Veterinary Microbiology 230 (2019) 7–13

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Immunization with subunits of a novel pilus produced by virulent *Clostridium perfringens* strains confers partial protection against necrotic enteritis in chickens

D. Lepp[a], S. Ojha[b], I. Mehdizadeh Gohari[b], B. Chakravarty[a], J.F. Prescott[b], J. Gong[a,*]

[a] Guelph Research and Development Centre, Agriculture and Agri-Food Canada, Guelph, Ontario N1G 5C9, Canada
[b] Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

ARTICLE INFO

Keywords:
Necrotic enteritis
Clostridium perfringens
Chickens
Pilus
Immunization

ABSTRACT

Necrotic enteritis (NE) is an economically important disease of broiler chickens that is caused primarily by *Clostridium perfringens* strains that produce the NetB toxin. It is controlled in North America principally through the application of in-feed antimicrobials, but alternative control methods, such as vaccination, are urgently needed. We previously identified a cluster of *C. perfringens* genes prevalent in disease-causing strains, denominated VR-10B, that is predicted to encode a pilus. The current study evaluated the ability of three predicted pilin structural subunits (CnaA, FimA, FimB) to protect against NE in two immunization studies. In the first study, young broiler chickens were immunized twice intramuscularly (i.m.) with CnaA or FimA, which resulted in only a weak serum antibody response, and no reduction in the severity of intestinal lesions following experimental challenge with *C. perfringens* strain CP1. In the second study, chickens were injected subcutaneously (s.c.) with CnaA, FimB, or a combination of all three proteins, on days 7, 14 and 19, which resulted in a marked antibody response specific to each antigen. Chickens immunized with either CnaA or FimB had significantly reduced NE lesion severity, whereas immunization with all three proteins in combination did not provide protection. Western blot experiments using serum from immunized birds were also performed, providing the first experimental evidence to suggest that this locus may in fact encode a functional pilus structure.

1. Introduction

Necrotic enteritis (NE) is a gastrointestinal disease of broiler chickens that occurs in both acute and subclinical forms, resulting in significant annual economic losses to the poultry industry (Wade and Keyburn, 2015). NE is principally caused by certain strains of *Clostridium perfringens* that produce the NetB pore-forming toxin, recently designated Type G strains (Rood et al., 2018). Under predisposing environmental conditions, these strains flourish in the small intestine where they eventually produce the characteristic intestinal lesions. In North America, NE has been primarily controlled through application of in-feed antibiotics, a practice that is increasingly discouraged due to concerns over the potential spread of antibiotic resistance. The use of growth-promoting antibiotics was banned in the European Union (EU) in 1999, and an accompanied increase in the incidence of NE was reported in some regions (Casewell et al., 2003). It is therefore urgent, from both a financial and public health perspective, to find non-antibiotic alternative approaches to control NE, the most promising of which is a vaccine against the disease.

The pathogenesis of NE is a complex, multifactorial process, with recent studies now just beginning to elucidate the full complement of virulence factors that enable certain *C. perfringens* strains to cause disease (Prescott et al., 2016). We previously identified, through comparative genomics, a number of genetic loci that are associated with NE-causing *C. perfringens* strains, and thus may potentially serve as vaccine candidates (Lepp et al., 2013, 2010). One such locus, designated VR-10B, is predicted to encode a novel pilus based on sequence similarity and synteny with Streptococcal pilus operons (Lepp et al., 2013), although this has yet to be experimentally demonstrated. VR-10B consists of six co-transcribed genes, predicted to encode three structural pilin subunits (CnaA, FimA, FimB), two sortases and a signal peptidase (Lepp et al., 2013; Wade et al., 2016). This locus was recently shown to be involved in binding to collagen (Wade et al., 2015), and hence renamed the collagen adhesion (CA) locus. Moreover, the CA locus was also demonstrated to be required for NE pathogenesis (Wade et al., 2016), presumptively via its role in adherence.

* Corresponding author.
E-mail address: joshua.gong@agr.gc.ca (J. Gong).

https://doi.org/10.1016/j.vetmic.2019.01.005
Received 27 April 2018; Received in revised form 5 January 2019; Accepted 6 January 2019

Table 1
Summary of immunization study designs.

Study	Antigens tested	Vaccination days	Injection site[1]	Serum collection days	CP1 challenge days	Day of euthanasia
1	CnaA, FimA	8, 20	i.m.	8, 20, 31	28, 29	31
2	CnaA, FimB, CnaA + FimA + FimB	7, 14, 19	s.c.	7, 19, 29	26, 27	29

1) i.m., intramuscular; s.c., subcutaneous.

In the current study, we investigated whether immunization with the three predicted pilin subunits was able to protect chickens that were experimentally infected with *C. perfringens* from developing NE. We also provide the first experimental evidence to suggest that the CA locus encodes a pilus structure.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

C. perfringens ATCC 13124 and Strain 13 (Shimizu et al., 2002) are both associated with human gas gangrene, while CP1 (Thompson et al., 2006), JGS4141 (Sawires and Songer, 2006) and JGS4120 (Cooper and Songer, 2009) are chicken NE isolates, although the latter was subsequently shown to be avirulent in an NE challenge model. *C. perfringens* strains were routinely grown anaerobically at 37 °C in Tryptone Glucose Yeast (TGY), Cooked Meat Medium (CMM), or Fluid Thioglycollate (FTG) broth (Difco). *E. coli* Top 10 and BL21 (DE3) (Millipore, Toronto, CA) were grown at 37 °C in Luria-Bertani (LB) broth or agar (Difco), supplemented with 50 μg/ml kanamycin as required.

