### Title

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# Focal duodenal necrosis in chickens: attempts to reproduce the disease experimentally and diagnostic considerations

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**Abstract.** Focal duodenal necrosis (FDN) is an intestinal disease of egg-layer chickens characterized by multifocal necrosis of the duodenal loop and proximal jejunum. Affected flocks usually have decreased egg weights and drops in egg production. Previous studies have associated this condition with *Clostridium perfringens* infection. We tried to reproduce FDN by experimental infection of egg-laying chickens using different *netB*-positive and *netB*-negative *C. perfringens* strains, and duodenal homogenate obtained from FDN lesions. Chickens challenged with *C. perfringens* and/or duodenal homogenate developed duodenitis after challenge. Gross lesions included mucosal erosions, hyperemia, mucosal hemorrhages, and watery intestinal content. Microscopic lesions included mild enterocyte degeneration and necrosis, and mild-to-moderate hemorrhage and lymphoplasmacytic and heterophilic infiltration of the lamina propria. Two *netB*-positive *C. perfringens* strains closely related to necrotic enteritis pathogenic strains, by genomic composition, were re-isolated from lesions. Necrosis of intestinal crypts was observed in chickens challenged with duodenal homogenate with or without *C. perfringens* coinfection. Characteristic microscopic FDN lesions with significant necrosis and loss of villus enterocytes were not reproduced.

**Key words:** *Clostridium perfringens*; egg-layer chickens; focal duodenal necrosis.

## Introduction

Focal duodenal necrosis (FDN) is an enteric disease of table egg-layer chickens characterized by decreased egg weights and drops in egg production. This disease was first identified in a cage-free layer flock in 1997 (Gingerich E. Focal duodenal necrosis (FDN) in table egg flocks, 2009. https://www.wattagnet.com/articles/519-focal-duodenal-necrosis-fdn-in-table-egg-flocks). Since then, the disease has been detected in most egg-producing states in the United States, and it is considered one of the top 10 disease concerns of the table egg industry (USAHA Committee on Poultry and Other Avian Species. US Egg Layer Health Report, 2019. https://www.usaha.org/transmissible-diseases-of-poultry-avian-species). Layers with FDN may not have clinical signs or may have a pale comb and decreased weight gain. Gross lesions are characterized by single-to-multiple, red-to-gray mucosal erosions that are commonly covered with a yellow pseudomembrane. These lesions are frequently located in the duodenal loop but can extend into the proximal jejunum, and in severe cases may be seen through the serosa (Franca M, et al. Duodenitis focal en ponedoras comerciales [Focal duodenal necrosis in commercial layers]. Avinews Latin America 2016;7:63–70. Spanish. Available at: https://avicultura.info/duodenitis-focal-ponedoras-comerciales/).

Previous studies have shown evidence that supports the role of clostridial species in the pathogenesis of FDN. One study described higher frequency of *Clostridium colinum* signature gene markers in duodenal samples from affected birds (Siragusa GR, et al. Molecular pathogenesis markers of focal duodenal necrosis in layer hens. Proc 80th Northeastern Conf Avian Dis; 2008 Sept 16–17; State College, PA). In another study, *C. perfringens* isolates encoding necrotic enteritis B–like (NetB) and beta2 (CPB2) toxins were isolated from duodenal lesions of laying hens with decreased egg production.[1] Our group at the University of Georgia

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**Table 1.** Toxinotyping results used in our study of *Clostridium perfringens* isolates from focal duodenal necrosis lesions.

| | Toxin genes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Isolate | *cpa* | *netB* | *etx* | *tpeL* | *cpb2* | *cpb* | *cpe* | *iA* |
| 1 | + | + | − | − | + | − | − | − |
| 2 | + | − | − | − | + | − | − | − |
| 3 | + | + | − | − | + | − | − | − |
| 4 | + | − | − | − | + | − | − | − |
| 5 | + | + | − | − | + | − | − | − |
| 6 | + | − | − | − | + | − | − | − |

+ = positive; − = negative; *cpa* = alpha-toxin gene; *cpb* = beta-toxin gene; *cpb2* = beta2-toxin gene; *cpe* = enterotoxin gene; *etx* = epsilon-toxin gene; *iA* = iota-toxin gene; *netB* = necrotic enteritis B toxin gene; *tpeL* = tpeL toxin gene.

