A Plant Based Vaccine for Necrotic Enteritis in Chickens

by

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ABSTRACT

Necrotic enteritis (NE) is caused by type A strains of the bacterium Clostridium perfringens, leading to an estimated 2 billion dollar global economic loss in the poultry industry annually. Traditionally, NE has been effectively controlled by antibiotics added to the diet of poultry. Concerns about increasing antibiotic resistance of poultry and human based pathogens have led to the consideration of alternative approaches for controlling disease, such as vaccination. NE causing strains of C. perfringens produce two major toxins, α-toxin and NetB. Immune responses against either toxin can provide partial protection against NE. We have developed a fusion protein combining a non-toxic carboxy-terminal domain of the α-toxin (PlcC) and an attenuated, mutant form of NetB (NetB-W262A) for use as a vaccine antigen to immunize poultry against NE. We utilized a DNA sequence that was codon-optimized for Nicotiana benthamiana to enable high levels of expression. The 6-His tagged PlcC-NetB fusion protein was synthesized in N. benthamiana using a geminiviral replicon transient expression system. The fusion protein was purified by metal affinity chromatography and used to immunize broiler birds. Immunized birds produced a strong serum IgY response against both the plant produced PlcC-NetB protein and against bacterially produced His-PlcC and His-NetB. However, the PlcC-NetB fusion had antibody titers four times that of the bacterially produced toxoids alone. Immunized birds were significantly protected against a subsequent in-feed challenge with virulent C. perfringens when treated with the fusion protein. These results indicate that a plant-produced PlcC-NetB is a promising vaccine candidate for controlling NE in poultry.

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INTRODUCTION

The Burden of Antibiotic Use

Since the 1940s the inconvenience of disease in livestock of all types was, and in many places is, controlled via the use of antibiotics (Gustafson and Bowen, 1997; Price *et al.*, 2015). Antibiotics and the poultry industry have enjoyed an intimate relationship since the discovery that antibiotics reduce the amount of feed needed to bring broilers to market weight (Gustafson and Bowen, 1997). However, just as concerns arose in the medical industry about overuse of antibiotics in humans the same concerns arose in agricultural sectors (Sneeringer *et al.*, 2017; Witte, 1998). Antibiotic resistance has been a topic of discussion for decades and, although antibiotics are still used regularly, there has been a notable decline in said use, especially in European countries (Butaye *et al.*, 2003; Levy, 2014).

A decline in antibiotic use presents its own issues since many major diseases, such as salmonellosis, bovine tuberculosis, brucellosis, and necrotic enteritis were controlled by antibiotics (Immerseel *et al.*, 2004; Wiethoelter *et al.*, 2015). As antibiotics can no longer be used as a long-term control method for disease another avenue must be perused, the most logical being vaccination. However, there are many challenges associated with vaccination of livestock such as vaccine cost, vaccine administration, vaccine coverage, and farmer compliance (McReynolds *et al.*, 2004a).

Poultry may very well be the most difficult of livestock to vaccinate as there are the most animals to vaccinate paired with the fact that broilers go to market between 5-7 weeks after hatching. Meat consumption is increasing worldwide with poultry as a growing source of animal protein, leading to an increased number of broiler birds in

need of vaccination yearly (Sans and Combris, 2015). Poultry is also accountable for 25% of foodborne illness outbreaks in the United States, reinforcing the need for vaccines against common poultry diseases (Chai *et al.*, 2017). *Clostridium perfringens* is a ubiquitous disease-causing bacteria in poultry, accounting not only for 26% of poultry based foodborne illness, but also resulting in economic losses of 2 billion dollars annually in the poultry industry (Chai *et al.*, 2017; McReynolds *et al.*, 2004b; Van der Sluis, 2000). *C. perfringens* causes necrotic enteritis (NE), necrosis of the intestinal mucosa, leading to decreased weight or death of broiler birds (Rood *et al.*, 2016; Rood and Cole, 1991; Uzal *et al.*, 2015). NE generally occurs in broiler birds at 2-6 weeks of age but can also occur in older birds (Cooper *et al.*, 2013; Cooper and Songer, 2010). This timeline of disease is important to note since broilers go to market as early as 5 weeks post hatch and their immune systems are not wholly developed at 2 weeks of age. Since this is the case any vaccine given to chickens must be done as early as possible, while still being effective with minimal boosts.

C. perfringens Toxins as a Path to Vaccination

To combat a disease its pathology must be understood to elicit antigenic targets for vaccination. However, NE pathogenesis is complex due to interactions of many *C. perfringens* genes paired with reduced intestinal health caused by food type, mycotoxins, environmental factors, and the presence of other pathogens (Prescott *et al.*, 2016; Shivaramaiah *et al.*, 2011). Toxins implicated in broad clostridium infections include; alpha toxin (CPA), beta toxin (CPB), epsilon toxin (ETX), iota toxin (ITX), enterotoxin (CPE), and necrotic B-like toxins (NetB) (Navarro *et al.*, 2018). Luckily, two toxins have been identified as favorable targets for vaccination in poultry; α-toxin and NetB.

α-toxin is encoded by the *plc* gene and is the major virulence factor for gas gangrene in humans (Navarro *et al.*, 2018; Titball *et al.*, 1999). The toxin is divided into two domains; the amino-terminal domain (encoding the catalytic site of phospholipase activity) and carboxy-terminal domain (encoding the sites involved in interactions with phospholipids which target the toxin to host cell membranes) (Guillouard *et al.*, 1996, 1997; Naylor *et al.*, 1998; Titball *et al.*, 1991). A nontoxic carboxy-terminal fragment of the α-toxin (aa 247-370), dubbed PlcC, has been shown to confer protection against *C. perfringens* when used as a vaccine target in a gas gangrene mouse model as well as induce neutralizing antibodies in rabbits (Siqueira *et al.*, 2018a; Williamson and Titball, 1993). Vaccination against *C. perfringens* infection using PlcC, delivered as purified protein or via live attenuated bacteria, has proven effective in protecting chickens against intestinal lesion challenge with pathogenic *C. perfringens* (Jiang *et al.*, 2015; Kulkarni *et al.*; Nagahama *et al.*, 2013; Siqueira *et al.*, 2018b; Zekarias *et al.*). The only commercially available vaccine for NE is based on an α-toxin derived from a specific A strain of *C. perfringens*, given that it is one of many causative factors for NE (such as NetB) an improved vaccine approach is desirable (Crouch *et al.*, 2010).