2.2. Cloning, expression and purification of clostridial proteins

To purify the recombinant pilin proteins, coding regions for the three pilin subunits (CnaA, FimA, FimB), excluding the predicted N-terminal signal peptides and C-terminal transmembrane domains, were each codon-optimized and synthesized by Integrated DNA Technologies (Coralville, IA, USA). The synthesized products were separately cloned into the pET28a expression vector (Millipore, Etobicoke, ON, CAN) by In-Fusion (Takara Bio USA, Mountain View, CA, USA) cloning according to the manufacturer's instructions, sequence-verified, and then transformed into *E. coli* BL21 (DE3) cells. The final expressed truncated CnaA, FimA and FimB protein sequences correspond to amino acids 30–658, 27–324 and 30–184 of RefSeq proteins WP_057230734.1, WP_057230739.1 and WP_057230742.1, respectively. Transformed colonies were grown at 37 °C for 18 h with shaking in 1 L LB broth supplemented with 50 μg/ml kanamycin and 1 mM IPTG. The culture was pelleted and resuspended in 20 ml of binding buffer (20 mM $NaPO_4$, 0.5 M NaCl, 30 mM imidazole) and lysed by sonication for 10 min on ice (10 s pulses, 20 s pauses, 50% amplitude) using a Q500 sonicator (Qsonica, Newtown, CT, USA) equipped with a 2 mm diameter Microtip probe. The cell lysate was loaded onto an AKTAprime plus system and the His-tagged proteins were purified under native conditions on a HisTrap FF Crude column (GE Healthcare, Montreal, CA) using a gradient of 50–500 mM imidazole. One ml fractions were collected, and fractions exhibiting a 280 nm peak were pooled and concentrated with Pierce Protein Concentrators (9 K MWCO) (Fisher Scientific, Unionville, ON, CAN) and desalted using Zeba Spin 7 K MWCO desalting columns (Fisher Scientific). Quantitation of the purified proteins was performed using the BCA Protein Assay kit (Fisher) according to the manufacturer's instructions.

2.3. Immunization with recombinant proteins

Two vaccination studies were carried out with the purified His-tagged recombinant proteins. For each study, commercial day-old male White Plymouth Rock broiler chickens were randomly divided into experimental groups (n = 15–17) and housed in separate rooms within an isolation unit. Birds were fed an antibiotic-free starter ration containing 20% protein until experimental induction of NE. The first study consisted of three groups: 1) Adjuvant-only control, 2) CnaA-immunized and 3) FimA-immunized. Each bird was injected intramuscularly (i.m.) in the pectoral muscle with 200 μl PBS containing QuilA adjuvant (50 μg) and recombinant pilin protein (50 μg) at days 8 and 20, and birds were euthanized on day 31. Serum was collected from five birds from each group at days 8, 20 and 31 to assess antibody titers against the recombinant proteins by ELISA. The second study consisted of four groups: 1) Adjuvant-only control, 2) CnaA-immunized, 3) FimB-immunized and 4) CnaA, FimA and FimB-immunized (in combination). In this study, each bird was immunized s.c. with 50 μg of recombinant protein at days 7, 14 and 19, and birds were euthanized on day 29. For group 4, each bird was immunized with a total of 150 μg of recombinant protein (50 μg of each protein). Serum was collected at days 7, 19 and 29 for antibody titer determination.

2.4. Experimental NE disease model

The experiments with chickens and the conditions of their use were approved by the University of Guelph Animal Care Committee (AUP No. 1936) in accordance with the guidelines of the Canadian Council on Animal Care. Birds were fasted for 24 h, and then fed an antibiotic-free turkey starter ration (28% protein) containing *C. perfringens* CP1 culture for two days prior to euthanasia (Table 1). The infected ration was prepared daily in the morning and afternoon by mixing with *C. perfringens* CP1 culture, grown in fluid thioglycolate (FTG) (Difco) medium at 37 °C for 15 h or 24 h, respectively, at a 2:1 (v/w) ratio. Following euthanasia, the small intestine (duodenum to ileum) was examined grossly for NE lesions and scored blindly from 1 to 6 using the system described by Keyburn et al (Keyburn et al., 2008) as follows: 0, no gross lesions; 1, thin or friable walls; 2, focal necrosis or ulceration (1–5 foci); 3, focal necrosis or ulceration (6–15 foci); 4, focal necrosis or ulceration (16 or more foci); 5, patches of necrosis 2–3 cm long; 6, diffuse necrosis typical of field cases. Statistical differences between NE scores among groups were determined by one-way ANOVA followed by Tukey's post-hoc test.