(UGA; Athens, GA) has reported the association of CPB2-positive *C. perfringens* type A in layers with typical FDN lesions in the United States.[4] FDN microscopic lesions resemble lesions of subclinical necrotic enteritis (NE) in broilers; however, FDN lesions are often associated with numerous long gram-negative filamentous bacteria in addition to intralesional *C. perfringens* identified by immunohistochemistry.[4]

The epidemiologic characteristics of egg layer flocks affected with FDN were recently investigated by our group at UGA.[16] Distiller's dried grains with solubles and animal protein meal were ingredients commonly added to the feed of flocks affected with FDN.[16]Additionally, most egg layer flocks affected with FDN received >12 different feed formulations from pre-lay to 60 wk of age.[16] Coccidiosis and roundworm parasitism were not reported as being a problem in FDN-affected flocks.[16] For most affected flocks, cages, walls, and ceilings were not washed prior to disinfection. *C. perfringens*–associated enteritides are multifactorial diseases that involve the combination of infection with virulent *C. perfringens* strains and the presence of environmental, dietary, and other pathogenic factors that may predispose to disease.[16] A better understanding of FDN and the development of a disease model are needed in order to design effective control measures to prevent FDN-associated egg production losses.

We investigated the ability of different *C. perfringens* strains and duodenal homogenates to reproduce FDN experimentally in chickens. Experimental challenges were performed using various experimental designs and different feed ingredients as possible predisposing factors for FDN development.

## Materials and methods

### Bacterial strains

The *C. perfringens* type A or G strains used in our study were originally isolated from field cases of FDN (Table 1).

### Inoculum preparation and titration

Challenge strains were streaked onto trypticase soy agar (TSA) with 5% sheep red blood cells (Remel, Lenexa, KS) and incubated anaerobically overnight at 37°C. The strains were harvested using 1 mL of sterile thioglycollate broth and suspended in an equal volume of sterile glycerol and kept frozen at −80°C until use. A volume of 0.5 mL of this culture was inoculated into 500 mL of thioglycollate media containing 2% beef extract and incubated at 37°C for 18 h for inoculum preparation. The inoculum was titrated, and the number of colony-forming units (CFU) per mL was determined.

For duodenal homogenate preparation, samples of duodenum from birds with macroscopic lesions of FDN were collected, placed into sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI), and maintained in anaerobic conditions (Gas Pak anaerobe pouches; BD, Sparks, MD) at 4°C for 1–2 d prior to processing. The duodenal mucosa was scraped with a sterile spatula, and mucosal scrapings were transferred to sterile Petri dishes, following a previously described protocol with some modifications.[15] Briefly, sterile phosphate-buffered saline (PBS) was added (0.5 mL of PBS per 0.1 g of sample), and samples were centrifuged at $4{,}000 \times g$ for 15 min at 4°C. Mucosal homogenate pellets were harvested, mixed with freezing stock medium containing 15% glycerol, and stored at −80°C until use.

### Experimental trials

For the experimental trials, birds were housed at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, UGA. Water and feed were provided ad libitum. A standard lighting program for commercial layers was followed. Clinical signs were monitored twice a day. All birds were euthanized with carbon dioxide followed by cervical dislocation. Autopsy was performed, gross lesions were evaluated, and intestinal samples were collected for histopathology and bacteriology.

Bird care was provided according to animal use protocols approved by the Institutional Animal Care and Use Committee (IACUC) at UGA (A2014 02-001 and A2016 11-001).

*Experimental trial 1.* Fifty-two laying hens (Hy-Line W-36; Hy-Line International, Dallas Center, IA) obtained from a local commercial flock at 22 wk of age were randomly divided into treatment groups 1 and 2 of 18 birds per group, and a control group (group 3) with 16 birds. Birds were reared in cages, with 2 chickens per cage. All treatment groups in this experiment received a standard corn–soy diet containing 9% distiller's dried grain with solubles (DDGS) and limestone particles (50% fine and 50% coarse particles). DDGS is a common ingredient used in egg layer flocks affected with FDN, and this ingredient can enhance intestinal proliferation of *C. perfringens*[16] (Macklin K, et al. The effects of different levels of DDGS on NE development in broiler chickens. Proc

60th Western Poultry Dis Conf; 2011 Mar 20–23; Sacramento, CA). At 24 wk of age, chickens in group 1 were inoculated daily for 8 d via crop gavage with 1 mL of $10^8$–$10^9$ CFU/mL of a *netB*-positive *C. perfringens* isolate (isolate 1); chickens in group 2 were inoculated with a *netB*-negative *C. perfringens* isolate (isolate 2). Chickens in group 3 (control) were inoculated with 1 mL of sterile thioglycollate broth daily and for 8 d via crop gavage. Euthanasia, autopsy, and sample collection were performed on day 9, the day after the last inoculation day.

