Research Note: Prevalence and molecular characteristics of *Clostridium perfringens* in "no antibiotics ever" broiler farms

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ABSTRACT *Clostridium perfringens* (***C. perfringens***) is the etiological agent of necrotic enteritis and gangrenous dermatitis; 2 diseases that cause significant economic and welfare concerns to the broiler industry. Previously, *Clostridium*-related diseases were managed with the use of antimicrobial growth promoters fed to broilers that improved gut health and performance. The recent shift to no antibiotics ever (**NAE**) production has increased the incidence of *Clostridium*-related diseases. The objective of this study was to identify *C. perfringens* prevalence and toxinotypes in NAE farms. Samples of litter, feces, and cloacal swabs were collected from 4 NAE broiler farms in the summer of 2019, on d 28 and d 56 of one flock cycle. A total of 734 presumptive isolates were obtained from 192 samples collected in the study. Irrespective of the age of flock and sample type, all 192 samples contained at least one colony presumptively identified as *C. perfringens* on Perfringens agar plate with morphology *as* a single, round colony with opaque ring and black center. All isolates were further screened using PCR for confirmation, toxinotyping, and identification of virulence-associated genes. Only 9 isolates among the 734 presumptive isolates were confirmed as *C. perfringens* and all confirmed isolates were toxinotype A with variation in presence of *netB, cpb2*, and *tpeL*. More extensive studies are required to assess the prevalence and virulence of *C. perfringens* in NAE farms.

Key words: *Clostridium perfringens*, broiler, poultry, no antibiotics ever

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INTRODUCTION

Clostridium perfringens (***C. perfringens***) is a spore-forming, Gram-positive anaerobe found in the gastrointestinal tract (**GIT**) of animals and humans (Uzal et al., 2014). It is the etiological agent of gangrenous dermatitis (**GD**) and necrotic enteritis (**NE**) in poultry. Gangrenous dermatitis is characterized by subcutaneous soft tissue damage with clinical signs of fever, ataxia, anorexia, and lateral recumbency (Shivaprasad, 2016). Clinical NE can cause sudden mortality, lesions in the ileum, reluctance to move, and the possibility of diarrhea, dehydration, and anorexia (Ficken and Wages, 1997; Brennan et al., 2003; Van Immerseel et al., 2004; Lee et al., 2011). Within broilers, GD and NE cause a significant decrease in average daily feed intake by 40% and average daily gain by 16% (Remus et al., 2014). The economic loss associated with *Clostridium* is estimated between $2 and $6 billion every year for the global poultry industry (Wade and Keyburn, 2015).

C. perfringens is classified into 5 toxinotypes (A, B, C, D, and E) based on their ability to produce major toxins (α, β, ε, and ι; Uzal et al., 2014). Toxinotype A is most associated with GD and NE in poultry (Wilder et al., 2001; Uzal et al., 2014). It has been suggested that α-toxin is the major virulence factor for NE in broilers (Wages, 2003); however, α-toxin-lacking mutants produce NE lesions (Keyburn et al., 2006). The pore-producing toxin, NetB, has been proposed as the new virulence factor for NE in poultry and has been detected in NE outbreaks in the United States and Canada (Chalmers et al., 2008; Keyburn et al., 2008). However, gene *netB*-positive isolates have also been recovered from healthy broilers, and *C. perfringens* can be *netB*-positive but fail to produce the NetB toxin,

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leaving its definitive role in *C. perfringens* unanswered (Chalmers et al., 2008; Abildgaard et al., 2010). Other virulence genes associated with *C. perfringens* include *cpb2* and *tpeL*; however, their exact correlation with NE virulence in broiler chickens is unclear (Abildgaard et al., 2010; Yang et al., 2018; Duff et al., 2019).

