Preliminary studies on development of a novel subunit vaccine targeting *Clostridium perfringens* mucolytic enzymes for the control of necrotic enteritis in broilers

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ABSTRACT Necrotic enteritis (**NE**) is a pervasive enteric disease responsible for large scale economic losses within the global poultry industry. The etiologic agent of NE is *Clostridium perfringens* (**CP**), an opportunistic pathogen that utilizes numerous extracellular toxins and glycoside hydrolases (**GH**) as key virulence and nutrient acquisition factors. Notably, some GH, mucinases, degrade components of mucin in the gastrointestinal tract as an energy source. Targeting this mechanism may serve to reduce the incidence of disease associated with CP. Two experiments were completed that evaluated mucinase vaccine targets sourced from conserved peptide sequences of carbohydrate binding module 32 of CP mucinases. In experiment 1, 37 antigen peptides were synthetically generated and used to produce hyper-immune sera, which was then evaluated for ability to obstruct CP growth in vitro. Total CFU of CP were measured at 4, 6, and 8 h incubation to determine growth rate. Peptides 4, 5, 22, 24, and 30 were selected for further in vivo testing based on conservation or the ability to inhibit CP growth by over 50% at 6 and 8 h. In experiment 2, the aforementioned peptides were conjugated to an agonistic, CD40-targetting antibody and evaluated in vivo. Broilers were given an *Eimeria maxima* and CP in order to induce NE and assess vaccine efficacy. Treatments included a non-vaccinated non-inoculated control, non-vaccinated inoculated control (**NVIC**), vaccination with peptide 4, 5, 22, 24, or 30 (VP4-VP30), or a combination of all 5 peptides (MC). There was a significant increase ($P < 0.05$) in the percent change in BWG relative to NVIC for vaccination with peptide 22 and MC of 18.54 and 17.43%, respectively. MC vaccinated group had the lowest lesions with a mean score of 0.63 ± 0.18. These results suggest the MC combination was the most successful in alleviating overall performance losses associated with NE-infected broilers and encourage future testing of MC in the development of an NE vaccine.

Key words: necrotic enteritis, *Clostridium perfringens*, mucinase, immunization, broiler

INTRODUCTION

Clostridium is a genus of Gram-positive bacteria which includes several significant animal and human pathogens capable of causing numerous enteric infections. *Clostridium perfringens* (**CP**) is the most critical clostridial pathogen of poultry and is recognized as the primary etiological agent of necrotic enteritis (**NE**). This multifactorial disease has a detrimental impact on intestinal integrity, performance, and can result in substantial economic losses linked to increased flock morbidity and mortality (Timbermont et al., 2010; Mot et al., 2014). When certain predisposing factors such as coccidiosis or improper nutrition affect a flock, the immune status, microbiota, and intestinal environment may be altered or damaged in a manner that creates a predisposing environment ideal for the colonization, proliferation, and prolonged toxin production of CP. (Williams, 2005; Shojadoost et al., 2012; Rodgers et al., 2015). The capacity to generate a minimum of 16 exotoxins alongside numerous additional extracellular enzymes is key to the virulence of CP and responsible for the affiliated lesions and symptoms of disease (Li et al., 2013).

Traditionally, NE infections in poultry have been prevented and treated through feed supplementation with antibiotics. In response to the emergence of antibiotic-resistant pathogens that threaten human and animal health, this practice has been phased out of livestock production (Dahiya et al., 2006). Production systems that control coccidiosis via live oocyst vaccination are

particularly susceptible to NE, and control of this disease is especially limited in antibiotic-free production systems (Shirley et al., 2005). Similarly, non-antibiotic strategies available for prevention of NE, such as feed additives and vaccines, are limited in number and efficacy (Dahiya et al., 2006; Timbermont et al., 2010). Vaccination could be a promising alternative control strategy regarding NE, particularly subunit vaccines, which combine select antigens or portions of antigens into a single vector.