NetB is a β-like, pore-forming toxin and virulence factor in *C. perfringens* mediated NE (Rood *et al.*, 2016). The NetB toxin gene is located on large conjugative plasmids similarly related to other toxin carrying plasmids and is transcribed as a monomer that forms heptameric pores (Keyburn *et al.*, 2010; Rood *et al.*, 2016). NetB was identified in *C. perfringens* type A strains which were parthenogenic and not generally found in healthy birds (Keyburn *et al.*, 2008; Rood *et al.*, 2016). Both purified native NetB and recombinant NetB exhibited cytotoxic activity against chicken leghorn male hepatoma cells (LMH) inducing lysis (Keyburn *et al.*, 2008). Given its importance

in the pathology of NE, NetB has become an antigen of interest concerning *C. perfringens* vaccination. NetB functions by binding cholesterol in membranes, forming heptameric pores of at least 1.6 nm (Savva *et al.*, 2013; Yan *et al.*, 2013). To attenuate the toxin for vaccination purposes several single amino acid substitutions in the rim loop region of NetB, believed to interact with the host membrane, have been identified to significantly reduce toxicity. Single amino acid substitutions include; Y191A, R200A, W257A W262A S254L, R230Q and W287R (Savva *et al.*, 2013; Yan *et al.*, 2013). NetB, like the α-toxin or PlcC portion of the α-toxin, has been used successfully to protect broiler birds against *C. perfringens* challenge (Fernandes da Costa *et al.*, 2013; Keyburn *et al.*, 2013; Yan *et al.*, 2013). Non-toxic rim mutants, W262A and S254L, have also been shown to generate a protective immune response in broiler birds (Fernandes da Costa *et al.*, 2013; Keyburn *et al.*, 2013).

In a recent study that examined serum antibody levels against *C. perfringens* α-toxin and NetB toxin of commercial birds involved in field outbreaks of NE, it was found that serum antibody levels against both α-toxin and NetB toxin were significantly higher in healthy birds compared with those showing clinical signs of NE (Lee *et al.*, 2012). Higher antibody titers against these toxins in healthy birds indicate that they play a key role in NE. These field results, paired with the clinical studies showing that vaccination with either α-toxin, PlcC, NetB toxin, or NetB rim domain mutants make a vaccine utilizing both toxins, preferably their non-toxic variants, a logical next step. Previously, a live attenuated *salmonella* vaccine platform was used to deliver PlcC, NetB, or both to broiler birds. This study found that broilers immunized against both PlcC and NetB had fewer severe lesions when challenged with *C. perfringens* than birds immunized with either antigen alone (Jiang *et al.*, 2015). Given these finding an approach combining

both the truncated PlcC region of *C. perfringens* α-toxin and a W262A rim mutant of NetB via a short, 15 amino acid, linker was perused. A 6-histadine tag was added to the fusion protein to allow metal affinity chromatography purification, a process by which a cobalt bead is used to capture the 6-histadines via ionic bonds which can be broken in downstream elution steps (Karakus *et al.*, 2016). The final fusion protein was dubbed PlcC-NetB.

Plant Based Transient Expression of a Fusion Protein

Nicotiana benthamiana was selected as the appropriate expression vector for many reasons, one of which was *salmonella's* inability to produce the fusion protein. However, *N. benthmiana* is a desirable expression vector for a multitude of other reasons, including safety, cost effectiveness, and scalability (Diamos *et al.*, 2016; Klimyuk *et al.*, 2012). Transient plant expression systems, unlike prokaryotic expression systems, allow for complex protein production and protein glycosylation which in turn makes the production of monoclonal antibodies and recombinant immune complexes (RICs), as well as other proteins requiring eukaryotic post-modification, a possibility (Cook *et al.*, 2018; Kim *et al.*, 2015; Mason, 2016; Schähs *et al.*, 2007; Strasser *et al.*, 2014). Transient plant expression is also optimal compared to transgenic expression as large amounts of a desired protein can be produced within 4 days rather than the long periods of time required to create a transgenic plant (Huang and Mason, 2004; Kim *et al.*, 2015; Marillonnet *et al.*, 2005; Sparkes *et al.*, 2006).

The use of *N. benthmiana* is reliant on the natural capability of *Agrobacterium tumefaciens* (Agro) to insert its transfer DNA or T-DNA into plant cells (Marillonnet *et al.*, 2005). A. *tumefaciens* contains a tumor inducing (Ti) plasmid, which encodes all the machinery responsible for transduction of a portion of our bean yellow dwarf virus

(BeYDV) derived vector plasmid into plant nuclei (Diamos *et al.*, 2016; Diamos and Mason, 2018; Huang *et al.*, 2009). The section between the left and right border regions is transduced with a the section of the plasmid between a pair of long intergenic regions (LIRs) forming a circular plasmid in the plant nucleus which undergoes rolling circle replication (Diamos *et al.*, 2016; Diamos and Mason, 2018; Huang *et al.*, 2009; Kim *et al.*, 2015). Introduction of an additional LIR allows this geminiviral replication system to produce multiple proteins from a single vector, such as antibodies or RICs which encode both the heavy and light chains on a singular plasmid (Diamos *et al.*, 2016; Diamos and Mason, 2018; Klimyuk *et al.*, 2012; Mason, 2016). Not only are the abilities of the geminiviral transient expression system convenient but, due to recent improvements, they are currently capable of producing the largest amount of protein known for a transient plant expression system (Diamos *et al.*, 2016; Diamos and Mason, 2018).

The recombinant PlcC-NetB fusion protein was produced using the briefly described geminiviral transient expression system above. This lead to the production of a large amount of extractable, soluble, and acid stable protein. Once purified via metal affinity chromatography the fusion protein was used to immunize broiler birds in three separate experiments. Due to concerns of the glycosylated nature of PlcC-NetB which was shuttled to the ER, a non-glycosylated (PlcC-NetB-NG) fusion protein targeted to the cytosol was also produced. Immunization experiments were also performed for the PlcC-NetB-NG fusion protein, however due to protein solubility issues only general conclusions about its immunogenicity can be made. Results of broiler bird immunizations with ER targeted PlcC-NetB resulted in statistically significant antibody titers against PlcC and NetB antigen. Immunization also conferred protection against C. *perfringens* challenge which was determined via lesion scoring of individual birds.

METHODS

Expression Vector Construction.

Experiments described in this section were performed by Hugh Mason.

A PlcC-NetB fusion protein was designed and codon optimized for expression in *N. benthamiana* (Geyer *et al.*, 2010). Amino acid sequences were based on GenBank accessions **AAP15462.1** for PlcC and **ABW71134.1** for NetB. Targeting of the fusion protein to the ER was facilitated by a barley alpha amylase signal peptide, which was followed by the 6-His tag for purification. The signal peptide and 6-His tag were added to the N-terminus of PlcC. A polypeptide linker, GGSGGSGGPSGGSGG, was inserted between PlcC and NetB (**Fig. 1**). The gene encoding the complete fusion protein, with 6-His tag, was synthesized commercially with flanking XbaI and SacI sites. The resulting 1402 bp XbaI-SacI fragment was ligated with the geminiviral vector pBYR2eK2M resulting in the pBYR2eK2M-6H-PlcC-NetB vector (Diamos *et al.*, 2016). Removal of the 417 bp BamHI fragment, containing the PlcC and linker sequences, yielded ER-targeted 6H-NetB, pBYR2eK2M-6H-NetB. To construct an ER-targeted 6H-PlcC vector an end-tailored PlcC sequence was subjected to PCR and a stop codon at a SacI site, at its 3' end, introduced using the primer PlcC-Sac-R: (5'-CCCGAGCTCCTATTTGATGTTGTAAGTGGAGTTTCC). Inserting the resulting product into the pBYR2eK2M vector yielded pBYR2eK2M-6H-PlcC.