The use of antimicrobial growth promoters (**AGPs**) was the main barrier against *Clostridium* infections in commercial broilers. They protected birds by direct antibacterial effects, modifying the gut microbiota, reducing GIT inflammation, and improving the overall physical health of the GIT (Elwinger et al., 1998; Lin et al., 2013; Costa et al., 2017; Ritter et al., 2019). However, due to concerns about antimicrobial resistance, broiler operations have limited their antibiotic usage. In 2011, the annual Agricultural Resource Management Survey concluded that 48% of grow-out operations raised broilers without antibiotics and only provided antibiotics when birds were sick (MacDonald, 2014). Recently, it was estimated that more than 50% of the industry raises broilers without any antibiotics (National Chicken Council, 2019). This type of broiler production is referred to as no antibiotics ever (**NAE**). Broilers reared within NAE facilities are not allowed to receive any antimicrobials in feed, water, supplementation, or injection, at any point in the bird's lifetime (Newman, 2018). The removal of AGPs has increased the mortality in NAE broiler production by 25 to 50%, compared to conventional production (Salois, 2017). On average, NAE mortality stands at 4.2%, with conventional broiler production at only 2.9% (Ritter et al., 2019). With greater disease-incidence and mortality rates, it is crucial to understand the prevalence and virulence characteristics of *C. perfringens* within NAE farms.

Presently, limited information is available on *C. perfringens* in commercial NAE broiler production. Therefore, the objective of this study was to determine the prevalence and virulence characteristics of *C. perfringens* in NAE commercial broiler farms.

MATERIALS AND METHODS

Experimental Design

Four commercial poultry farms from the same integrator under the NAE policy were selected in Mississippi. At each farm, 2 houses of mixed-sex broilers were chosen for sample collection. Sample collection was performed for one flock grow-out, 61 days in length, during the summer months (April–July 2019). Sample collection occurred on d 28 and d 56, consisting of litter, feces, and cloacal swabs. A power analysis was conducted to determine sample size.

All samplings in this trial were in compliance with the Guide for the Care and Uses of Agriculture Animals in Research and Teaching (Federation of Animal Science Societies, 2010) and the Mississippi State University Institutional Animal Care and Use Committee (**IACUC**, Animal welfare assurance #17-224).

Sample Collection

Eight litter samples, approximately 20 g each, were aseptically collected with a gloved hand from 4 quadrants of 2 poultry houses per farm on each sampling day. Litter was collected at no more than approximately 2.5 cm in depth in the house and placed into a 200 mL Whirl-Pak bag (Nasco Sampling/Whirl-Pak, Madison, WI). Each sample was pooled from random spots within a quadrant and gloves were changed between samples. Similar sample collection was conducted for fecal samples collecting approximately 15 g per sample using sterile tongue depressors (SKU:25-705, Puritan, Guilford, ME) as forceps. Feces samples were collected from undisturbed droppings on the litter floor.

Using a similar sampling plan, 8 cloacal swabs per farm were collected on each sampling day. A random, apparently healthy bird that was bright, alert, and active was selected in each quadrant and was swabbed using a sterile cotton swab (SKU:25-806, Puritan, Falmouth, ME). The swab was placed into the cloaca of the bird, gently rotated clockwise around the inside of the cloaca approximately 3 times and was immediately placed in a sterile culture tube (Cat No:149569C Fisherbrand, Fisher Scientific, Pittsburgh, PA) containing 5 mL of Reinforced Clostridial Medium (**RCM**; CM0149B, Fisher Scientific).

Isolation of Clostridium Perfringens

From collected litter, 10 g of litter was aseptically weighed and placed into a new 200 mL Whirlpak plastic bag with 90 mL of buffered peptone water (**BPW**), and the bag was stomached for 60 s. For fecal samples, 5 g of feces and 15 mL of BPW were used. A volume of 2 mL of the solution was transferred into a sterile culture tube containing 8 mL of RCM. Cloacal swabs with 5 mL of RCM were vortexed with swabs remaining in the tube.

All culture tubes containing the inoculated RCM were incubated anaerobically at 37°C for 24 h. Subsequently, culture tubes were gently vortexed, and a sterile loop was used to transfer and streak a loopful of RCM suspension onto 2 Perfringens agar (Fisher Scientific) plates. Plates were incubated anaerobically at 35°C for 24 h. A single, round colony with an opaque ring and black center was identified as presumptive *C. perfringens*. Each colony was transferred into 3 mL of Brain Heart Infusion (**BHI**; Bacto, Sparks, MD) and incubated anaerobically for 24 h at 37°C. A volume of 1.5 mL of the vortexed culture was transferred into a labeled CryoELITE cryogenic vial containing 400 μL of glycerol 80% v/v and stored at −80°C for later use.