Utilizing CP toxins as immunogens is one method of increasing antibodies and resistance to CP infection. In addition to toxins, CP produces a range of extracellular enzymes that could serve as valuable immunogens for vaccination, namely glycoside hydrolases (**GH**) like glucosaminidases and galactosidases (mucinases) which are involved in the breakdown of mucin in the host intestine (Ficko-Blean and Boraston, 2009). Degradation of mucin provides CP with an energy source while simultaneously decreasing the protective mucus layer in the gastrointestinal tract (**GIT**). Jiang and coauthors (2009) investigated vaccination with whole endo-β-N-acetylglucosaminidase (**enβNGlcase**) as protection against NE. The vaccine was able to reduce lesion scores (**LS**) associated with 1 of 2 CP inoculum doses. The authors tested a second enzyme that was successful against both strains of CP, but noted that enzyme production in quantities sufficient for vaccination was difficult. In addition to enβNGlcase, endo-β-galactosidase (**enβGalase**) has been identified and characterized in CP and is well-conserved amongst strains. While this enzyme is not uncommon amongst bacteria, enβGalase of CP appears to have a broader range of activity than typically found in nonpathogens (Ashida et al., 2008; Koutsioulis et al., 2008)

Various families of polysaccharide-recognizing modules known as carbohydrate-binding modules (**CBM**) can be found within GH. Though the CBM are not enzymatically active, they promote the anchoring of the enzyme in the proper orientation and proximity to carbohydrate substrates. Numerous families of CBMs have been characterized, but members of the CBM32 family are particularly abundant in the GH of CP (Ficko-Blean and Boraston, 2009). A search for conserved domains on NCBI revealed GH family 16, and active sites are known. Records for CP GH16 are present in both CAZy and UniProt, which also show that GH16 possesses CBM32. Multiple pathogens have been reported to possess mucolytic enzymes with CBM32 as a key virulence factor, therefore this domain may be a key antigen for vaccine production.

Conserved portions or peptides of CBM32 in mucinases were targeted to produce antibodies against a variety of CP strains. The studies presented here describe selection of immunogenic target antigens against CBM32. If antibodies in generated hyper-immune serum could bind to regions of GH enzymes important for activity in vitro, the ability of CP to break down mucin as an energy source would be inhibited, and a reduction in CP growth would be observed. Following the selection of the optimal antigens and incorporation into an antibody-guided vaccine complex, this strategy was further evaluated in vivo via an *Eimeria maxima* (**EM**) and CP-induced NE model.

MATERIALS AND METHODS

Mucinase Peptide Selection

Peptide sequences were chosen using Immune Epitope Database and Analysis Resource open-source predictive algorithms to identify potential B- and T-cell epitopes (Vita et al., 2015). Linear stretches of the GH and CBM32 domains that appeared to be immunogenic and simple to synthesize were selected with a preference towards hydrophilic linear segments with a slight bend for B-cell epitopes. Several of the chosen peptides, however, were hydrophobic segments with a slight bend for towards T-cell epitopes. Selection using these criteria theoretically increased the likelihood of the peptide being immunogenic. A total of 37 peptide antigens were selected for testing (Table 1).

Immunization

Hyper-immune sera was generated against selected CBM32 antigenic regions using the methods previously described by Chen et al. (2012). The immunization complex incorporated mouse anti-chicken CD40 antibody 2C5 for immune-stimulation. The biotinylated CD40 receptor-targeting antibodies were joined to select biotinylated peptides using streptavidin as a scaffold. Antibody-guided complexes were stoichiometrically produced to contain a molar ratio of two antibodies and two (identical) peptides. Each immunization complex was administered to two broilers each via subcutaneous (**SC**) injection on d7 (prime) and d14 (boost). Sera samples were collected one week after each injection. Briefly, serum samples were diluted in phosphate buffered saline at a ratio of 1:100 and incubated overnight on an orbital shaker at 4°C before the addition of peroxidase-conjugated rabbit anti-chicken IgG secondary antibody was applied at a 1:5000 dilution. Levels of peptide-specific IgG in each sample were determined according previously published literature (Chen et al., 2012; Vuong et al., 2017) and presented below (Figure 1).

In vitro Growth Inhibition Assay

A total of 74 hyper-immune sera were developed against 37 different peptides. Each serum was tested in replicates of 5, with 3 replicate experiments, and all data are presented as a composite of replications. In sterile borosilicate glass tubes (12 × 75 mm; Fisher Scientific, Hampton, NH), 10 μL of 1 × 10^6 CFU/mL of CP was added to 1 mL of 0.5X Luria-Bertani broth (Becton and Dickenson, Sparks, MD) containing 1.00%

Table 1. Comprehensive list of mucinase peptides tested with the growth inhibition assay. Peptides that were selected for further testing generated antibodies that were able to decrease the growth of CP by more than 50% at either or both time points (6 and 8 h).