2.5. Measurement of serum antibody levels by ELISA

C. perfringens recombinant proteins were diluted to 10 μg/ml in 50 mM carbonate/bicarbonate coating buffer, pH 9.6 and 100 μl was added to each well of a 96-well MaxiSorp Immuno plate (Fisher Scientific). Wells were coated for 1 h at 37 °C, followed by overnight at 4 °C, washed three times with wash buffer (phosphate-buffered saline [PBS] containing 0.05% Tween 20), and then blocked in wash buffer containing 1% bovine serum albumin (BSA) (Sigma) for 2 h at 37 °C. Two-fold serial dilutions of each serum sample diluted in wash buffer containing 1% BSA (1/64 to 1/65,536) were incubated in separate wells for 2 h at 37 °C and then washed three times in wash buffer. Wells were incubated with goat anti-chicken IgY horseradish peroxidase (HRP)-conjugated polyclonal antibody, diluted 1:5000 in wash buffer for 1 h at room temperature, and then washed three times in wash buffer. Substrate solution (0.2 mg/ml 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Sigma) in 1X ABTS buffer (Sigma)) was added to each well and incubated for 30 min at room temperature. After

the reaction was stopped with 0.5% sodium dodecyl sulfate (SDS), the absorbance was measured in a BioTek plate reader at 405 nm. Titers were calculated as the $\log_2$ value of the lowest serum dilution with an absorbance greater than twice that of the background wells, in which PBS containing 1% BSA was used in place of serum. Statistical differences between pre-immune and post-immune titers for each antigen among the different vaccination groups were determined by one-way ANOVA followed by Tukey's post-hoc test.

2.6. Extraction of C. perfringens cell surface proteins for pilus identification

Total surface protein was extracted from *C. perfringens* strains essentially as described by Chang et al (2013). Strains were grown overnight in TGY medium (3% tryptone, 2% glucose, 1% yeast extract) anaerobically at 37 °C, subcultured 1:100 into 10 ml TGY medium and grown to an OD_{600} ~ 1. Cells were pelleted at 6000 × g for 5 min and washed once in SMM buffer, pH 6.8 (0.5 M sucrose, 10 mM $MgCl_2$, 10 mM maleate). The bacterial pellet was resuspended in 1 ml SMM buffer, to which was added 60 µl of 5U/µl of mutanolysin (Sigma) in muramidase buffer (2 mM acetic acid, 48 mM sodium acetate) and 10 µl of 0.1 M phenylmethylsulfonyl fluoride (PMSF) (Sigma). Following at least 4 h incubation at 37 °C with constant rotation, protoplasts were pelleted at 20,000 × g for 5 min, and the supernatant fraction containing cell wall proteins was removed. Proteins were precipitated by addition of 81 µl 100% (w/v) trichloroacetic acid (TCA) (Sigma) per ml and incubation at 4 °C overnight. Following centrifugation at 20,000 × g at 4 °C for 20 min, the protein pellet was washed with acetone and slowly resuspended in 50 µl sample loading buffer (62.5 mM Tris − HCl, pH 6.8, 2% SDS, 20% glycerol, 4% β-mercaptoethanol, 3 M urea, 0.01% bromophenol blue) at room temperature for at least 15 min.

2.7. Western blotting

Surface protein extracts (5 µl) were loaded onto two Novex NuPAGE 3–8% Tris-Acetate gel (Fisher Scientific) and electrophoresed at 150 V for 1 h. One gel was used for staining with Biosafe Coomassie stain (BioRad), and the second gel was transferred onto a PVDF membrane at 350 V for 1 h in 1X transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.1% SDS). Chemiluminescent detection was performed with the WesternBreeze Chemiluminescent kit (Life Technologies) according to the manufacturer's instructions, using chicken anti-FimA serum (1:200) as primary antibody, and a goat anti-chicken IgY alkaline phosphatase (AP)-conjugated secondary antibody (1:2000). The serum used as primary Ab was obtained at sacrifice from a FimA-immunized chicken that subsequently exhibited a high anti-FimA titer.

3. Results

3.1. Purification of recombinant pilin subunit proteins

The full-length coding sequence of each pilin subunit was initially cloned into pET28a and expressed in *E. coli* BL21(DE3), but despite several attempts to optimize expression, very low amounts of expressed protein were obtained. All three pilin subunits were predicted to contain an N-terminal signal peptide and a C-terminal cell wall sorting signal (CWSS), the latter of which consists of a hydrophobic region that is ultimately cleaved during pilus assembly. Cloning of the pilin subunits with these signal sequences removed, based on their predicted cleavage sites, resulted in high levels of expression of the recombinant proteins (Fig. 1), presumably due to the increased solubility resulting from removal of the C-terminal hydrophobic regions.

3.2. Immunization with pilin proteins and serum antibody response

Two animal studies were carried out to assess the ability of the pilin subunits to protect against NE in an experimental disease model (Table 1). In the first study, two groups of birds were immunized i.m. twice with either CnaA or FimA recombinant proteins, and a third group with adjuvant alone. Serum IgY titers against CnaA and FimA were determined in five birds from each group before (d8) and after (d20 and 31) immunization, which revealed a variable response (Fig. 2A, B). The average serum response against CnaA in the CnaA-immunized group was significantly higher only at d31, compared to the pre-immune birds (d8), but the variability among birds was high (Fig. 2A). In the FimA-immunized group, the average FimA response did not significantly increase at either time point after immunization, although two of the three birds exhibited a strong response by d31 (Fig. 2B). These results indicated that the antigen preparations were immunogenic, but other factors were limiting antigen delivery in the majority of birds.

The design of the second study was therefore modified to increase the likelihood of successfully delivering the antigens and eliciting an antibody response. Birds were immunized s.c., instead of i.m., as this route has proven reliable in past studies, and a third immunization was added. Three immunization groups were included in this study, in addition to the adjuvant-only control: 1) CnaA, 2) FimB, and 3) a combination of CnaA, FimA and FimB. A significant and robust serum antibody response was observed at both days 19 and 29 in all of the immunized groups (with the exception of FimB at d19) compared to the pre-immune controls, and the magnitude of response was also greater than in the first study (Fig. 3A–C). To confirm that this response was specific to the pilin proteins themselves, and not to the His-tag regions, we also measured the day 29 antibody response to pilin proteins other than those against which the birds had been immunized. The response to the heterologous antigens was negligible in each case (Fig. 3A–C).