DNA Isolation of Suspected C. perfringens

Creating a Cell Pellet Using a sterile inoculating loop, CryoELITE cryogenic vial stocks were scraped and placed into a sterile culture tube containing 9 mL of BHI. Culture tubes were incubated anaerobically at 37°C for 48 h and centrifuged at 3,220 rcf at 25°C for 5 min

(Centrifuge 5810R, Eppendorf, Hamburg, Germany). The supernatant was removed to leave 2 to 3 mL of solution and the cell pellet. The remaining solution was vortexed and transferred to a microcentrifuge tube (Fisherbrand, Fisher Scientific) and centrifuged again for 3 min at 15,244 rcf (Centrifuge 5417R, Eppendorf). The supernatant was discarded, leaving approximately 0.1 g of cell pellet to be stored at −20°C for later use.

DNA Extraction With SpeedBeads Field collected isolates failed to produce acceptable DNA when using the typical DNA extraction kits. The following method described below had greater DNA yield and purity and therefore, it was used to extract DNA from presumptive *C. perfringens* isolates (Zhang, unpublished data). A microcentrifuge tube containing a 0.1 g cell pellet was combined with 300 μL of lysis buffer (1X TE + 1% SDS) to dissipate cell pellets into solution. The solution was transferred to a sterile, screw-top microcentrifuge tube containing 200 mg of 0.1 mm glass beads (Cell Disruption Media, Scientific Industries, Inc., Bohemia, NY) that were used to shear the cells. The solution was vortexed at 1,800 rpm at room temperature for 5 min (CRP-18X, CAPP Plate Shaker, Nordhausen, Germany) and incubated at 56°C (Cat No. 88870002, Dry Bath Standard 2-Block 100-120V, Fisher Scientific) for 30 min to lyse cells. After incubation, the solution was gently vortexed and 10 μL of 10 mg/mL RNase A was added to remove RNA materials (Cat No. 19101, Qiagen, German Town, MD). The solution was incubated again at 37°C for 10 min (Dry Bath Standard 2-Block 100-120V, Fisher Scientific) and centrifuged for 60 s at 15,244 rcf (Centrifuge 5417R, Eppendorf, Enfield, CT). A volume of 200 μL of the supernatant was transferred into a new microcentrifuge tube without disturbing the glass beads. At room temperature, a 200 μL of Sera-Mag Magnetic SpeedBeads (09-981-123, Fisher Scientific) in a prepared solution was added to the supernatant for incubation at room temperature for 5 min to allow the DNA materials to adhere to the SpeedBeads. After that, the microcentrifuge tube was placed on a magnetic rack (#1614916, 16-Tube SureBeads Magnetic Rack, Bio-Rad Laboratories, Inc., Hercules, CA) for 2 min for the supernatant to become clear. The magnetic rack affixes the Sera-Mag Magnetic SureBeads containing the DNA materials to the microcentrifuge tube wall. The supernatant was subsequently discarded and the SpeedBeads were washed with 400 μL of 70% molecular grade ethanol twice while the microcentrifuge tube remained on the magnetic stand to remove the remaining foreign materials. The microcentrifuge tubes were incubated without a cap at 37°C until the SpeedBeads were dry. On a regular stand, 100 μL of Tris-EDTA buffer was added and the solution was vortexed to dislodge the dried SpeedBeads and release the DNA materials. The microcentrifuge tubes were placed back on the magnetic stand for 2 min for the supernatant to become clear. The supernatant now containing DNA was transferred to a new microcentrifuge tube. The DNA solution was tested for purity and concentration (Thermo Scientific NanoDrop One, Fisher Scientific) and confirmed with a 0.7% agarose gel.