Peptide	Peptide sequence	Accession #	6 h Reduction (%)	8 h Reduction (%)
1	EHPDVGNEGLAK	BAB80572.1	201.90	−12.45
2	NPVEELPQS	BAB80572.1	103.80	−19.74
3	PRSGAKNGNIT	BAB80572.1	−70.69	NS
4*	SEDGNNFRK	BAB80572.1	−75.51	−55.00
5*	WDDSGSLKTLKFNS	BAB80572.1	−77.30	−52.88
6	SSGLVPRGSH	BAB80572.1	−61.67	NS
7	SNSQSGNPLSNITDGDLSSLWISDNGAMP	BAB80572.1	−36.40	−31.42
8	DMTSNTEDNKKSYNIPVKKE	BAB80572.1	−67.56	−35.36
9	KAPGGSFDQAW	BAB80572.1	308.07	51.00
10	SNNSEANNLRDGNENTLWVPGQEEEKSV	BAB80572.1	143.75	52.99
11	SKGNSPLKYS	BAB80572.1	24.73	−0.29
12	SNDGTEWTKIVDENNNEENKAV	BAB80572.1	38.54	5.32
13	NSENVKIGEIK	BAB80572.1	−15.49	NS
14	PRGSHMASNPSLIRSESWQVYEGNE	P26831.2	85.68	20.93
15	DKTGAPAGKDVIEESFETP	P26831.2	11.69	24.89
16	SDELENAGNKEN	P26831.2	22.18	76.55
17	KGIDPFTNPR	BAB80940.1	−23.05	52.83
18	SDGDMNTFWHSKWSSPAHEGPHH	BAB80940.1	3.31	139.38
19	PRQDSKNGR	BAB80940.1	395.72	251.11
20	SDQANGRGKF	BAB80940.1	18.84	121.41
21	KKAGSEFALDSSK	ABG84084.1	−64.96	−39.29
22*	SEADRDYKENAVDGDENT	ABG84084.1	−81.24	−72.14
23	QAADKLP	ABG84084.1	−37.27	78.90
24*	PRQNSRNGH	ABG84084.1	−77.52	−66.01
25	DNENWTEVR	ABG84084.1	5.98	1.52
26	NLEVNEAGNALANRGYNP	ABG84084.1	−60.91	−16.11
27	GDTNNKY	ABG84084.1	870.81	16.79
28	SSEHPNIGSEG	ABG83298.1	−57.81	−44.69
29	IDGDESTIWHTKWSP	ABG83298.1	−59.84	−81.50
30*	RQDALNGSISKYE	ABG83298.1	−31.56	−88.98
31	DGENFTKVLEGD	ABG83298.1	−16.94	−64.81
32	KFDPVEATHVR	ABG83298.1	853.03	0.23
33	VPGQEEEKSVTFDLSKEKDISAIDIVSK	BAB80572.1	70.61	−43.35
34	TKYNPVEELPQSITLELGGSYEINKFTYLPRSGAKNG	BAB80572.1	288.89	4.68
35	YKTLNGDTSLAGEFIGLDLGKEIKLDGIRFVIGKNGGGSSD	P26831.2	230.42	8.04
36	HSKWSSPAHEGPHHLTLELDNVYEINKVKYAPRQDSKNG	BAB80940.1	−70.87	45.59
37	WHSAYQAADKLPVSITIKLDKAYDLNQIDYLPRQNSRNG	ABG84084.1	827.51	74.41

*Peptides selected for continued in vivo testing.
NS = No sample

porcine stomach mucin (Sigma Aldrich, St. Louis, MO) and 20 μL of chicken hyper-immune serum for a final concentration of 1×10^4 CFU/mL of CP in each tube. All tubes were then incubated in anaerobic jars at 37°C. At the 4, 6, and 8 h marks of incubation, samples were collected for quantification of CFU by serial dilution plating on tryptic soy agar (**TSA**) with 0.25% sodium thioglycolate (VWR, Radnor, PA) and incubated in anaerobic jars at 37°C for 24 h. Growth (CFU/mL) of CP was calculated as a sample over negative ratio relative to the mean negative control and converted to a percentage.