*Experimental trials 2–4.* Two hundred 1-d-old Hy-Line W-36 chicks were obtained from a local commercial hatchery. In experimental trials 2 and 3, we evaluated the ability of different *C. perfringens* strains isolated from FDN lesions to cause disease in pullets using 2 different experimental designs. In trial 2, we followed an experimental model that caused mild NE lesions in young chickens challenged with a *netB*-positive *C. perfringens* as described previously,[7] with some modifications in inoculum dose, age of the chickens at inoculation, chicken breed used, and housing methods. The pullets were randomly distributed into 6 treatment groups (groups 1–6) and 1 sham-inoculated control group (group 7), with 10 birds per group. Each treatment group was inoculated with one *C. perfringens* isolate (isolate 1–6, respectively). All pullets were housed in cages with 2 birds per cage and received a starter diet containing 20% crude protein for 14 d. Thereafter, the pullets were provided with a corn–soy, high-protein diet containing 50% fishmeal. This high-protein diet with 50% fishmeal is a known predisposing factor for *C. perfringens*–induced NE in broiler chickens.[9] All chickens were feed-restricted for 12 h prior to inoculations. On days 20 and 21, chickens in groups 1–6 were inoculated with $10^8$–$10^9$ CFU/mL of *C. perfringens* culture via crop gavage. On day 21, these chickens received feed mixed with *C. perfringens* culture in a ratio of 1:10 (v:w). Chickens in the negative control group were also feed-restricted prior to receiving sham inoculum via crop gavage on days 20 and 21, and received feed mixed with sterile culture medium in a ratio of 1:10 (v:w) on day 21. All chickens were euthanized on day 24 for autopsy and sample collection.

In experimental trial 3, we evaluated the ability of different *netB*-positive *C. perfringens* strains isolated from FDN lesions to cause disease in pullets by following an experimental model that can induce more severe intestinal necrosis in chickens as described previously,[3] with some modifications in housing method, chicken breed used, age at inoculation, and feeding schedule. The pullets were randomly distributed into treatment groups 1–3 and a negative control group (group 4) containing 10 birds per group, and were housed in cages, with 2 birds per cage. Three days prior to challenge, the feed was changed to a corn-soy–based feed containing 50% fishmeal. All birds were feed-restricted for 18 h prior to challenge. Chickens in challenge groups 1–3 received an in-feed challenge containing $10^8$–$10^9$ CFU/mL *netB*-positive *C. perfringens* (isolate 1, 3, or 5) serially passed culture as described previously,[3] which was mixed with the feed in a ratio of 3:4 (v:w) and provided for 4 d. A separate culture was prepared daily for each challenge feeding. Chickens in the negative control group received feed mixed with sterile culture broth in a ratio of 3:4 (v:w). All chickens were euthanized on day 5, the day after the last inoculation day, for autopsy and sample collection.

Experimental trial 4 was conducted to evaluate the ability of a combination of 2 *C. perfringens* isolates and duodenal homogenate to induce more significant microscopic lesions in the duodenum of challenged chickens fed 2 different diets. Four groups of 23-wk-old Hy-line W-36 chickens were housed in cages, with 2 birds per cage. The experiment had a $2 \times 2$ factorial design with 2 challenge and control groups receiving 2 different diets. Diet 1 was provided to experimental groups 1 and 2; diet 2 was provided to groups 3 and 4. Diet 1 was a standard corn–soy layer diet with 50% fine and 50% coarse limestone particles. Diet 2 was a standard corn–soy diet containing 10% DDGS, 5% meat and bone meal, with 20% fine and 80% coarse limestone particles. We used a higher percentage of coarse limestone particles in diet 2 to increase gastrointestinal retention of calcium particles because previous studies have reported that the cytotoxic activity of *C. perfringens* requires calcium for the specific binding of alpha-toxin to cell membranes.[11]

Chickens were feed-restricted for 18 h prior to inoculations via crop gavage. Twelve chickens in groups 1 and 3 were inoculated with 2 mL of sham inoculum (sterile medium) for up to 7 d. Twenty chickens in groups 2 and 4 were inoculated for up to 7 d with $10^8$–$10^9$ CFU/mL of a *C. perfringens* inoculum containing a mixture of *netB*-negative and *netB*-positive isolates (isolates 2 and 5) and 1 mL of duodenal homogenate obtained from FDN-affected birds. Chickens were euthanized on day 4 or 8 for autopsy and sample collection.