3.3. Protection against experimental induction of NE

Protection of immunized birds against NE was assessed by gross pathology of the small intestinal mucosa at necropsy, following two days of in-feed challenge with virulent *C. perfringens* strain CP1. In study 1, no significant differences in the average NE lesion scores were observed between the immunized and adjuvant-only control groups, with all groups exhibiting substantial disease (Fig. 2C). The average NE scores for the adjuvant-only control, CnaA-immunized and FimA-immunized groups were 3.1, 3.0 and 3.3, respectively.

In the second study, however, both the CnaA- and FimB-immunized groups had significantly (Tukey's; $p = 0.02$ and $p = 0.004$, respectively) lower NE scores (2 and 2.06, respectively) compared to the adjuvant-only control (3.75), indicating that these antigens partially protected against the onset of NE (Fig. 3D). Immunization with the three proteins in combination, however, did not significantly reduce the severity of disease (average NE score = 3.7).

3.4. Detection of high molecular weight (HMW) pilus proteins in C. perfringens isolates

The CA locus has been predicted to encode a pilus, based on evidence from *in silico* analyses, though this has yet to be shown experimentally. Western blotting of *C. perfringens* surface proteins was performed, using serum from a FimA-immunized bird as primary antibody, to: 1) determine if *C. perfringens* strains that carry the CA locus produce of a high-molecular weight (HMW) polymer indicative of a pilus, and 2) verify the specificity of the chicken serum antibody response in the FimA-immunized birds. Surface proteins were extracted from three poultry (CP1, JGS4141 and JGS4120) and two non-poultry (Strain 13, ATCC13124) *C. perfringens* strains and subjected to Western blot analysis. The two strains known to carry the CA locus (CP1 and JGS4141), as determined previously by both microarray analysis and PCR (Lepp et al., 2013), produced a HMW ladder-like pattern consistent with that produced by other Gram-positive pilus-producing species (Mora et al., 2005; Ton-That and Schneewind, 2003), while the remaining three CA-

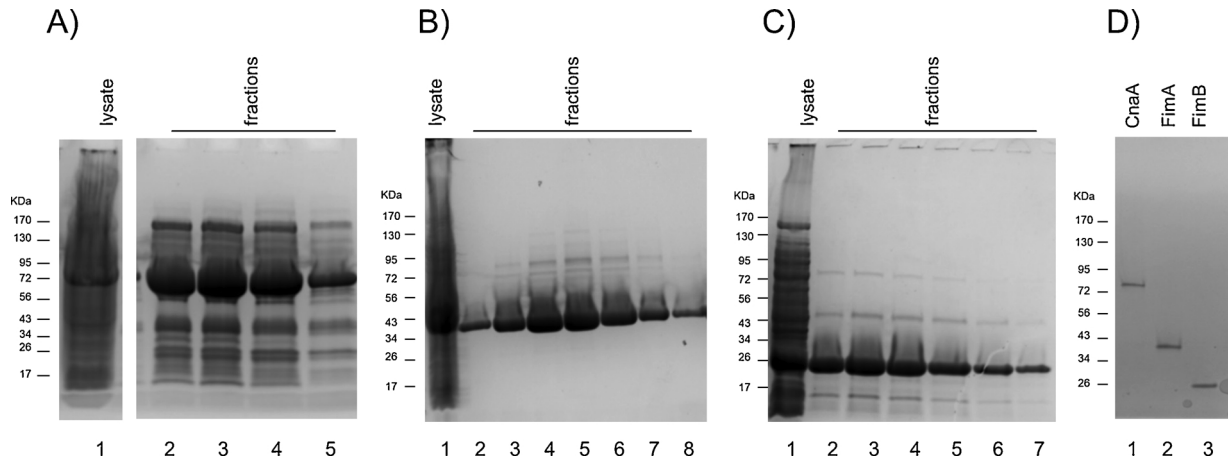

Fig. 1. Purification of recombinant pilus subunit proteins. His-tagged recombinant proteins were eluted from a HisTrap FF column using a linear imidazole gradient and fractions exhibiting absorbance at 280 nm were collected. Ten µl of crude lysate (lane 1) and fractions (lanes 2–8) of A) CnaA, B) FimA or C) FimB were visualized by SDS-PAGE and Coomassie staining. D) Pooled fractions (100 ng) of each purified protein following concentration and desalting.