Cytosol-targeted variants of the above ER-targeted constructs were produced by removing the signal peptide by introducing an XbaI site and start codon 5' of the 6-His sequence via PCR with primer 6H-Xba-F: (5' GACTCTAGAACAATGGCTCACCATCACCATCATCAC). Resulting XbaI-SacI fragments were ligated with pBYR2e3K2Mc-GFP digested with XbaI and SacI, yielding the vectors

pBYR2e3K2Mc-c6H-PlcC-NetB, pBYR2e3K2Mc-c6H-PlcC, and pBYR2e3K2Mc-c6H-NetB (Diamos and Mason, 2018).

Protein Production and Purification

Expression vectors outlined above were inserted into *A. tumefaciens* EHA105 via electroporation. The transformed agrobacterium was plated on LB + kanamycin (50 mg/L) plates and individual colonies selected. Resulting colonies were screened using PCR and restriction digestion to confirm transformation. Agrobacterium strains were grown overnight at 30°C in YENB + 50 mg/L kanamycin and 2.5 mg/L rifampicin. Bacteria were pelleted for 10 minutes at 5,000 g, then resuspended in infiltration buffer [10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5 and 10 mM $MgSO_4$] with a final OD600 = 0.2. Needleless infiltration, a process by which a small puncture is made in the leaf tissue and a needleless syringe used to force agrobacterium suspensions into the leaf, of *N. benthamiana* leaves at 5- to 6-weeks of age was performed (Huang and Mason, 2004). Infiltrated leaves were harvested at 4- to 5-days post infiltration (DPI).

Protein Extraction, Acid Precipitation, and Filtration

Total protein was extracted from agro-infiltrated leaf samples using a 1:3 (w:v) ratio of extraction buffer (50 mM sodium phosphate, 200 mM sodium chloride, pH 8.1) at 4˚C with 50 mM sodium ascorbate and 1 mM PMSF added. Using a two speed Conair™ Waring™ Laboratory Blender (Fisher Scientific, Waltham, MA) leaf samples were homogenized for 5-10 min until an even consistency was obtained. Homogenized

leaf tissue was stirred at high speed for 10 minutes at 4°C to improve protein solubility before acid precipitation.

Acid precipitation was performed by adding 1 M phosphoric to the crude extract with constant mixing until a pH of 4.4-4.6 was reached, as measured by a VWR Sb70 Symphony Ph Meter (VWR, Radnor, PA). The acidified extract was stirred for 10 min at 4°C. After this 10 minute period, 2 M Tris base was added to the extract until a pH of 7.5-7.7 was reached. The mixture was stirred for another 10 min at 4°C before being filtered through Miracloth and centrifuged at 17,000g, 4°C, for 30 min. The extract was then subjected to a second filtration through Miracloth resulting in a clarified extract, which was passed through a 0.45-micron filter for sterilization before metal affinity chromatography.

Metal Affinity Chromatography and Dialysis

3 mL of Talon metal affinity resin (Clontech/Takara Bio, Mountain View, CA) was spun down at 10,000 *g*, 4°C, for 5 min and the supernatant removed from the bed volume. Resulting bed volume (1.5 mL) was washed using 15 mL of extraction buffer (referred to below as wash buffer) before being spun down at 10,000 g, 4°C, for 5 min. Supernatant was discarded and another 15 mL of wash buffer added to resuspend the resin bed volume before it was added to a 20 mL column and washed with an additional 20 mL wash buffer. Once wash buffer had been passed through the column the clarified, filtered extract was added to the column. Once filtered extract was passed through the column 60 mL of wash buffer was used to remove any loosely bound protein contaminants. Five elutions, each of 1 mL PBS + 150 mM imidazole, pH 7.4, were added to the column and collected individually.

Elution samples were dialyzed overnight in 1 L sterile PBS using either a 3 mL or 10 mL 3,500 MWCO Slide-A-Lyzer G2 dialysis cassette (Thermo Scientific, Waltham, MA), following manufacturer's instructions. A280 absorption was utilized to determine protein concentration of elutions both before or after dialysis, using PBS + 150 mM Imidazole and PBS as blanks, respectively. Protein concentrations were confirmed by Coomassie staining of SDS-PAGE gels analyzed with ImageJ (Schneider *et al.*)

SDS-PAGE and Western Blot

Crude extracts, clarified extracts, and eluted samples were mixed with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue) under reducing conditions (0.5 M DTT). Samples were then boiled for 10 min and separated on 4-15% polyacrylamide gels (Bio-Rad, Hercules, CA). Gels were stained with Coomassie stain (Bio-Rad) or electroblot transferred to a PVDF membrane. PVDF membranes were blocked with 5% PBSTM (PBS with 0.05% Tween-20 and 5% nonfat dry milk) overnight at 4°C. Membranes were then washed with PBST before incubation with a 1:10,000 dilution of rabbit anti-PlcC or rabbit anti-NetB antibodies in 1% PBSTM for 1 hour at 37°C (Jiang *et al.*, 2015). After probing with the relevant antibody, membranes were washed again before being incubated with a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase conjugate in 1% PBSTM. Bound antibody was detected with ECL reagent (Amersham).

Stripping-for-reprobing was performed using a modified Abcam protocol (Stripping for reprobing). PVDF membrane was washed for 10 min in mild stripping buffer (for 1 liter: 15 g glycine, 1 g SDS, 10 mL Tween20, pH 2.2) per Abcam's recipe. PVDF membrane was then rinsed twice in 20 mL of PBS for 10 minutes followed by two

5 min rinse steps in 20 mL PBST. 5% PBSTM was used to block the membrane before re-probing which followed the above Western blotting steps.

Production of Recombinant Proteins in E. *coli* for Immune Assays

Experiments described in this section were performed by members of the Roland lab.

His-tagged recombinant PlcC (rPlcC) and NetB (rNetB) were cloned by PCR. The resulting PCR fragments were digested with BamHI and SalI and ligated to BamHI/SalI-digested plasmid pQE30. Plasmids carrying the desired inserts were electroporated into E. *coli* strain M15(pREP4) (Qiagen). Protein synthesis was induced by the addition of IPTG according to the manufacturer's instructions. rPlcC and rNetB were purified by passage over a TALON affinity column (Clonetech Laboratories, Inc, Mountain View, CA) following the manufacturer's recommendations.

Vaccination of Broiler Birds

Experiments described in this section were performed by members of the Roland lab.

Jumbo Cornish X rock chicks were immunized subcutaneously in three separate experiments. In experiment 1 birds were immunized with 50 μg of fusion protein and 50 μg of Quil-A adjuvant. Birds were given three immunizations at weekly intervals beginning at 11 days of age, with the final immunization at 25 days of age.