PCR Confirmation of* C. perfringens *Isolates PCR was performed in a Mastercycler to test for *cpa*, which is present in all *C. perfringens* toxinotypes. The reaction mixture contained 5 μL of GoTaq Green 2X, 0.5 μL of 10 μM *cpa* forward primer, 0.5 μL of 10 μM *cpa* reverse primer, and 3 μL of nuclease-free water for a total of 9 μL of template per reaction and 1 μL of DNA template. PCR parameters consisted of initial denaturation at 95°C for 60 s, 35 cycles of denaturation for 95°C for 60 s, annealing at 61°C for 60 s and extension at 72°C for 60 s, and a final extension at 72°C for 5 min. Isolates were confirmed with 1.5% agarose gel electrophoresis.

PCR Identification of* C. perfringens *Toxinotypes Isolates positive for *cpa* were tested for the remaining toxinotype related genes. Primers and genes tested on *Clostridium* isolates are listed in Table 1. All genes except *netB* were tested using standard PCR techniques.

Table 1. Genes and corresponding primers used for the identification and toxinotyping of *C. perfringens* isolated from litter, feces, and cloacal swab samples collected from commercial NAE broiler farms.

Gene	Gen Bank accession number	Primers	Sequence (5'-3')	Annealing temp (°C)	Product (bp)	Reference
cpa	L43545	CPA5L	AGTCTACGCTTGGGATGGAA	61	900	(Fan et al., 2016)
		CPA5R	TTTCCTGGGTTGTCCATTTC			
cpb	X83275	CPBL	TCCTTTCTTGAGGGAGGATAAA	48	611	(Fan et al., 2016)
		CPBR	TGAACCTCCTATTTTGTATCCCA			
cpb2	L77965	CPB2L	AGATTTTAAATATGATCCTAACC	55	567	(Yang et al., 2018)
		CPB2R	CAATACCCTTCACCAAATACTC			
etx	M95206	CPETXL	TGGGAACTTCGATACAAGCA	55	396	(Fan et al., 2016)
		CPETXR	TTAACTCATCTCCCATAACTGCAC			
iap	X73562	CPIL	AAACGCATTAAAGCTCACACC	55	293	(Fan et al., 2016)
		CPIR	CTGCATAACCTGGAATGGCT			
cpe	X81849	CPEL	GGGGAACCCTCAGTAGTTTCA	55	506	(Fan et al., 2016)
		CPER	ACCAGCTGGATTTGAGTTTAATG			
tpeL	EU848493	TPELF	ATATAGAGGCAAGCAGTGGAG	50	466	(Coursodon et al., 2012)
		TPELR	GGAATACCACTTGATATACCTG			
netB	GU433338	NETBL	TGATACCGCTTCACATAAAGGTTGG	60	169	(Yang et al., 2018)
		NETBR	ATAAGTTTCAGGCCATTTCATTTTTCCG			

Abbreviation: NAE, no antibiotics ever.

Reaction mixture consisted of 5 μL of GoTaq Green 2x, 0.25 μL of 10 μM forward primer, 0.25 μL of 10 μM reverse primer, and 3.5 μL of nuclease-free water. PCR cycles began with an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 60 s, annealing temperature was adjusted for each primer's calculated annealing temperature (Table 1) for 60 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min (Yang et al., 2018). All isolates were confirmed in a 1.5% agarose gel using electrophoresis.

For more sensitive detection of *netB* abundance, real-time PCR was performed, and a 10 μL of reaction mixture was prepared as follows: 5 μL of SYBR Green, 0.5 μL of 10 μM *netB* forward primer, 0.5 μL of 10 μM *netB* reverse primer, 2 μL of nuclease-free water, and 2 μL of template DNA (100 ng). Real-time PCR was performed in a Quant Studio 3 with an initial denaturation at 95°C for 2 min, followed by PCR stage of 45 cycles of denaturation at 95°C for 5 s, annealing, and extension at 60°C for 30 s, with a melting curve analysis performed in a range of 60°C to 95°C at 0.5°C per 5 s increments as described by Yang et al. (2018). All isolates were confirmed in a 1.5% agarose gel using electrophoresis.

RESULTS AND DISCUSSION

The farms used in this study had converted to NAE system 6 months before sampling and had no clinical history of NE or GD. Sample types of litter, feces, and cloacal swabs were used to establish the prevalence of bacteria within the broiler environment.