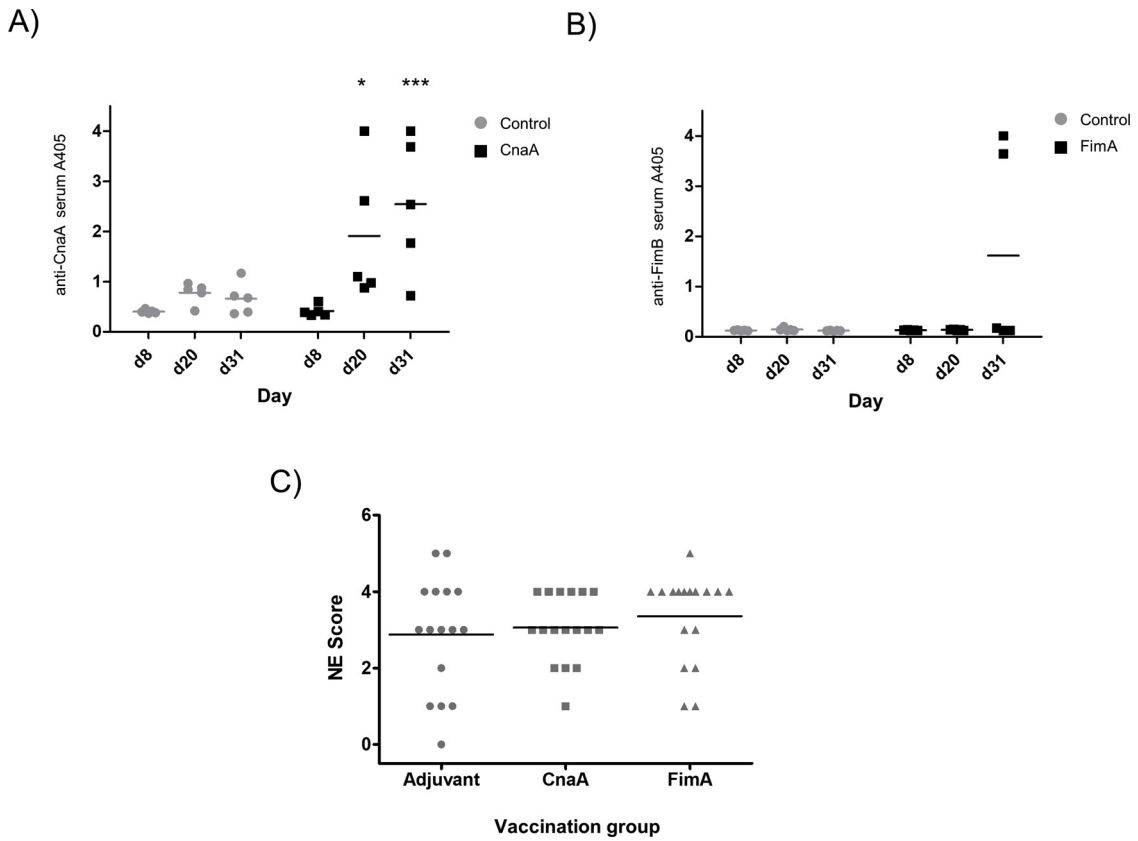

Fig. 2. Study 1 IgY response and NE scores. Serum IgY response against A) CnaA or B) FimA recombinant proteins from birds immunized i.m. with 50 µg adjuvant alone (grey circle), CnaA (black square) or FimA (black square) at days 8 and 20. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's) versus the pre-immune sample (d 8) from each group. Abs405 measurements of 1/1024 dilutions are shown as this dilution provided maximum discrimination between the treatment groups. C) NE scores from birds immunized with adjuvant alone, CnaA, or FimA, following two days of in-feed challenge with *C. perfringens* CP1. Each dot represents a single individual and horizontal lines represent means.

negative strains did not. Several low molecular weight (LMW) bands were also observed in each strain, regardless of CA locus carriage. Western blots probed with serum from non-vaccinated birds exhibited similar LMW banding patterns (data not shown), suggesting that these bands likely result from non-specific antibody reactions. The lack of a 36 kDa band corresponding to the FimA monomer was anticipated, as the monomeric protein is located intracellularly, whereas isolated surface-associated proteins were examined in this experiment.

4. Discussion

NE imposes a significant economic burden on poultry producers (Wade and Keyburn, 2015) and is currently the primary target of in-feed antibiotics; the development of a vaccine to control the disease would thus have an enormous positive impact on the industry. The current work sought to evaluate three putative *C. perfringens* pilus subunits (CnaA, FimA and FimB) as protective antigens against NE in two vaccination studies. In an initial study, in which CnaA and FimA