In experiment 2 birds were immunized with 100 μg of fusion protein and 50 μg of Quil-A adjuvant. Similarly to experiment 1, birds received 3 doses starting on day 11 with the final immunization at 25 days of age. For both experiments 1 and 2 blood was taken for serum analysis one week after final boost. Three days later, when the birds were 33 days of age, birds were given an in-feed challenge with virulent C. *perfringens* for 5 days. The birds were scored for intestinal lesions the following day.

For experiment 3, birds were injected with 50 μg of PlcC-NetB at 7 days of age and again at 12 days of age. Blood and tissues were taken for immunological analysis ten days after the boost. Birds were challenged two weeks after the final boost, when the birds were 26 days of age.

Challenge with C. *perfringens*

Experiments described in this section were performed by members of the Roland lab.

Chickens were fed an antibiotic-free starter feed containing 21% protein for 20 days, at which time the feed was switched to a high protein (28% protein), wheat-based feed containing 36% fish meal and zinc at 400 ppm (customized by Reedy Fork Farm, NC) to predispose the birds to NE (Coursodon *et al.*, 2012; Shojadoost *et al.*, 2012). Birds were challenged with virulent C. *perfringens* strain CP4 in feed for 5 days with some modifications (Thompson *et al.*, 2006). Feed was withdrawn on the day prior to challenge for 15 h. The following day, chickens were orally gavaged with 1.0 mL of an overnight culture of C. *perfringens* CP4 grown in CMM medium. Immediately after gavage, infected feed was provided thereafter for 5 consecutive days. To prepare infected feed, C. *perfringens* was grown in CMM medium for 24 h at 37°C, which was then

inoculated into FTG medium at a ratio of 0.3% (v/v) and incubated at 37°C for 15 h (morning challenge) or 23 h (evening challenge). The C. *perfringens* culture was mixed with feed at a ratio of 1:1 (v/w). Infected feed was prepared fresh twice daily. All birds were euthanized and necropsied the day following the final challenge.

Lesion Scoring

Experiments described in this section were performed by members of the Roland lab.

Protection against C. *perfringens* challenge was assessed based on gross intestinal lesion scores at necropsy. On day 33, birds were euthanized with CO2 and their small intestines (defined herein as the section between the gizzard and Meckel's diverticulum) were examined for visible gross lesions. Intestinal lesions were scored as follows: 0 = no gross lesions; 1 = thin or friable wall or diffuse superficial but removable fibrin; 2 = focal necrosis or ulceration, or non-removable fibrin deposit, 1 to 5 foci; 3 = focal necrosis or ulceration, or non-removable fibrin deposit, 6 to 15 foci; 4 = focal necrosis or ulceration, or non-removable fibrin deposit, 16 or more foci; 5 = patches of necrosis 2 to 3 cm long; 6 = diffuse necrosis typical of field cases (Shojadoost *et al.*, 2012). Birds that died during challenge were assigned a score of 6.

Statistical Analysis

Experiments described in this section were performed by members of the Roland lab.

All statistics were carried out using GraphPad Prism 6.0 (Graph-Pad Software, San Diego, CA). Antibody titers and lesion scores were analyzed using one-way ANOVA followed by Tukey's posttest. The values were expressed as means ± SEM, and differences were considered significant at $P < 0.05$

RESULTS

Construction of a PlcC-NetB Fusion Protein

A fusion protein comprising an N-terminal 6-His tag, PlcC, linker, and NetB was designed (**Fig. 1**). The polypeptide linker, GGSGGSGGPSGGSGG, was used to avoid steric hindrance, allowing for proper protein folding, and allow ready access to all conformational epitopes. The PlcC-NetB fusion protein contains 5 eukaryotic Asn-linked glycosylation sites (N-X-S/T), which could result in addition of glycans to the Asn residue when the protein is targeted to the ER in plant cells. Expression vectors were constructed to target proteins to the ER (pBYR2eK2M-6H-PlcC-NetB, -6H-PlcC, and 6H-NetB), or to the cytosol (pBYR2e3K2Mc-c6H-PlcC-NetB, -c6H-PlcC, and -c6H-NetB). It was expected that the cytosol targeted proteins would be unglycosylated while the ER targeted proteins would be glycosylated in any combination of, or all, the 5 Asn-linked glycosylation sites.

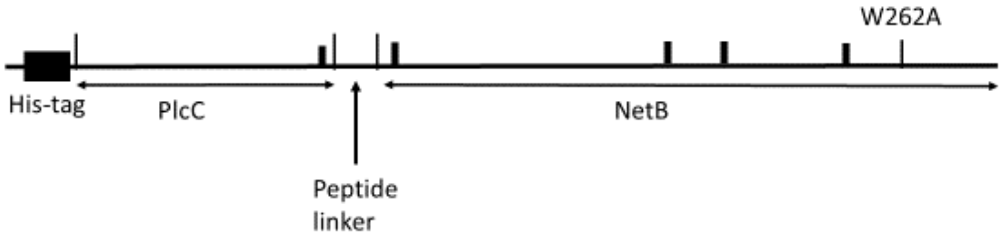

Figure 1. Schematic of the His-tagged PlcC-NetB Fusion Protein. Potential Asn-glycosylation sites are indicated by a short line (▌). The site of the W262A mutation is indicated.

The fusion protein detailed in Figure 1 was inserted into a geminiviral expression vector containing many different and important elements. These elements are outlined

in figure 2. The most important of these elements are the LIRs, SIR, C1/C2, and boarder regions.

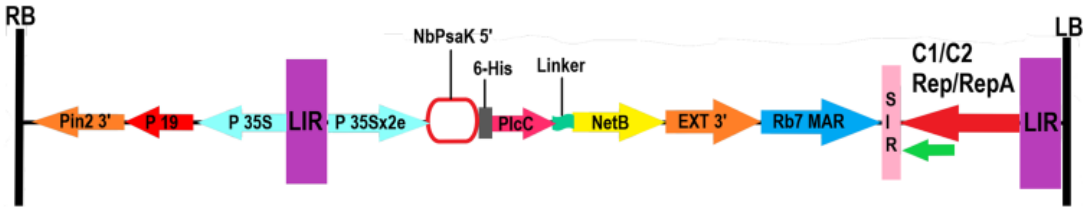

Figure 2. Outline of Geminiviral Expression Vector. Pin2 3' mediates polyadenylation. P 19 is an RNAi suppressor. P 35S is a promoter from cauliflower mosaic virus. LIRs function as a bidirectional promoter. P 35SxX2e is a promoter from cauliflower mosaic virus that increases the frequency of transcription initiation as it was doubled. NbPsaK 5' a 5′ UTR from N. *benthamiana* psaK gene. 6-His a six Histidine tag. PlcC a truncated form of C. perfringens alpha-toxin. Linker is a polypeptide linker. NetB is an attenuated B-like toxin. EXT 3' adds a poly-A-tail. Rb7 MAR is a matrix attachment region which promotes stability. SIR is the short intergenic region and contains polyadenylation elements for the preceding protein. C1/C2 encode Rep/RepA depending on splicing. RB and LB indicate left and right border regions from Agrobacterium.