*Experimental trial 5.* A total of 40, 17-wk-old Lohmann pullets were obtained from a local commercial flock. The chickens were housed in cages with 2 birds per cage. At 23 wk of age, the chickens were randomly distributed into treatment groups 1–3 and a negative control group (group 4), with 10 birds per group. All birds were provided a corn–soy diet containing 10% DDGS, 5% protein meal, with 20% fine and 80% coarse limestone particles, and received inocula via crop gavage. Chickens in group 1 were inoculated with $10^8$–$10^9$ CFU/mL of *C. perfringens* culture containing *netB*-positive bacteria (isolate 5). Chickens in group 2 were inoculated each day for 7 d with 1 mL of duodenal homogenate obtained from FDN-affected birds. Chickens in group 3 were inoculated for 7 d with $10^8$–$10^9$ CFU/mL *C. perfringens* culture containing *netB*-positive bacteria (isolate 5) and also received 1 mL of duodenal homogenate. Chickens in group 4 (control group) were inoculated for 7 d with 1 mL of sham inoculum (sterile medium). All birds were

euthanized on day 8, the day after the last inoculation day, for autopsy and sample collection.

### Histopathology

Segments of duodenum from all autopsied chickens were fixed in 10% neutral-buffered formalin, processed routinely, and stained with hematoxylin and eosin. Duodenal samples were examined blindly by M. França. Intestinal samples were evaluated for the presence of lymphoplasmacytic inflammation, heterophilic inflammation, hemorrhage, necrosis of enterocytes, cystic crypts and/or crypt necrosis, and inflammatory infiltrate in the lumen. Each of these lesions was scored as 0 = no lesion, 1 = mild, 2 = moderate, and 3 = marked; the sum of lesion scores was used to determine the total microscopic lesion score per chicken. Kruskal–Wallis one-way ANOVA and Dunn multiple comparison tests were used to evaluate statistical significance and to compare intestinal microscopic lesion scores between groups. Analyses were performed using commercial statistical software (GraphPad Software, La Jolla, CA); $p \leq 0.05$ was considered statistically significant.

### Bacteriology

Duodenal samples were collected aseptically, incubated anaerobically at 37°C for 24 h in thioglycollate broth medium, and plated onto blood agar media as described previously.[4] Colonies with a double zone of hemolysis, resembling *C. perfringens* colonies, were selected and plated on selective phenylethyl alcohol blood agar media for aerobic and anaerobic incubation. Gram-positive anaerobic colonies resembling *C. perfringens* were selected for identification (RapID ANA II system; Thermo Scientific, Carlsbad, CA).

### DNA bacterial extraction and PCR

Colonies of *C. perfringens* recovered from duodenal lesions were subcultured and incubated anaerobically at 37°C for 48 h in pre-reduced TSA with 5% sheep red blood cells. Three colonies were suspended in 500 µL of nuclease-free water (Thermo Scientific). Bacterial DNA was extracted (DNA extraction kit; Qiagen, Germantown, MD) according to the manufacturer's recommendations.

Multiplex PCR for the presence of genes coding for alpha (*cpa*) and NetB (*netB*) toxins was performed. Final reaction volume was 28 µL and consisted of 15 µL of 2× GeneAmp Fast PCR master mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA), 0.75 µL of each pair of primers with final concentration of 0.25 µM (*cpa*, *netB*), 10 µL of water, and 2 µL of DNA template. PCR reaction was amplified (MyCycler Thermal Cycler; Applied Biosystems) with 25 cycles of 95°C for 10 s, 94°C for 1 s, 62°C for 40 s, and extension at 72°C for 10 s. PCR product was visualized by electrophoresis (100 V constant voltage) in 1.5% agarose gel with ethidium bromide; 1-kb plus DNA ladder was included as a molecular marker (Thermo Scientific).