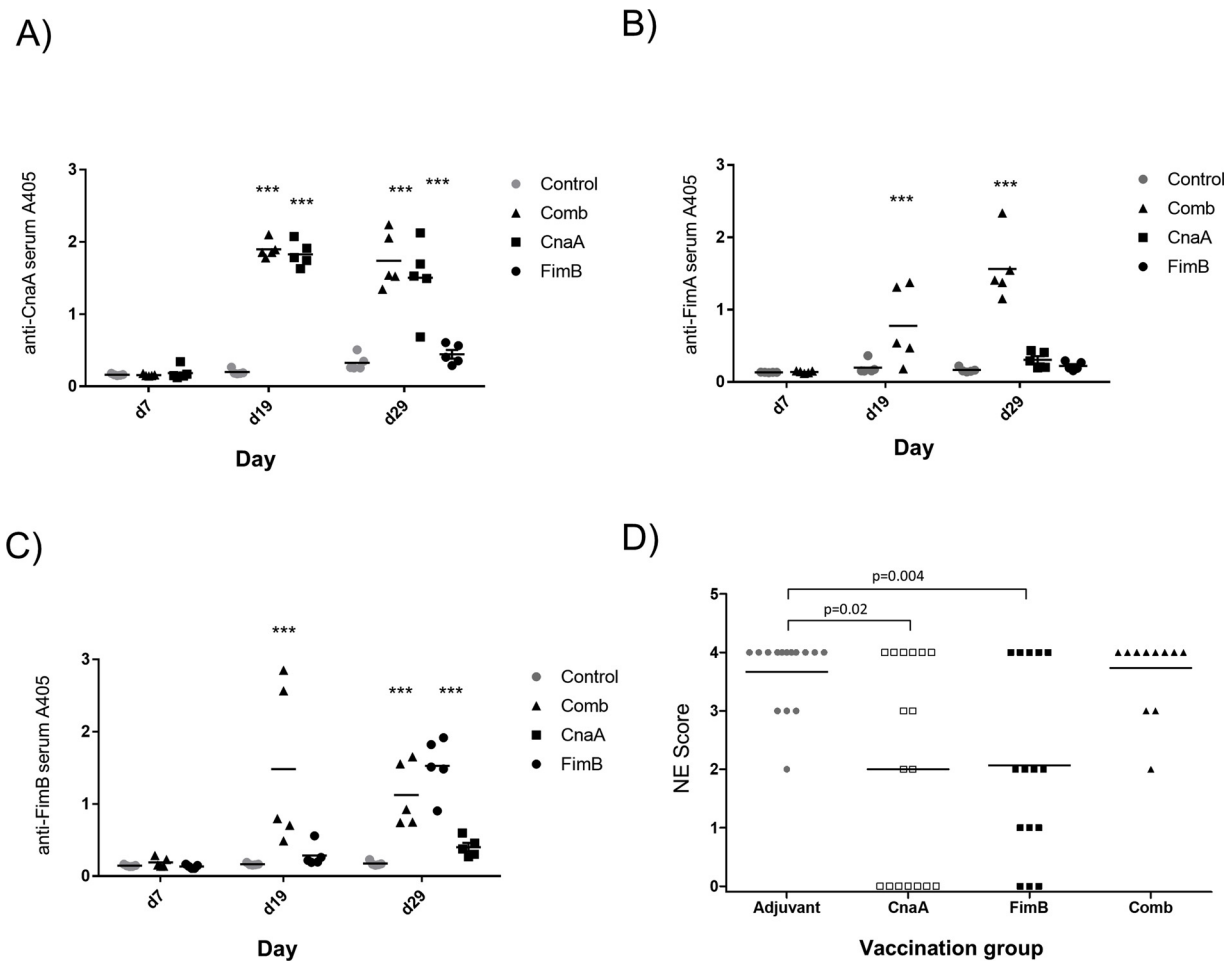

Fig. 3. Study 2 IgY response and NE scores. Serum IgY response against A) CnaA, B) FimA and C) FimB recombinant proteins from birds immunized s.c. with 50 µg adjuvant alone (grey circles), CnaA (black squares), FimB (black circles) or a combination of CnaA, FimA and FimB (black triangles) at days 7, 14 and 19. ***$p < 0.001$ (Tukey's) vs. the pre-immune sample (d 7) from each group. D) NE scores from birds immunized with adjuvant alone, CnaA, FimB or a combination of CnaA, FimA and FimB (Comb), followed by two days in-feed challenge with *C. perfringens* CP1. Significant differences in lesion scores between the adjuvant control and vaccination groups, as determined by Tukey's test, are indicated. Each dot represents a single individual and horizontal lines represent means.

were assessed, no reduction in disease was observed in the immunized birds following challenge with *C. perfringens* CP1. There was a correspondingly poor and inconsistent serum antibody response against these two antigens in the five birds examined per group. In particular, on day 31, one bird in the CnaA group did not exhibit any response and the other birds varied substantially, while three of the five birds in the FimA group did not exhibit any response. Extrapolated to the entire group, this uneven response likely resulted in the poor average protection observed, which we hypothesized was a result of inconsistent antigen delivery. A second study, adding a third vaccination day to improve the likelihood of delivery, resulted in a significant increase in IgY titers against each antigen compared to the pre-immune serum. More importantly, a significant decrease in NE score was observed in the CnaA- and FimB-immunized groups compared to the adjuvant-only control, indicating that these two proteins conferred partial protection against NE. It should be noted that this second study was performed only once and requires repetition before further studies are carried out.

A combination of all three subunits did not confer protection, despite eliciting a strong serum antibody response against all three antigens. The explanation for this result is unclear, although the current study did not examine the production of secretory IgA (sIgA), which is considered the primary effector of the mucosal immune response. Previous studies have shown that serum IgY and sIgA titres do not necessarily correlate (Kulkarni et al., 2007), and it is therefore possible that the "combination" group had a poor sIgA response, despite the high observed IgY response.

A wide range of *C. perfringens* recombinant proteins have been evaluated previously as vaccine candidates (Cooper et al., 2009; Fernandes da Costa et al., 2013; Jang et al., 2012; Jiang et al., 2009; Keyburn et al., 2013; Kulkarni et al., 2007; Lovland et al., 2004), and while many have conferred partial protection against NE, complete protection has yet to be demonstrated. In the current study, we were also only able to confer partial protection with the two pilus subunits. The severity of disease induced in the current study was relatively high in relation to many previous NE vaccination studies, with average NE scores in the adjuvant-only control group of 3.1 and 3.75 in studies 1 and 2, respectively. In comparison, a recent investigation using models of both "mild" and "severe" infection produced respective average lesion scores of 0.76 and 1.70 in the adjuvant-only control group (Fernandes da Costa et al., 2016), while other studies have reported scores of 1.07 (Fernandes da Costa et al., 2013), 2.6 (Keyburn et al., 2013) and 3.0 (Keyburn et al., 2013) in control groups, using the same scoring system as the present study (Fernandes da Costa et al., 2016). It is possible, therefore, that a milder challenge would result in stronger perceived protection. The vaccination scheme used in the current study would not be appropriate for field application, and thus alternative delivery routes, such as maternal, *in ovo* or oral vaccination, would need to be evaluated.