Production of PlcC-NetB and PlcC-NetB-NG Fusion Proteins in Plants

Using the methods outlined herein two different fusion proteins were produced. One, targeted to the endoplasmic reticulum (ER), was glycosylated (PlcC-NetB). The other, targeted to the cytoplasm, was non-glycosylated (PlcC-NetB-NG). Both fusion proteins were probed with rabbit anti-PlcC and rabbit anti-NetB antibodies which detected the same ~49 kDa protein on a western blot (**Fig. 3**). PlcC-netB also has incrementally increased band sizes up to ~63 kDa, with the smallest band aligning with the singular PlcC-NetB-NG band at approximately 49 kDa which is the expected relative size of the fusion protein (**Fig. 3**).

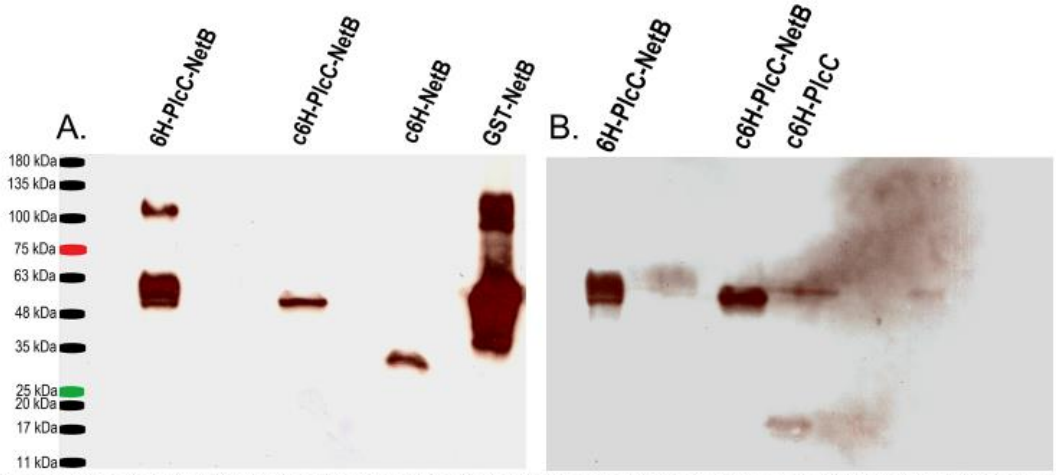

Figure 3. Western Blot Comparing Different Fusion Constructs Probed With Rabbit anti-NetB (A) Before Stripping and Re-probed with Rabbit anti-PlcC (B). 6H-PlcC-NetB shows a clear band in both the original and re-probed western with a higher band at ~110 kDa seen only in the western probed with rabbit anti-NetB. The GST-NetB standard, as well as cytosolically targeted c6H-NetB, are only seen in the western probed with rabbit anti-NetB. The c6H-PlcC-NetB (cytosolically targeted) fusion protein is also seen in both westerns and is a singular band that aligns with the lowest band seen in the 6H-PlcC-NetB (ER targeted) fusion protein sample. A faint ghost band at the size expected of PlcC may also be seen in blot B where a 6-His-PlcC fusion was loaded.

The process of purification for the PlcC-NetB protein is displayed in Figure 4A. Coomassie (**Fig. 4A**) shows an uninfiltrated, wild-type (WT) control followed by crude extract which has already been acid precipitated. Flow through contains bands of a similar profile to the crude extract on the Coomassie while both washes have no apparent banding. A GST-NetB standard is present followed by three elutions which contain bands at approximately 59 kDa. Anti-NetB antibodies (**Fig. 4B**) reacted with the standard and elution 2 primarily, while elution 1 and 3 have visible but less prominent bands. A bell curve relationship, where a middle elution has the highest protein concentration, is expected from a Talon resin elution protocol and is apparent in PlcC-NetB elutions. There is also a very light band present in the crude extract, giving an indication of relative concentrations.

When probed with anti-PlcC antibodies (**Fig. 4C**) similar bands can be seen in every lane except the blank and NetB standard lanes. Elution bands are as strong as those seen in the crude extract lane. Flow through and wash lanes have lighter but still

present bands when probed with anti-PlcC. Bands which reacted with anti-PlcC antibodies are also located at approximately 49 kDa.

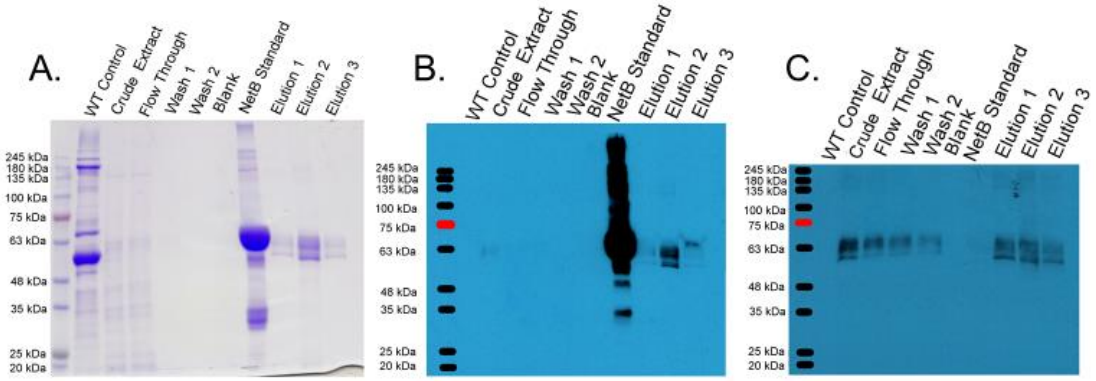

Figure 4. Purification of Glycosylated PlcC-NetB Fusion. (**A**) Coomassie stain of all purification steps. (**B**) Western blot of all purification steps, rabbit anti-NetB probe. (**C**) Western blot of all purification steps, rabbit anti-PlcC probe. Note: NetB standard is a GST-NetB fusion.

To determine whether or not A280 readings of the eluted and dialyzed protein samples were accurate enough to use for protein concentration determination a Coomassie gel (**Fig. 5B**) was run with sequential dilutions (1:1, 1:2, 1:4, 1:8) of both a 2 mg/mL BSA standard and a single PlcC-NetB-NG elution reading ~2 mg/mL. The dilutions of the BSA standard and elution were compared using ImageJ. Figure 5A shows the resulting graph which has two almost identical trend lines, with the exception of the final dilutions. These trend lines show that the A280 is an accurate estimate of protein concentration when acid precipitation and metal affinity chromatography are used in conjunction to remove an estimated 95% of native plant proteins.