*C. perfringens* isolates cultured from FDN lesions and inoculum strains were submitted for pulsed-field gel electrophoresis (PFGE; Molecular Epidemiology, Lake Forest Park, WA) to determine if cultured and inoculum strains had identical fingerprint patterns.

### Genome sequencing and analysis

Selected *netB*-positive *C. perfringens* strains were sequenced (Illumina MiSeq 2×300bp platform; Georgia Genomics Facility, UGA). Genomes were assembled using the Spades v.3.12 pipeline[2] and were annotated using prokka 1.13.3.[14] The outputs from prokka were used as input to Roary, the pan-genome pipeline.[12] To investigate the relationships of these 2 isolates in the broader species context, single nucleotide variants were called using snippy v.4.3.2 (https://github.com/tseemann/snippy) against the reference strain ATCC13124 (NC_008261.1). A maximum-likelihood tree was made using IQ-TREE[10] using the GTR+F+G4 model and rapid bootstrapping (-bb 1000).[5] The tree was visualized using iTOL.[6]

## Results

### Experimental trial 1

No clinical signs were observed in control or challenged birds. Gross lesions observed in the duodenal loop of chickens challenged with *netB*-positive *C. perfringens* (group 1) included mucosal hyperemia (10 of 18 birds), mucosal hemorrhage (4 of 18), mucosal erosions (2 of 18; Fig. 1A), and watery and/or frothy content in the lumen (2 of 18). Likewise, chickens challenged with *netB*-negative *C. perfringens* (group 2) had mild-to-moderate mucosal hyperemia (18 of 18 birds), watery or frothy intestinal content (5 of 18), small mucosal erosions (3 of 18), and mild mucosal hemorrhage (1 of 18; Fig. 1B). Mild mucosal hyperemia was observed in 12 of 16 sham-inoculated control chickens, and 4 of 16 control birds had small amounts of frothy content in the duodenum. Mucosal erosions and hemorrhage were not observed in the duodenum of control birds.

Histopathology revealed microscopic duodenal lesions in chickens from both challenged groups. Chickens challenged with *C. perfringens* had higher microscopic lesion scores than the control group ($p \leq 0.0001$; Supplementary Fig. 1). Lesions observed included moderate lymphoplasmacytic inflammation in the lamina propria with variable numbers of heterophils (Fig. 2A), as well as hyperemia and mild hemorrhages in the mucosa and lumen. Epithelial separation from the lamina propria and individual enterocyte necrosis (Fig. 2B) were observed in 3 of 18 birds inoculated with *netB*-negative *C. perfringens* (group 2). Characteristic microscopic lesions of FDN (necrosis and loss of enterocytes in villus tips with fibrinoheterophilic exudate in the lumen) were not observed in challenged birds.