Despite eliciting only partial protection, the pilus antigens possess several attributes that make them attractive candidates for further

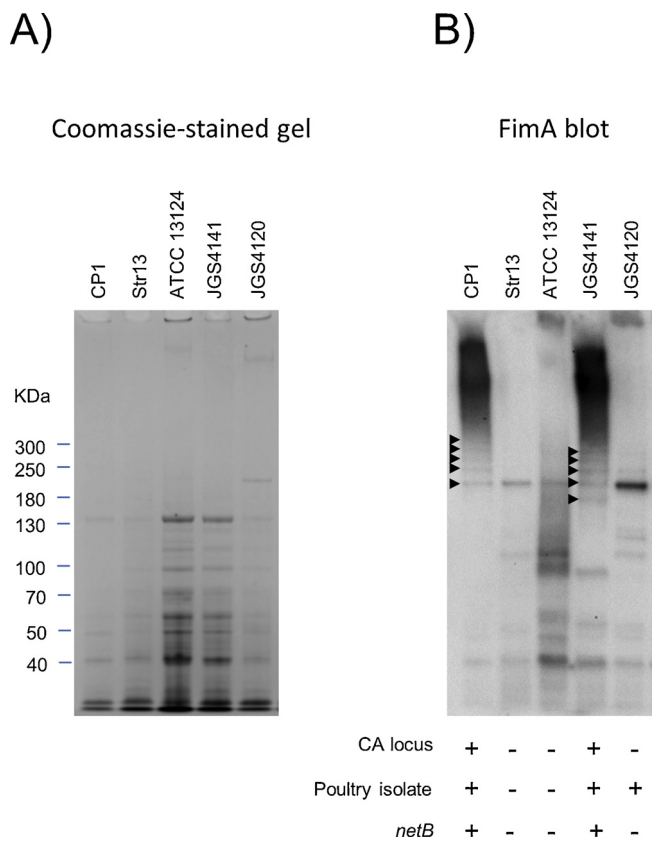

Fig. 4. Western blot analysis of surface protein extracts from different *C. perfringens* isolates using serum from a FimA-immunized chicken. Surface proteins isolated from 10 ml of log-phase culture grown in TGY were precipitated and resuspended in 50ul sample loading buffer. Five µl of each sample was loaded onto two 3–8% Tris-acetate SDS gels, which were either A) stained with BioSafe Coomassie (BioRad), or B) transferred to a PVDF membrane and detected using anti-FimA chicken serum. Below each lane is indicated whether the isolate possesses the CA locus and netB, as determined by PCR, if the source of the isolate was a chicken. The arrowheads highlight the location of the HMW ladder-like pattern.

study. Most notably, Gram-positive pili are covalently-linked to the bacterial cell surface, and therefore are susceptible to targeting by antibodies and host immune cells. In addition, the CA locus is predominantly associated with disease-causing strains (Lepp et al., 2013; Wade et al., 2016), and has been shown to be essential for NE pathogenesis (Wade et al., 2016). A subset of virulent strains do not carry the CA locus, and therefore a pilus-based vaccine would presumably be ineffective against these isolates. The previously demonstrated collagen-binding ability of CnaA, together with the reduced ability of a *cnaA*-null mutant to colonize chickens (Wade et al., 2016), suggests that this putative tip adhesin mediates adherence during NE pathogenesis. We hypothesize that the immune response induced by the pilus subunits interferes with this adherence, thereby blocking colonization either before or after cellular damage is induced.

This study is the first to provide experimental evidence that *C. perfringens* may produce a sortase-dependent pilus (Fig. 4B). One other pilus has thus far been identified in *C. perfringens*: a Type IV pilus involved in gliding motility and biofilm formation (Varga et al., 2008). Sortase-dependent pili are composed of variable numbers of covalently-linked pilin subunits, thus giving rise to a characteristic HMW ladder-like pattern upon visualization by Western blotting. This indicative pattern was observed in *C. perfringens* isolates that carry the CA locus, but absent from those that do not, suggesting that this locus may encode a functional pilus. Some additional lower MW bands were also observed in both the CA-positive and CA-negative strains, which likely result from reactions with other antibodies present in the crude chicken serum used as primary antibody in these experiments. Additional studies, such as transmission electron microscopy of immuno-gold labelled cells, are still needed to conclusively demonstrate the production of this novel pilus.

In summary, this study demonstrates the potential of recombinant *C. perfringens* pilus subunits to protect against NE, and provides the first experimental evidence for the production of a pilus by the CA locus. Further studies are required to enhance protection, as well as to understand more precisely how this pilus contributes to NE pathogenesis.

Conflict of interest

None.

Funding

This research was supported by Agriculture and Agri-Food Canada and Canadian Poultry Research Council through the Poultry Cluster II Program (Project # J-000263, Dr. Joshua Gong).

References

Casewell, M., Friis, C., Marco, E., McMullin, P., Phillips, I., 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52, 159–161.

Chang, C., Huang, I.-H., Hendrickx, A.P.A., Ton-That, H., 2013. Visualization of gram-positive Bacterial Pili. In: Delcour, H.A. (Ed.), Bacterial Cell Surfaces: Methods and Protocols. Humana Press, Totowa, NJ, pp. 77–95.