A total of 733 presumptive isolates were obtained from 192 samples collected in the current study. Of the 733 isolates, 57 exhibited *C. perfringens* morphology upon second streaking on Perfringens agar. After PCR testing for the presence of *cpa* (alpha-toxin gene), 9 isolates were confirmed as *C. perfringens*. These nine isolates were toxinotyped and tested for the presence of *cpb2, netB*, and *tpeL*. The prevalence of each gene tested and each isolate classification can be seen in Table 2. All identified *C. perfringens* isolates tested negative for remaining toxinotype classifying genes, and therefore, all were classified as Toxinotype A. The prevalence of virulence-related genes varied. Of the 9 isolates, 7 tested positive for *cpb2*, 4 tested positive for *netB*, and 2 tested positive for *tpeL*. No isolates had a prevalence of all 3 genes, and only one isolate had no prevalence of any virulence-related genes.

Recovery of *C. perfringens* isolates was lower than anticipated as other *C. perfringens* field studies had higher recovery rates in drug-free flocks. Gaucher et al. (2015) reported that 22% of the fecal samples collected from antibiotic-free flocks contained *C. perfringens*. The lower recovery observed in the current study may be explained by no known history of *C. perfringens* related outbreaks in these farms. In Gaucher et al. (2015), they demonstrated a higher recovery of 13 *C. perfringens* strains from NAE flocks raised under NAE guidelines as compared to 8.5 strains in conventional flocks. Sample collection during the Summer season (April−July) might have contributed to the low *C. perfringens* prevalence due to the warm and dry season. Previous studies have reported that *C. perfringens* infections are more common in the cold season and early winter (Kaldhusdal and Skjerve, 1996; Lovland and Kaldhusdal, 2001). Litter collected was objectively noted as dry and dry litter might reduce the prevalence of active *C. perfringens* as high litter moisture can have up to 3 times the bacterial load as its dry counterpart (Wadud et al., 2012).

All *C. perfringens* isolates identified were classified as Toxinotype A, which is consistent with previous reports of Toxinotype A being the most prevalent in poultry and its environment (Crespo et al., 2007; Yang et al., 2018).

C. perfringens relies on plasmid-encoded toxins to induce NE (Crespo et al., 2007). Plasmid encoded genes *cpb2, netB*, and *tpeL* are thought to play a role in the virulence of *C. perfringens* by the production of toxins that result in NE. The beta2-toxin gene, *cpb2,* is found in a variety of animals suffering from *C. perfringens* enteric-related disease (Van Asten et al., 2010). In the current study also *cpb2* was found in 7 out of 9 isolates. However, it has been found to exist in both non-NE and NE isolates (Crespo et al., 2007; Tolooe et al., 2011). Crespo et al. (2007) reported that cpb2 gene was detected by PCR in majority (75%) of the *C. perfringens* isolates from NE-affected and healthy birds, however, the CPB2 toxin was produced by only 54.5% of these isolates (western blotting). They also found that 90% of

Table 2. Prevalence of genes *cpa, cpb, etx, iap, cpe, cpb2, netB, and tpeL* and the toxinotypes of nine confirmed *C. perfringens* isolates from commercial NAE farms.

Isolate	Sample type	*cpa*	*cpb*	*etx*	*iap*	*cpe*	*cpb2*	*netB*	*tpeL*	Toxinotype
9081	Litter	+	−	−	−	−	+	+	−	**A**
9278	Litter	+	−	−	−	−	−	+	−	**A**
9347	Cloacal Swab	+	−	−	−	−	+	+	-	**A**
9528	Cloacal Swab	+	−	−	−	−	−	−	−	**A**
9556	Litter	+	−	−	−	−	+	+	−	**A**
9641	Litter	+	−	−	−	−	+	−	−	**A**
9643[1]	Litter	+	−	−	−	−	+	−	+	**A**
9645[1]	Litter	+	−	−	−	−	+	−	+	**A**
9653	Litter	+	−	−	−	−	+	−	−	**A**

Abbreviation: NAE, no antibiotics ever.
[1]From the same sample; two separate isolated colonies.

the cpb2 positive isolates from healthy birds produced CPB2 toxin as opposed to only 38.5% from NE-affected birds, suggesting that CPB2 toxin probably does not play an important role in the pathogenesis of NE in birds. The toxin-inducing ability of *cpb2* and its NE-producing ability is yet to be fully elucidated and may not play as strong of a role as expected.