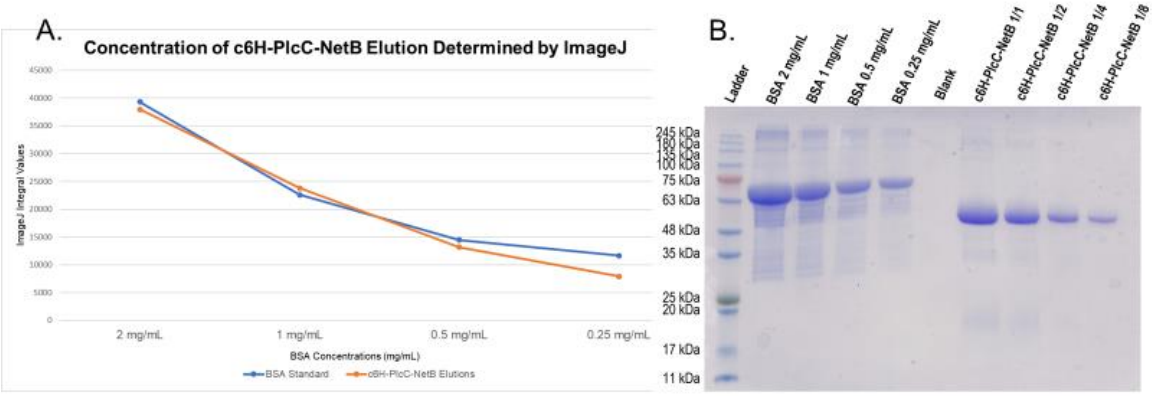

Figure 5. Comparison of PlcC-NetB-NG Elution Concentrations to BSA 2 mg/mL Standard Using Coomassie Stain Gel and ImageJ. The graph (A) shows a comparison of the integer values obtained from the Coomassie gel (B) using ImageJ.

Serum Antibody Responses to Fusion Protein Antigens

Serum antibody responses against PlcC-NetB were evaluated using recombinant rPlcC and rNetB produced in bacteria as well as the plant made fusion protein. Anti-PlcC-NetB titers were significantly higher than controls in all experiments (**Fig. 6**). In experiments 1 and 2 titers against rPlcC and rNetB were also elevated and significant (**Fig. 6A, 6B**). In experiment 3, when broiler birds were only immunized twice, at day 7 and 12, only the titers against the fusion protein were significant (**Fig. 6C**).

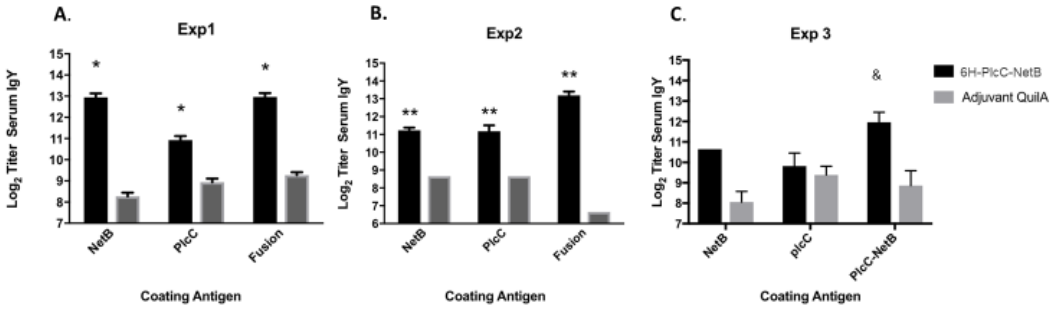

Figure 6. IgY Serum Titers From Immunized and Non-immunized Birds. All *P* values are compared to adjuvant only controls. Fusion refers to his-tagged, Glycosylated PlcC-NetB.
(**A**) Experiment 1, * $P \leq 0.009$ (**B**) Experiment 2, ** $P \leq 0.0025$. (**C**) Experiment 3, & $P < 0.0001$

Cellular Responses Against PlcC-NetB in Experiment 1 and 3

Immunization with PlcC-NetB led to variable cellular responses. The fusion protein elicited weak but significant cellular responses against rNetB and rPlcC-NetB in splenocytes during experiment 1 (**Fig. 7**). This was shown again in experiment 3 (**Fig. 8**). However, no significant antigen-specific stimulation was detected in lymphocytes except for an odd case of PlcC-NetB-NG vaccinated chickens having a response to PlcC.

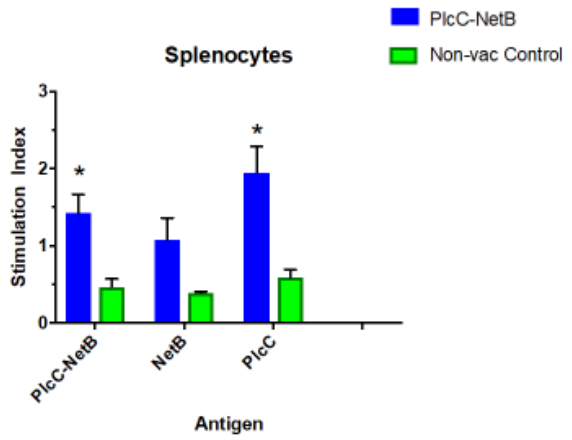

Figure 7. Cellular Responses to PlcC-NetB, PlcC and NetB After Immunization with Glycosylated PlcC-NetB.

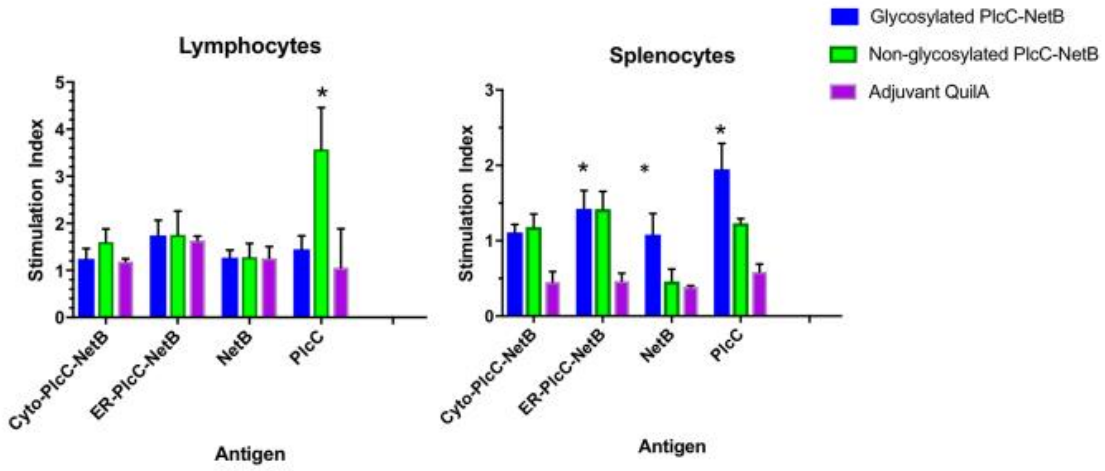

Figure 8. Cellular Responses to PlcC-netB Fusion Proteins, PlcC and NetB After Immunization.
*, $P < 0.02$