Cooper, K.K., Songer, J.G., 2009. Virulence of *Clostridium perfringens* in an experimental model of poultry necrotic enteritis. Vet. Microbiol. 142, 323–328.

Cooper, K.K., Trinh, H.T., Songer, J.G., 2009. Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with Clostridium perfringens. Vet. Microbiol. 133, 92–97.

Fernandes da Costa, S.P., Mot, D., Bokori-Brown, M., Savva, C.G., Basak, A.K., Van Immerseel, F., Titball, R.W., 2013. Protection against avian necrotic enteritis after immunisation with NetB genetic or formaldehyde toxoids. Vaccine 37, 4003–4008.

Fernandes da Costa, S.P., Mot, D., Geeraerts, S., Bokori-Brown, M., Immerseel, F.V., Titball, R.W., 2016. Variable protection against experimental broiler necrotic enteritis after immunisation with the C-terminal fragment of *Clostridium perfringens* alpha-toxin and a non-toxic NetB variant. Avian Pathol. 45, 1–26.

Jang, S.I., Lillehoj, H.S., Lee, S.H., Lee, K.W., Lillehoj, E.P., Hong, Y.H., An, D.J., Jeong, W., Chun, J.E., Bertrand, F., Dupuis, L., Deville, S., Arous, J.B., 2012. Vaccination with *Clostridium perfringens* recombinant proteins in combination with Montanide ISA 71 VG adjuvant increases protection against experimental necrotic enteritis in commercial broiler chickens. Vaccine 30, 5401–5406.

Jiang, Y., Kulkarni, R.R., Parreira, V.R., Prescott, J.F., 2009. Immunization of broiler chickens against *Clostridium perfringens* induced necrotic enteritis using purified recombinant immunogenic proteins. Avian Dis. 53, 409–415.

Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Di Rubbo, A., Rood, J.I., Moore, R.J., 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog. 4, e26.

Keyburn, A.L., Portela, R.W., Ford, M.E., Bannam, T.L., Yan, X.X., Rood, J.I., Moore, R.J., 2013. Maternal immunization with vaccines containing recombinant NetB toxin partially protects progeny chickens from necrotic enteritis. Vet. Res. 44, 108.

Kulkarni, R.R., Parreira, V.R., Sharif, S., Prescott, J.F., 2007. Immunization of broiler chickens against *Clostridium perfringens*-induced necrotic enteritis. Clin. Vacc. Immunol. 14, 1070–1077.

Lepp, D., Roxas, B., Parreira, V.R., Marri, P.R., Rosey, E.L., Gong, J., Songer, J.G., Vedantam, G., Prescott, J.F., 2010. Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. PLoS One 5, e10795.

Lepp, D., Gong, J., Songer, J.G., Boerlin, P., Parreira, V.R., Prescott, J.F., 2013. Identification of accessory genome regions in poultry *Clostridium perfringens* isolates carrying the netB plasmid. J. Bacteriol. 195, 1152–1166.

Lovland, A., Kaldhusdal, M., Redhead, K., Skjerve, E., Lillehaug, A., 2004. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Pathol. 33, 83–92.

Mora, M., Bensi, G., Capo, S., Falugi, F., Zingaretti, C., Manetti, A.G., Maggi, T., Taddei, A.R., Grandi, G., Telford, J.L., 2005. Group A Streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc. Natl. Acad. Sci. U. S. A. 102, 15641–15646.

Prescott, J.F., Parreira, V.R., Mehdizadeh Gohari, I., Lepp, D., Gong, J., 2016. The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know. Rev. Avian Pathol. : J. W.V.P.A 45, 1–21.

Rood, J.I., Adams, V., Lacey, J., Lyras, D., McClane, B.A., Melville, S.B., Moore, R.J., Popoff, M.R., Sarker, M.R., Songer, J.G., Uzal, F.A., Van Immerseel, F., 2018. Expansion of the Clostridium perfringens toxin-based typing scheme. Anaerobe In Press.

Sawires, Y.S., Songer, J.G., 2006. *Clostridium perfringens*: insight into virulence evolution and population structure. Anaerobe 12, 23–43.

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S., Hayashi, H., 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. U. S. A. 99, 996–1001.

Thompson, D.R., Parreira, V.R., Kulkarni, R.R., Prescott, J.F., 2006. Live attenuated vaccine-based control of necrotic enteritis of broiler chickens. Vet. Microbiol. 113, 25–34.

Ton-That, H., Schneewind, O., 2003. Assembly of pili on the surface of Corynebacterium diphtheriae. Mol. Microbiol. 50, 1429–1438.

Varga, J.J., Therit, B., Melville, S.B., 2008. Type IV Pili and the CcpA protein are needed for maximal biofilm formation by the gram-positive anaerobic pathogen *Clostridium perfringens*. Infect. Immun. 76, 4944–4951.

Wade, B., Keyburn, A.L., 2015. The true cost of necrotic enteritis. World Poultry 9, 2.

Wade, B., Keyburn, A.L., Seemann, T., Rood, J.I., Moore, R.J., 2015. Binding of *Clostridium perfringens* to collagen correlates with the ability to cause necrotic enteritis in chickens. Vet. Microbiol. 180, 299–303.

Wade, B., Keyburn, A.L., Haring, V., Ford, M., Rood, J.I., Moore, R.J., 2016. The adherent abilities of Clostridium perfringens strains are critical for the pathogenesis of avian necrotic enteritis. Vet. Microbiol. 197, 53–61.