Production of toxin NetB was recently identified as a virulence factor for NE-inducing *C. perfringens* (Keyburn et al., 2010) and gene *netB* was found in 4 out of 9 *C. perfringens* isolates in the current study. In *netB* studies, *netB* prevalence is typically higher in diseased birds and exists in both healthy and diseased broilers (Chalmers et al., 2008; Keyburn et al., 2010; Park et al., 2015; Mwangi et al., 2019). Healthy birds have a more diverse population of *C. perfringens*, which may explain the lower prevalence of *netB* in healthy individuals (Engstrom et al., 2003; Abildgaard et al., 2010). Recently, Zhou et al. (2017) determined that while *netB* is essential in the virulence of NE, it cannot induce NE alone and requires other genes to induce full virulence. Therefore, the *netB* gene alone is not a sufficient virulence factor for determining the potential of NE-causing isolates.

Gene *tpeL* was found in 2 out of nine isolates. Gene *tpeL* contributes to NE pathogenesis and is considered highly virulent (Coursodon et al., 2012). It is usually found in combination with *cpb2* and *netB* in NE-inducing isolates; therefore, *tpeL* is thought to play a role in NE induction together with other virulence genes (Engstrom et al., 2003; Wei et al., 2020). No isolates in this study had a combination of all 3 virulence-related genes and only the combinations of *cpb2* and *netB,* or *cpb2* and *tpeL* were detected. Gene prevalence of virulence-related genes in field samples was similar to other studies (Abildgaard et al., 2010; Gaucher et al., 2017) and *tpeL* is found in lower frequency compared to *netB* (Victoria et al., 2010). Most of these virulence genes of *C. perfringens* are located on large plasmids, except cpa, which is chromosomally located. The enterotoxin gene (cpe) can be present in chromosome as well as plasmid (Petit et al., 1999). Ultimately, these geneses can be transferred between the strains resulting in different strains of varying pathogenicity. In conclusion, even though the prevalence of *C. perfringens* was very low in NAE samples, the isolates contained major virulence genes that may induce *Clostridium*-related disease. *Clostridium* disease should continue to be closely monitored in NAE facilities year-round, especially in facilities that have chronic *Clostridium*-related outbreaks. By monitoring the prevalence of disease-causing *C. perfringens* in the NAE environment, NAE facilities could limit future outbreaks.

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DISCLOSURES

The authors declare no conflicts of interest

REFERENCES

Abildgaard, L., T. E. Sondergaard, R. M. Endberg, A. Schramm, and O. Højberg. 2010. *In vitro* production of necrotic enteritis toxin B, NetB, by *netB*-positive and *netB*-negative *Clostridium perfringens* originating from healthy and diseased broiler chickens. Vet. Microbio. 144:231–235.

Brennan, J., J. Skinner, D. A. Barnum, and J. Wilson. 2003. The efficacy of bacitracin methylene disalicylate when fed in combination with narasin in the management of necrotic enteritis in broiler chickens. Poult. Sci. 82:360–363.

Chalmers, G., S. W. Martin, D. B. Hunter, J. F. Prescott, L. J. Weber, and P. Boerlin. 2008. Genetic diversity of *Clostridium perfringens* isolated from healthy broiler chickens at a commercial farm. Vet. Microbiol. 127:116–127.

Costa, M. C., J. A. Bessegatto, A. A. Alfieri, J. S. Weese, J. A. Filho, and A. Oba. 2017. Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. PLoS One 12:e0171642.

Coursodon, C. F., R. D. Glock, K. L. Moore, K. K. Cooper, and J. G. Songer. 2012. *Tpel*-producing strains of *Clostridium perfringens* type A are highly virulent for broiler chicks. Anaerobe 18:117–121.

Crespo, R., D. J. Fisher, H. L. Shivaprasad, M. E. Fernandez-Miyakawa, and F. A. Uzal. 2007. Toxinotypes of *Clostridium perfringens* isolated from sick and healthy avian species. J. Vet. Diagn. Invest. 19:329–333.