Vaccine Preparation

The anti-*Clostridium* mucinase vaccine complexes used for in vivo testing were prepared as described above with 2C5 antibodies. A total of 6 vaccines were developed based on successful growth inhibition results from preliminary in vitro trials. Five of the tested vaccines were constructed from 5 individual polypeptides–

4, 5, 22, 24, and 30, while the sixth vaccine consisted of a combination of all five aforementioned proteins.

Eimeria Maxima Preparation

Purified cultures of EM Guelph strain oocysts were floated in a saturated salt solution, quantified in a Mc-Master chamber, and the number of sporulated oocysts were calculated as number of oocysts per mL solution. Oocysts were then resuspended to an inoculum of 2.0×10^4 oocysts/bird in distilled water.

Bacterial Culture Preparation

For the in vivo experiment, *Salmonella enterica* serovar Enteritidis (**SE**) was prepared and administered to all chicks on day of hatch as described by Shivaramaiah and co-authors (2011). Briefly, approximate concentration of SE was quantified spectrophotometrically (Spectronic 200E, Thermo Scientific, Madison, WI), followed by serial dilutions in sterile saline to reach an approximate concentration of 10^4 CFU/chick.
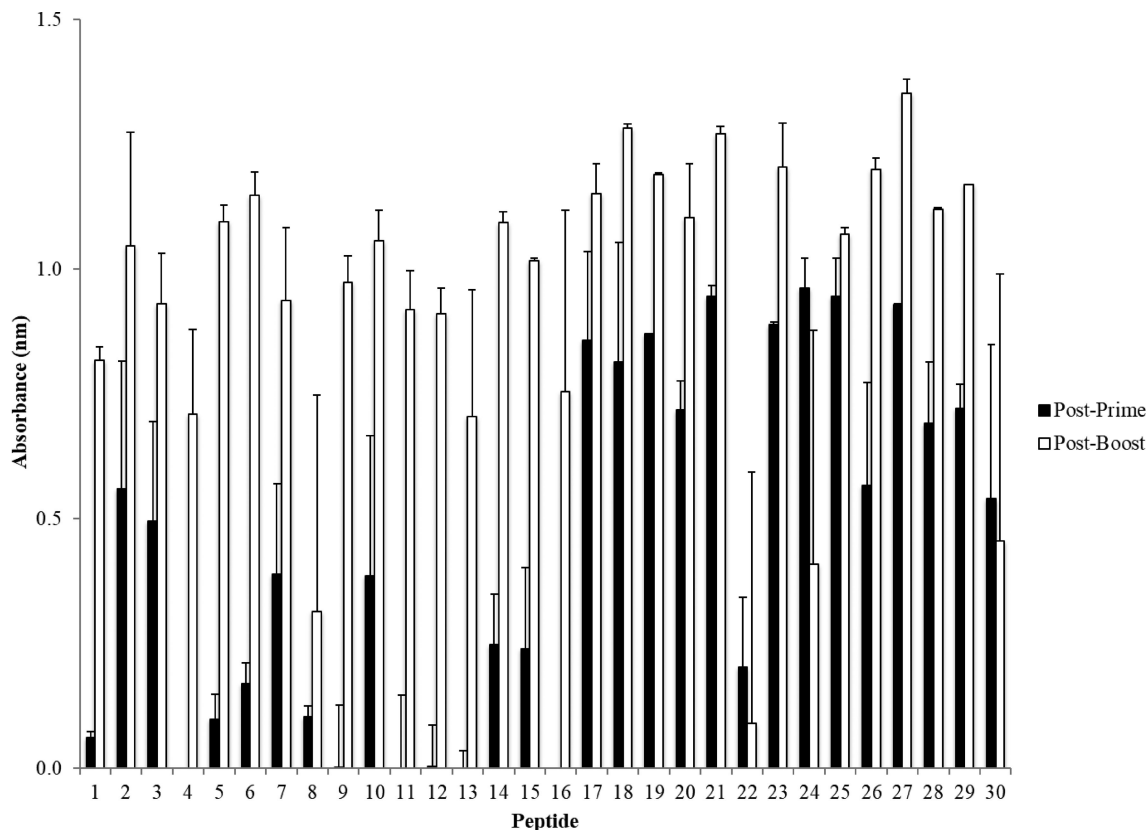
Figure 1. Difference between post-immunization antibody response and pre-immunization response. Each peptide immunization complex was administered to two broilers each via SC injection. The prime injection occurred on day 7 and boost injection on day 14. Sera samples were collected 1 wk after each injection and levels of peptide-specific IgG in each sample were determined and reported as the mean A(450) values $\pm$ standard deviation.

