideration when immunising poultry flocks. Previously, other workers have expressed *C. perfringens* fructose-biphosphate-aldolase, pyruvate-ferredoxin oxidoreductase, hypothetical protein, or alpha-toxoid in attenuated strains of *S. enterica* serovar Typhimurium and have reported the induction of protective immunity against experimental NE [36,37]. These *Salmonella* based vaccines have been given to poultry orally and immunisation resulted in both serum and mucosal antibody responses to the *C. perfringens* antigens [36,37].

Alternatively, we have previously reported the expression of alpha-toxoid on the surface of *Bacillus subtilis* spores, and these spores have been shown to be immunogenic in mice, inducing mucosal antibody responses after oral dosing [38]. The immunogenicity of the recombinant spores in poultry has not been investigated. However, *B. subtilis* spores have been shown to be effective as competitive exclusion agents in poultry, limiting colonisation of the gut by *C. perfringens* [39]. Oral dosing with *B. subtilis* spores expressing NetB W262A might therefore offer short term benefits as the spores act as competitive exclusion agents and also induce protective antibody responses in the longer term.

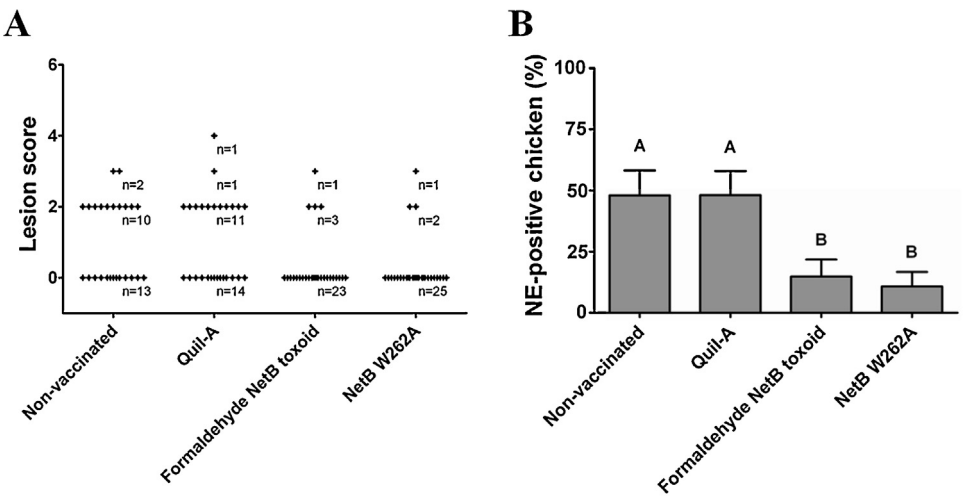

Fig. 3. *In vivo* NE model. (A) Lesion scores of individual broiler chickens. According to severity, lesions in the small intestine were scored from 0 (no gross lesions) to 6 (diffuse necrosis). Individual broiler chickens are marked as (+). *n* = number of animals. (B) NE-positive chickens. Animals with lesion scores of 2 or higher were classified as NE-positive. Black bars represent the SEM. Groups not sharing the indicated letters are significantly different ($p < 0.01$) according to the binary logistic regression analysis.

An alternative approach to devising an NE vaccine that provides more complete protection against disease might lie in using a combination of protective antigens, such as NetB toxoid with alpha-toxoid, glyceraldehyde-3-phosphate dehydrogenase, pyruvate-ferredoxin oxidoreductase, fructose 1,6-biphosphate-aldolase, hypothetical protein, elongation factor-Tu, TpeL, endo-beta-*N*-acetylglucosaminidase, or phosphoglyceromutase which have all been shown to provide partial protection against disease.

In conclusion, this study demonstrates that a formaldehyde NetB toxoid or NetB W262A could be used in subsequent work to devise a vaccine to control NE. Compared to the production method of formaldehyde NetB toxoid, the NetB W262A mutant is easily produced in *E. coli*. In addition, toxoiding using formaldehyde can show batch to batch variation and reversion to toxicity has been reported in other bacterial toxins in the past [40,41]. Furthermore, it is important to minimise any free formaldehyde in vaccine preparations to avoid possible side effects [42]. As a NetB formaldehyde toxoid might suffer from these problems, the use of a NetB mutant would allow a more reliable and simple production of a vaccine. However, further work is required to establish how such a toxoid vaccine could be effectively delivered, suitable for use in the poultry industry.

Conflict of interests

The authors have no competing interests.

Authors' contributions

SFdC carried out most of the experiments and drafted the manuscript. DM carried out the *in vivo* NE model and drafted the manuscript. CS helped to purify NetB and drafted the manuscript. MB-B helped with experiments and interpreted the data. RT devised this project and drafted the manuscript, FVI, AB designed research and revised the manuscript. All authors read and approved the final manuscript.

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