Duff, A. F., C. N. Vuong, K. L. Searer, W. N. Briggs, K. M. Wilson, B. M. Hargis, L. R. Bergham, and L. R. Bielke. 2019. Preliminary studies on development of a novel subunit vaccine targeting *Clostridium perfringens* mucolytic enzymes for the control of necrotic enteritis in broilers. Poultry Sci. 98:6319–6325.

Elwinger, K., E. Berndtson, B. Engström, O. Fossum, and L. Waldenstedt. 1998. Effect of antibiotic growth promoters and anticoccidials on growth of Clostridium perfringens in the caeca and on performance of broiler chickens. Acta Vet. Scand. 39:433–441.

Engstrom, B. E., C. Fermer, A. Lindberg, E. Saarinen, V. Baverud, and A. Gunnarsson. 2003. Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. Vet. Microbiol. 94:225–235.

Fan, Y. C., C. L. Wang, C. Wang, T. C. Chen, C. H. Chou, and H. J. Tsai. 2016. Incidence and antimicrobial susceptibility to *Clostridium perfringens* in premarket broilers in Taiwan. Avian Dis. 60:444–449.

Ficken, M., and D. Wages. 1997. Necrotic enteritis.Pages 264−267 in Disease of Poultry. BW Calnex, ed. 10th ed. Iowa State University Press, Ames, IA.

Gaucher, M-L, G. G. Perron, J. Arsenault, A. Letellier, M. Boulianne, and S. Quessy. 2017. Recurring necrotic enteritis outbreaks in commercial broiler chicken flocks strongly influence toxin gene carriage and species richness in the resident *Clostridium perfringens* population. Front. Microbiol. 8:881.

Gaucher, M-L., S. Quessy, A. Letellier, J. Arsenault, and M. Boulianne. 2015. Impact of a drug-free program on broiler chicken growth performances, gut health, *Clostridium perfringens* and *Campylobacter jejuni* occurrences at the farm level. Poult. Sci. 94:1791–1801.

Kaldhusdal, M., and E. Skjerve. 1996. Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway. Preventive Vet. Med. 28:1–16.

Keyburn, A. L., S. A. Sheedy, M. E. Ford, M. M. Williamson, M. M. Awad, J. I. Rood, and R. J. Moore. 2006. Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. Infect. Immun. 74:6496–6500.

Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, and D. Parker. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog 4:26.

Keyburn, A. L., T. L. Bannam, R. J. Moore, and J. J. Rood. 2010. NetB, a pore-forming toxin from necrotic enteritis strains of *Clostridium perfringens*. Toxins 2:1913–1927.

Lee, K. W., H. S. Lillehoj, W. Jeong, H. Y. Jeoung, and D. J. An. 2011. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poult. Sci. 90:1381–1390.

Lin, J., A. A. Hunkapiller, A. C. Layton, Y. J. Chang, and K. R. Robbins. 2013. Response of intestinal microbiota to antibiotic growth promoters in chickens. Foodborne Pathog Dis 10:331–337.

Lovland, A., and M. Kaldhusdal. 2001. Severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated hepatitis. Avian Path. 30:73–81.

MacDonald, J. M. 2014. Technology, organization, and financial performance in US broiler production. United States Department of Agriculture, Economic Research Service. EIB 126. Accessed Mar. 2021. https://www.ers.usda.gov/webdocs/publications/43869/48159_eib126.pdf?v=2343.6.

Mwangi, S., J. Timmons, S. Fitzcoy, and S. Parveen. 2019. Characterization of *Clostridium perfringens* recovered form broiler chickens affected by necrotic enteritis. Poult. Sci. 98:128–135.

Newman, L. 2018. New strategies in coccidiosis control to meet customer demands. Int. Poult. Prod. 26:15–17.

Park, J. Y., S. Kim, J. Y. Oh, H. R. Kim, I. Jang, H. S. Lee, and Y. K. Kwon. 2015. Characterization of *Clostridium perfringens* isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. Poult. Sci. 94:1158–1164.

National Chicken Council. https://www.nationalchickencouncil.org/questions-answers-antibiotics-chicken-production-2/#:~:text=As%20of%20April%202019%2C%20more%20than%2050%25%20of,those%20questions%20and%20address%20some%20of%20those%20concerns. Posted February 8, 2019. Accessed Feb. 2021.