Exact concentration was retrospectively determined by serial dilution plating on TSA and determined to be 1.5 $\times$ 10^4 CFU/chick. To prepare CP inoculum, a single frozen aliquot of wild-type CP TXAM0108 was inoculated into individual tubes with TSB containing 0.25% sodium thioglycolate (VWR, Radnor, PA), and incubated under anaerobic conditions at 37°C for up to 24 h. Post-incubation, cells were washed 3 times in sterile saline by centrifugation at 1,800 $\times$ g for 15 min. The approximate concentration of CP was quantified spectrophotometrically, followed by serial dilutions in sterile saline for the desired inoculum of 10^7 CFU/chick. Exact concentration was determined retrospectively by serial dilution plating on TSA with sodium thioglycolate and determined to be 3.0 $\times$ 10^7 CFU/chick.

Animal Housing and Handling

The in vivo NE experiment was completed under approved animal care protocols approved by the Ohio State University Institutional Animal Care and Use Committee. Chicks were kept in a single room in separate floor pens with fresh pine shaving litter. Feed and water were provided *ad libitum* per the nutritional requirements established by the Nutrient Requirements of Poultry: Ninth Revised Edition (1994), and ambient temperature was maintained within an age-appropriate

range for the duration of the experiment. Throughout the first week, birds were provided 24 h light, and darkness was increased by 1 h per week until a 20 h light: 4 h dark cycle was reached. All chicks were initially placed on a basal, starter diet that contained an anticoccidial (Salinomycin; 50 ppm), and all groups except the non-vaccinated non-inoculated control (**NVNC**) were switched to non-medicated starter three days prior to EM inoculation for the remainder of the experiment.

In vivo Vaccine Testing

A total of 140 Ross 708 broilers were randomly assigned into 1 of 8 treatment groups. These treatments included NVNC, a non-vaccinated inoculated control (**NVIC**), vaccination with peptide 4 (**VP4**), vaccination with peptide 5 (**VP5**), vaccination with peptide 22 (**VP22**), vaccination with peptide 24 (**VP24**), vaccination with peptide 30 (**VP30**), and vaccination with a combination of all five selected mucinase peptides (**MC**). All treatments, except NVNC and MC, had a sample size of 20 chicks, while NVNC and MC had 10 chicks each. On day of placement, all treatments except NVNC were inoculated with SE via oral gavage. On days 2 and 19, all vaccine treatments were injected SC with 0.2 mL of treatment-specific vaccine. On day 27, all treatments, except NVNC, were inoculated with

EM Guelph strain followed by inoculation with CP on day 32, as described above, in order to induce NE. Body weights were measured on day 27 prior to EM inoculation, and again on day 34, in order to calculate body weight gain (**BWG**) and percent change in BWG (**%ChangeBWG**) relative to NVIC according to Wilson and coauthors (2018). After final body weight collection, birds were killed by inhalation of carbon dioxide gas, and the small intestines (jejunum to ileum) were scored for gross necrotic lesions using the 0 to 4 point scale described by Prescott and coauthors (1978).

Statistical Analysis

The BW and BWG data were subjected to an Analysis of Variance in the JMP Pro 12 statistical software (SAS Institute Inc., Cary, NC, 2016), and statistical differences between means were determined using Tukey's Honestly Significant Difference (**HSD**) test. The %BWG was calculated as $(BWG/BW_{initial})$. The %ChangeBWG, relative to NVIC, was calculated as $\{(\%BWG/\text{Average }\%BWG\text{ of NVIC})-1\}\times 100$. LS were subject to an ANOVA as a completely randomized design using the Proc Mixed procedure in SAS 9.4 (SAS Institute Inc., Cary, NC 2016) according to Price et al. (2013) and statistical differences amongst the means were determined using Tukey's HSD at $P < 0.05$. The bird was the statistical unit for all analyses. All BW and LS values reported are expressed as treatment mean $\pm$ standard error.