Protection Against C. *perfringens* Challenge

Protection against C. *perfringens* challenge was assessed by scoring intestinal lesions using a six point scoring system (Thompson *et al.*, 2006). Immunization with PlcC-NetB and PlcC-NetB-NG resulted in statistically significant reduction in lesion scores across all three experiments (**Fig. 9**). In experiment 3 (**Fig. 9C**) birds were challenged a week earlier than those in experiment 1 (**Fig. 9A**) and experiment 2 (**Fig. 9B**). We speculate that this may have been the cause of the higher lesion scores in some birds. Note that two birds died due to challenge during experiment 3 and were thusly given a lesion score of 6. These were the only two birds to die from C. *perfringens* challenge. Note that the Y-axis, while aligned, are not the same. Though the amount of PlcC-NetB-NG delivered to broiler birds cannot be accurately assessed, the resulting protection from challenge was almost identical to the PlcC-NetB fusion protein. We speculate that this result tells us that the dose was similar to that of PlcC-NetB. It also suggests that the glycosylation of ER-targeted fusion protein does not interfere with presentation of protective epitopes of PlcC and NetB.

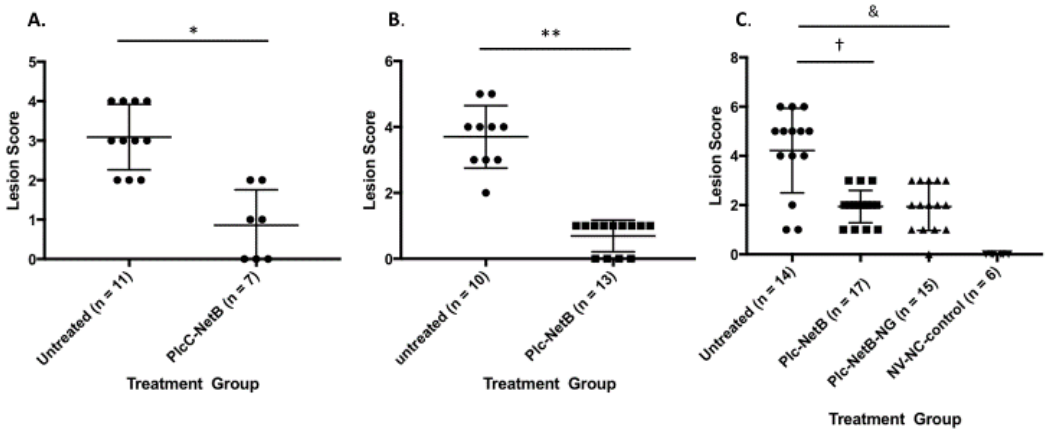

Figure 9. Intestinal Lesions Scores in Experiment 1 (**A**) Experiment 2 (**B**) and Experiment 3 (**C**). Plc-netB refers to Glycosylated variant while Plc-NetB-NG refers to non-glycosylated variant. Differences between immunized groups and controls are indicated.
* P < 0.001; ** P = 0.004; &*P* = 0,0004; †*P* , 0.0001

DISCUSSION

As nations and companies continue to reduce their reliance on antibiotics for protection against diseases such as NE the need for permanent alternatives will rise. Vaccination is a proven method for controlling disease and immunization with antigens based on toxins has routinely proven effective (Crouch *et al.*, 2010; Keyburn *et al.*, 2013; Yan *et al.*, 2013). However, immunizing with both the PlcC α-toxid and attenuated NetB provides better protection than when either antigen is used alone (Fernandes da Costa et al., 2016). Based on these observations, a novel fusion protein combining both PlcC and NetB was designed and shown to confer protection through immunization. Using a plant based expression system for the production of the PlcC-NetB fusion protein has many advantages including, high yields, ease of stockpiling, rapid and easy scaling, quick response to emerging pathogens, and lack of any animal components in the final vaccine product.

Production of a PlcC-NetB fusion protein was readily accomplished in N. *benthamiana* using a geminiviral replicon transient expression system. While other expression systems were unable to produce the fusion protein, namely salmonella, the plant expression system was shown to do so at a high capacity. High levels of protein production, 400 μg purified protein/g fresh leaf weight on average for both glycosylated and non-glycosylated fusion protein, were due to our use of the geminiviral replicon transient expression system which has been recently optimized and has undergone improvement since the production of the PlcC-NetB fusion protein (Cook et al., 2018; Diamos et al., 2016; Diamos and Mason, 2018). Due to these recent improvements our bean yellow dwarf virus geminiviral replicon transient expression system is now able to outproduce leading industry expression systems (Diamos et al., 2016; Diamos and Mason, 2018; Mason et al., 2014).

Acid precipitation (a process by which the isoelectric point of proteins, and their repulsive electrostatic forces, equal zero and results in protein aggregation) proved to be an effective clarification step, over other purification methods, as both the PlcC-NetB and PlcC-NetB-NG fusion proteins proved to be acid stable (Wilken *et al.*). This allowed for easy purification using metal affinity chromatography as the majority of plant protein contaminants were removed from solution while the fusion proteins remained soluble. While the metal affinity chromatography did purify the protein to a substantial degree, contaminants could be seen upon concentration of elutions (data not shown). However, contaminants were deemed not to be an issue for vaccination as they were in concentrations estimated to be 5-10%.

Since a plant expression system was used, native plant glycosylation of the ER-targeted fusion protein PlcC-NetB occurred. An illustration of this can be seen in both Figure 3 and Figure 4 as there are progressively higher bands above the lowest fusion protein band at 49 kDa only in the ER targeted variant. Glycosylation was a concern as an immune response could potentially be directed towards the glycans rather than the fusion protein itself (Maverakis et al., 2015; Schähs et al., 2007; Strasser et al., 2014), or the glycans could mask native protein epitopes. For this reason, a PlcC-NetB-NG fusion was pursued.

PlcC-NetB-NG proved to be troublesome as it precipitated after dialysis in 4˚C, a phenomenon that has been observed for rubisco, the most abundant plant protein contaminant. This may indicate that the glycoforms play a role in protein stability and solubility as PlcC-NetB did not have solubility issues under the same buffer, concentration, and purification conditions. It is also possible that the plant contaminants became insoluble and protein interactions between PlcC-NetB-NG and native plant

proteins lead to the non-glycosylated fusion protein becoming insoluble as well. PlcC-NetB Results indicate that, even with plant native glycosylation, the PlcC-NetB fusion promoted an anti-toxoid response. We speculate that the glycoforms could have possibly had an adjuvant effect as antibody titers for the fusion protein were greater than E. *coli* produced PlcC and NetB delivered simultaneously (Bosch and Schots, 2010). In any case, production of both the PlcC-NetB and PlcC-NetB-NG fusion proteins was found to be efficient on a small scale. Due to the nature of plant based transient expression systems, this process is readily scalable. Moreover, the fusion protein vaccine was found to be effective when injected into broiler birds as is discussed below.