Petit, L., M. Gibert, and M. R. Popoff. 1999. Clostridium perfringens: toxinotype and genotype. Trends Microbiol. 7:104–110.

Remus, A., L. Hauschild, I. Andretta, M. Kipper, C. R. Lehnen, and N. K. Sakomura. 2014. A meta-analysis of the feed intake and growth performance of broiler chickens challenged by bacteria. Poult. Sci. 93:1149–1158.

Ritter, G. D., G. R. Acuff, G. Bergeron, M. W. Bourassa, B. J. Chapman, J. S. Dickson, K. Opengart, M. J. Salois, R. S. Singer, and C. Storrs. 2019. Antimicrobial-resistant bacterial infections from foods of animal origin: understanding and effectively communicating to consumers. Ann. N. Y. Acad. Sci. 1441:40–49.

Salois, M. 2017. The cost of broiler welfare standard. *Chicken Marketing Summit. 2017 Elanco* GMABRLNON00638.

Shivaprasad, H. L. 2016. Gangrenous dermatitis in poultry.Pages 255−264 in Clostridial Diseases of Animals. F. A. Uzal, ed. eds. Wiley-Blackwell, Ames, IA.

Tolooe, A., B. Shojadoost, and S. M. Peighambari. 2011. Molecular detection and characterization of Cpb2 gene in *Clostridium perfringens* isolates from healthy and diseased chickens. J. Venom Anim. Toxins Includ. Trop. Dis. 17:59–65.

Uzal, F. A., J. C. Freedman, A. Shrestha, J. R. Theoret, J. Garcia, and M. M. Awad. 2014. Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. Fut. Microbiol. 9:361–377.

Van Asten, A. J., G. N. Nikolaou, and A. Gröne. 2010. The occurrence of cpb2-toxigenic *Clostridium perfringens* and the possible role of the β2-toxin in enteric disease of domestic animals, wild animals and humans. Veter. J. 183:135–140.

Van Immerseel, F., J. De Buck, F. Pasmans, G. Huyghebaert, F. Haesebrouck, and R. Ducatelle. 2004. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol. 33:537–549.

Victoria, J. N., P. Cornelis, R. P. Valeria, J. Yan-Fen, R. Richard, and F. P. John. 2010. *Clostridium perfringens* in retail chicken. Anaerobe 16:314–315.

Wade, B., and A. Keyburn. 2015. The true cost of necrotic enteritis. World Poult. 31:16–17.

Wadud, S., A. Michaelsen, E. Gallagher, G. Parcsi, O. Zemb, R. Stuetz, and M. Manefield. 2012. Bacterial and fungal community composition over time in chicken litter with high or low moisture content. Br. Poult. Sci. 53:561–569.

Wages, D. P., and K. Opengart. 2003. Necrotic enteritis.Pages 781−785 in Disease of Poultry. Y. M. Saif, ed. 11th ed. Iowa State Press, Ames, IA.

Wei, B., S. Y. Cha, J. F. Zhang, K. Shang, H. C. Park, J. W. Kang, K. J. Lee, M. Kang, and H. K. Jang. 2020. Antimicrobial susceptibility and association with toxin determinants in *Clostridium perfringens* isolates from chickens. Microorganisms 8:1825.

Wilder, T. D., J. M. Barbaree, K. S. Macklin, and R. A. Norton. 2001. Differences in the pathogenicity of various bacterial isolates used in an induction model for gangrenous dermatitis in broiler chickens. Avian Dis. 45:659–662.

Yang, W. Y., C. H. Chou, and C. Wang. 2018. Characterization of toxin genes and quantitative analysis of *netB* in necrotic enteritis (NE)-producing and non-NE-producing *Clostridium perfringens* isolated from chickens. Anaerobe 54:115–120.

Zhou, H., D. Lepp, Y. Pei, M. Liu, X. Yin, R. Ma, J. F. Prescott, and J. Gong. 2017. Influence of pCP1NetB ancillary genes on the virulence of *Clostridium perfringens* poultry necrotic enteritis strain CP1. Gut Pathog. 9:6.