RESULTS

In vitro CP Growth Inhibition

A total of 37 antigens were tested and shown to induce an antibody response in broilers (Figure 1). These peptides were further evaluated based on ability to obstruct the growth of CP in vitro. Two birds were immunized per peptide and the mean growth was calculated. Colony forming units of CP were measured at 4, 6, and 8 h to determine growth rate in the presence of anti-mucinase hyper-immune serum, and negligible differences in CFU were measured at 4 h of incubation (data not shown). Growth rate was presented as percent change in growth to express growth reduction compared to normal growth of CP in the control group, and was calculated as $\{(\text{Total CFU/Average CFU of negative control})-1\}\times 100$. Antibodies generated against 14 peptides inhibited growth of CP by more than 50% at either 6 or 8 h post-incubation (Table 1). Furthermore, culture treatment with six of these 14 peptides–4, 5, 22, 24, 28, and 29, resulted in decreased growth of CP by more than 50% at both 6 and 8 h time points (Table 1). Peptides 4, 5, 22, 24, and 30 were selected for further testing. Although peptide 30 did not exhibit a greater than 50% reduction in growth at both time points, it was preferred over peptide 29 based on overlap with

Table 2. Body weight gain and percent BWG days 27 to 34. BW was measured on day 27 prior to EM inoculation, and on day 34. BWG and %BWG data are presented as mean $\pm$ standard error, and %ChangeBWG is presented as mean $\pm$ standard deviation. For %ChangeBWG, positive percentage values indicate greater relative percentage of body weight gain compared to NVIC.

Group	BWG (g)	%BWG	%ChangeBWG
NVNC	538.20 $\pm$ 24.77[a]	52.18 $\pm$ 1.80[a]	–
NVIC	359.90 $\pm$ 32.10[b]	33.24 $\pm$ 2.51[b]	0.00 $\pm$ 31.17[a,b]
VP4	370.60 $\pm$ 27.64[b]	35.64 $\pm$ 2.79[b]	7.23 $\pm$ 37.56[a,b]
VP5	354.40 $\pm$ 20.62[b]	33.37 $\pm$ 1.73[b]	0.39 $\pm$ 23.23[a,b]
VP22	416.10 $\pm$ 29.97[a,b]	39.40 $\pm$ 2.49[b]	18.54 $\pm$ 33.52[a*]
VP24	343.10 $\pm$ 24.98[b]	31.57 $\pm$ 2.21[b]	−5.01 $\pm$ 28.17[b]
VP30	411.20 $\pm$ 23.86[a,b]	38.38 $\pm$ 2.18[b]	15.47 $\pm$ 28.63[a†]
MC	428.80 $\pm$ 37.99[a,b]	39.03 $\pm$ 1.65[a,b]	17.43 $\pm$ 14.05[a*]
SEM	22.38	2.30	3.61

[a,b]Mean values with different superscript letters within a column indicate a significant difference ($P < 0.05$)

[*]significantly different from positive control ($P < 0.05$)

[†]Different from positive control at $P = 0.0661$

NVNC = non-vaccinated non-inoculated control, NVIC = non-vaccinated inoculated control, VPn = vaccination with peptide n, MC = combination of all five mucinase peptides.

other sequences, immunogenicity, and literature that suggested peptide 30 was more highly conserved among different CP isolates (Ficko-Blean and Boraston, 2009; Ficko-Blean et al., 2012).

In vivo Vaccination with Mucinase Polypeptides Conjugated with 2C5 Antibody-Guided Complex in Broilers

Compared to NVIC, no significant differences were measure in BWG during the disease period of days 27 to 34, though greater numerical values were reported for birds vaccinated with VP22, VP30, and MC when compared to NVIC. Birds vaccinated with MC exhibited the greatest BWG of all vaccinated groups with a value of 428.80 ± 37.99 g which was 68.9 g greater than the average BWG measured in NVIC (Table 2). When analyzing BW data in terms of %ChangeBWG, which negates any BW differences at the start of the disease period, vaccination with VP22 and MC were significantly increased by 18.54 and 17.43%, respectively (Table 2). Notably, there was a significant difference between the BWG of NVNC and NVIC indicating subclinical NE was induced (Table 2). Mean LS were low for all inoculated groups, and numerically lowest ($P = 0.7786$) for birds vaccinated with the MC complex which exhibited a mean score of 0.63 ± 0.18 (Table 3).