In Experiments 1 and 2, three weekly injections with the fusion protein generated strong serum IgY responses (**Fig. 6**). Vaccinated birds had geometric mean titers of around 2,000 against recombinant NetB and PlcC proteins purified from E. *coli* (**Fig. 6**). Titers against the PlcC-NetB fusion protein produced in plants were around 8,000 in immunized birds. The difference in titers between single proteins and the fusion protein is speculated to be due to enhanced immune response to the fused protein, since PlcC and NetB are both present during infection, or responses against the carbohydrate moieties on the glycosylated protein leading to an enhanced immune response.

PlcC-NetB and PlcC-NetB-NG vaccination led to variable lymphocyte and splenocyte responses when tested with recombinant PlcC and NetB. The fusion protein elicited relatively weak responses in splenocytes (**Fig. 7**). This was seen in experiment 3 as well, for chickens vaccinated with either the glycosylated and non-glycosylated fusion protein (**Fig. 8**). However, no significant antigen-specific stimulation was detected in lymphocytes in experiment 3 except for PlcC in broilers that were vaccinated with an unknown amount of PlcC-NetB-NG (**Fig. 8**). This is an odd outlier and it is concerning

that no other antigen elicited a response as PlcC did. Background was also seen to be high in all these experiments. This may be explained by C. *perfringens* being native to the gut microbiome of chickens. We speculate that some amount of antibodies/immune cell recognition may always occur due to this fact. All broilers, across all experiments, when vaccinated with either PlcC-NetB or PlcC-NetB-NG did exhibit vaccination mediated protection from C. *perfringens* challenge (**Fig. 9**).

In Experiment 3 birds were immunized at 7 and 12 days of age. We speculate that this lead to a reduced immune response as the chicken adaptive immune system does not fully mature until approximately two weeks post-hatch, although humoral responses can be detected after immunization with a protein antigen at 12 days of age (Mast et al.). It has been shown that an adjuvanted NetB toxoid was ineffective when injected into day-of-hatch chicks (Mot et al., 2013). Therefore, it is possible that immunization at day 7 was ineffective and the immunization at day 12 was effectively the priming dose. It has been shown that immunization at 12 days of age, but not 7 days of age, can be boosted by injection at 29 days of age (Mast et al.). Even though the chickens were effectively not boosted, we infer that the birds were adequately primed since, despite the low serum antibody responses, they were protected against challenge (**Fig. 9C**).

We have demonstrated that this novel fusion protein produced in plants is not only efficient to produce but immunogenic and protective in chickens. However, we acknowledge that a more practical immunization approach is needed in order to have a positive impact on the broiler industry. There are several options for immunization on an industrial scale that will not only protect chickens, but will also promote farmer compliance. Immunization of parent stock is likely to induce maternal antibodies to protect chicks during the first few weeks of life. This approach has been demonstrated to

be effective with an α-toxoid (Crouch *et al.*, 2010). However, maternal antibodies are no longer circulating en mass by 2-4 weeks post hatch. Alternatively, protein antigens can be successfully delivered via in ovo injection with an adequate adjuvant. Delivery of the fusion protein via cornmeal could also be a cheap and effective mass delivery method for vaccine produced in corn seeds. The effectiveness of these approaches will be assessed in future studies.

FUTURE WORK

The first step in continuing the PlcC-NetB project would be to solve the issue of PlcC-NetB-NG solubility. While it has been hypothesized that the insolubility issue of the non-glycosylated protein could be due to its lack of glycans, paired with plant protein contaminants having an aggregating effect, it is worth pursuing due to greater protein volume per leaf gram produced than the glycosylated variant. One simple option would be to purify as has already been outlined and simply dialyze/store at 16˚C - 25˚C. However, this may become an issue if protein stability is suboptimal at temperatures above 4˚C or if the PlcC-NetB-NG aggregates at these temperatures in the presence of plant proteins as well.

An additional cause of the insolubility of PlcC-NetB-NG may be stripping of metal ions from both PlcC and NetB that are normally present. According to the crystal structure of PlcC it likely binds calcium though due to difficulties in creating the crystal structure cadmium was substituted (Naylor *et al.*, 1998). NetB has been shown to bind magnesium in its crystal structure (Yan *et al.*, 2013). These metal ions could be removed during the final dialysis step, which might affect protein structure and solubility. These ions could also be removed at another step in the purification process. Therefore, adding Ca^{2+} and Mg^{2+} in sub-mM concentrations to all steps may solve the issue of solubility.

Another option is using an additional purification method similar to those used industrially to both remove all protein contaminants and test the feasibility of the selected technique. Purification techniques include two dimensional gel electrophoresis, size exclusion chromatography, ion exchange chromatography, or immunoaffinity chromatography which would utilize the already present 6-His tag (Gräslund *et al.*, 2008). Any of these techniques could be applied to a small-scale purification and are

used, on one scale or another, industrially. While testing for the optimal large scale purification technique is important, performing a purification is key to discovering if the PlcC-NetB-NG fusion protein is becoming insoluble due to plant protein contaminants or due to self-interactions and aggregation.

While discovering the reason for PlcC-NetB-NG insolubility is important, using an isolated protein may not be the best option for vaccination. While this would be needed for in-ovo vaccination, and perhaps for maternal vaccination, targeting the PlcC-NetB fusion protein to seeds, namely from corn, could be the optimal approach for wide spread broiler bird vaccination. It has been shown that the use of the zein signal peptide routinely shuttles a target protein to protein bodies in N. *benthamiana* (Conley *et al.*, 2011; Hofbauer *et al.*, 2016). Zein signal peptides have also been shown to shuttle proteins to corn seeds where large amounts of the zein protein are generally directed (Nuccio, 2018). Since milled corn is regularly used to feed broiler birds it would be simple to supplement their diets with corn that contains PlcC-NetB. There are some drawbacks to this approach, the main being the use of transgenic corn rather than performing transient expression in N. *benthamiana*. Creation of transgenic plants requires a substantial amount of time and resource investment. Small scale experiments of this type therefore pose a bigger investment risk. That being said, there are many risks this approach avoids including: expensive industrial purification, farmer compliance issues, vaccine coverage, and ease of vaccine administration.

However, in ovo vaccination could also avoid most of the issues already mentioned with the exclusion of industrial protein purification. If in ovo vaccination is shown to work, the companies who hatch the chicks before they are shipped to farms could perform the vaccination easily. This would remove farmer compliance and vaccine

coverage issues completely. The main downside is the upfront investment for equipment, which is avoided in the corn-based delivery method, and cost of purifying the fusion protein.

Finally, additional research should be performed to optimize the time course of vaccine administration in any form as well as the optimal amount of antigen to be delivered. Further work should also be done to determine whether or not glycosylation of the PlcC-NetB fusion lead to higher antibody titers and greater protection against challenge. This is simple enough to perform in conjunction with testing the solubility of PlcC-NetB-NG if the non-glycosylated protein can be routinely purified while retaining solubility. If protein using zein as a signal peptide is produced its solubility and immunogenicity must also be tested in similar studies as those performed in this study.

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