DISCUSSION

Due to the complex multifactorial nature of NE, there has been an emphasized need to develop non-antibiotic prophylactic strategies such as vaccination. Documented predisposing factors for NE have included suboptimal nutrition, stress, concurrent coccidiosis infection, and poultry pathogenic CP strains (Timbermont et al., 2011). Toxin production by pathogenic CP plays a major role in the onset and intestinal damage seen in NE infections. However, other components for survival

Table 3. Lesion Scores. Lesion scores were determined using a 0 to 4 rank scale. Macroscopic NE lesion score scale is reported as number of birds per respective score/total birds lesion scored on a per treatment basis. Mean LS are reported as mean ± standard error.

Treatment	Macroscopic NE lesion score scale					Mean
	0	1	2	3	4	
NVNC	5/9	4/9	0/9	0/9	0/9	0.44 ± 0.18[1]
NVIC	8/20	10/20	2/20	0/20	0/20	0.70 ± 0.15
VP4	8/20	11/20	1/20	0/20	0/20	0.65 ± 0.13
VP5	8/20	11/20	0/20	1/20	0/20	0.70 ± 0.16
VP22	6/20	8/20	6/20	0/20	0/20	1.00 ± 0.18
VP24	4/18	10/18	4/18	0/18	0/18	1.00 ± 0.16
VP30	2/19	14/19	3/19	0/19	0/19	1.05 ± 0.12
MC	3/8	5/8	0/8	0/8	0/8	0.63 ± 0.18

[1]Mean values did not exhibit significant differences ($P > 0.05$)
NVNC = non-vaccinated non-inoculated control, NVIC = non-vaccinated inoculated control, VPn = vaccination with peptide n, MC = combination of all 5 mucinase peptides.

and growth in the GIT, such as mucinases, may contribute to pathogenicity and serve as potential immunogenic targets (Jiang et al., 2009; Rodgers et al., 2015; Lacey et al., 2016). Rather than producing a vaccine that targets whole enzymes, which may require multiple vaccine components to address mucolytic constituents produced by CP, greater success may be achieved through the development of a vaccine against conserved enzymatic domains. Because CBM32 is important for localization and orientation of multiple CP enzymes, such as enβNGlcase and enβGalase, this region may be an ideal target to initiate the production antibodies against a variety of CP strains. However, other CBMs related to sialic acid and glycogen binding should not be ruled out as potential antigens for incorporation into subunit vaccine complexes. Antibody guided antigens have thus far provided a reliable and efficient method for testing vaccine antigens against multiple pathogens (Chen et al., 2012; Chou et al., 2016; Vuong et al., 2017). Vaccination with CD40 agonistic antibody-conjugated peptides in the in vivo experiment, especially with the combined MC, showed a potentially promising and positive impact on %ChangeBWG when subclinical NE was induced.

Inhibition of CP growth in the mucin assay tests and modest improvements in growth performance presented here suggest that an approach to limit growth of CP within the GIT may have potential for control of NE in chickens. This in vivo study involved SC injection of antigens on the 2C5 antibody complex, which, though known to elicit acquired immune response, has not been optimized for mucosal immunity. Thus, these studies should perhaps serve as preliminary data to justify further exploration of the vaccine's potential, especially in the context of necessary mucosal immune response to limit growth of CP within the GIT. Further possibilities include combination with other pathogens and antigens commonly considered predisposing factors to NE, such as alpha toxin, *Eimeria*, and even *Salmonella*.

Notably, CP is only one component in the multifactorial development of NE which and can over prolifer-

ate in response to a variety of predisposing conditions. As previously stated, coccidiosis has been recognized as one of the major predisposing factors for NE which can be attributed to enterocyte lysis, leakage of plasma proteins, and overproduction of mucus in the GIT (Collier et al., 2008). The subsequent enteric damage creates a predisposing environment that is ideal for the colonization, proliferation, and prolonged toxin production by CP. Therefore, these findings are particularly relevant when regarding production systems that control coccidiosis via live oocyst vaccination which can increase the susceptibility of flocks to coccidiosis and therefore NE (Shirley et al., 2005). As such, continued work surrounding this anti-clostridial vaccine has been focused on the incorporation of CP toxin peptides and *Eimeria* antigens into a single vector thereby targeting multiple components that lend to the development of NE. A subunit vaccine of this class has the potential to provide a more encompassing range of protection against NE than is currently available for broiler flocks elsewhere on the market.

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