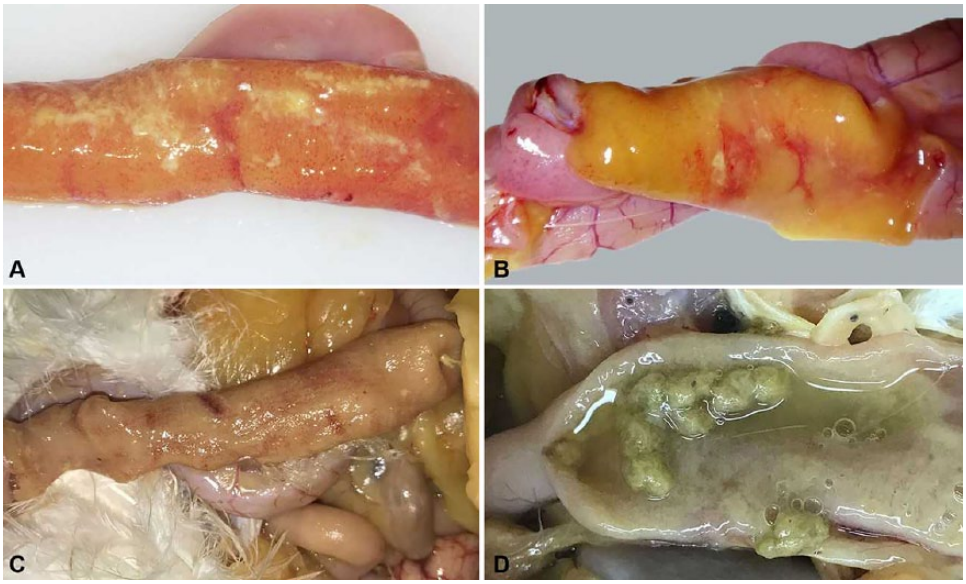


**Figure 1.** Duodenal lesions in chickens inoculated with *Clostridium perfringens*. **A.** Red mucosal erosions and yellow pseudomembranes in the duodenum of a laying hen inoculated with *netB*-positive *C. perfringens* isolate 1 (experiment 1, group 1). **B.** Mucosal hemorrhages and small amounts of yellow pseudomembrane in the duodenum of a laying hen inoculated with *netB*-negative *C. perfringens* isolate 2 (experiment 1, group 2). **C.** Red mucosal erosions in the duodenum of a laying hen inoculated with *C. perfringens* and duodenal homogenate (experiment 4, group 2). **D.** Jejunitis characterized by thickening of the intestinal wall and watery, mucoid, and frothy content in a laying hen inoculated with *C. perfringens* and duodenal homogenate (experiment 4, group 2).

*C. perfringens* was isolated from duodenal lesions from one bird in group 1. The isolate was confirmed as *C. perfringens* by RapID ANA II, and the *cpa* and *netB* genes were detected by PCR. This isolate was analyzed by PFGE and the restriction-fragment length polymorphism pattern was compared against the *C. perfringens* strain present in the inoculum. The isolate recovered from lesions was identical, as determined by PFGE, to the *netB*-positive *C. perfringens* isolate 1 used in the inoculum. Analysis of the genome suggests *C. perfringens* isolate 1 was a typical pathogenic poultry isolate, closely related to NE-causing *C. perfringens* stains, such as C26 and C31 (Fig. 3). This isolate encodes *netB*, perfringolysin O gene (*pfoA*), *cpa*, an atypical *cpb2* gene, as well as the *VR-10B* adhesion locus and the collagen-binding protein gene, *cnaA*. The *VR-10B* adhesion locus is highly correlated with virulence in NE pathogenic strains and has been identified as important for *C. perfringens* adherence to intestinal cells and extracellular matrix proteins.[8,17]

## Experimental trial 2

No overt clinical signs were observed in control and challenged birds. Mucosal erosions were not observed in control and treatment groups. Mucosal hyperemia was observed in 1 of 10 pullets from group 1 (inoculated with isolate 1), in 3 of 10 pullets from group 2 (inoculated with isolate 2), in 4 of 10 pullets from group 3 (inoculated with isolate 3), in 6 of 10 pullets from group 4 (inoculated with isolate 4), in 5 of 10 pullets from group 5 (inoculated with isolate 5), and in 6 of 10 pullets from group 6 (inoculated with isolate 6). Small intestines contained watery content in 4 of 10 pullets from group 2. Microscopic lesions observed in control and challenged chickens included mild-to-moderate lymphoplasmacytic and heterophilic inflammation in the lamina propria, mucosal hyperemia, mucosal hemorrhages, and mild-to-moderate parasitism by *Eimeria acervulina*. Characteristic microscopic lesions of FDN (necrosis and loss of enterocytes from villus tips, with fibrinoheterophilic exudate in the lumen) were not observed in challenged birds. There were no statistically significant differences in microscopic lesion scores between groups ($p > 0.05$; Supplementary Fig. 2). All experimental groups were negative for *C. perfringens* by culture.

## Experimental trial 3

No overt clinical signs were observed in control and challenged birds. Small foci of mucosal erosion in the duodenal loop were observed in a few pullets inoculated with *netB*-positive *C. perfringens* isolates in all groups; 1 of 10 chickens in group 2 and 2 of 10 chickens in groups 1 and 3 had mucosal erosions in the duodenum upon autopsy. Other lesions observed included mucosal hyperemia in birds from group 1 (2 of 10 birds) and group 3 (6 of 10), and the presence of watery and frothy intestinal content in birds from group 1 (1 of 10), group 2 (5 of 10), and group 3 (6 of 10). Some sham-inoculated control birds (group 4) had mild mucosal hyperemia (1 of 10 birds) and small amounts of watery intestinal

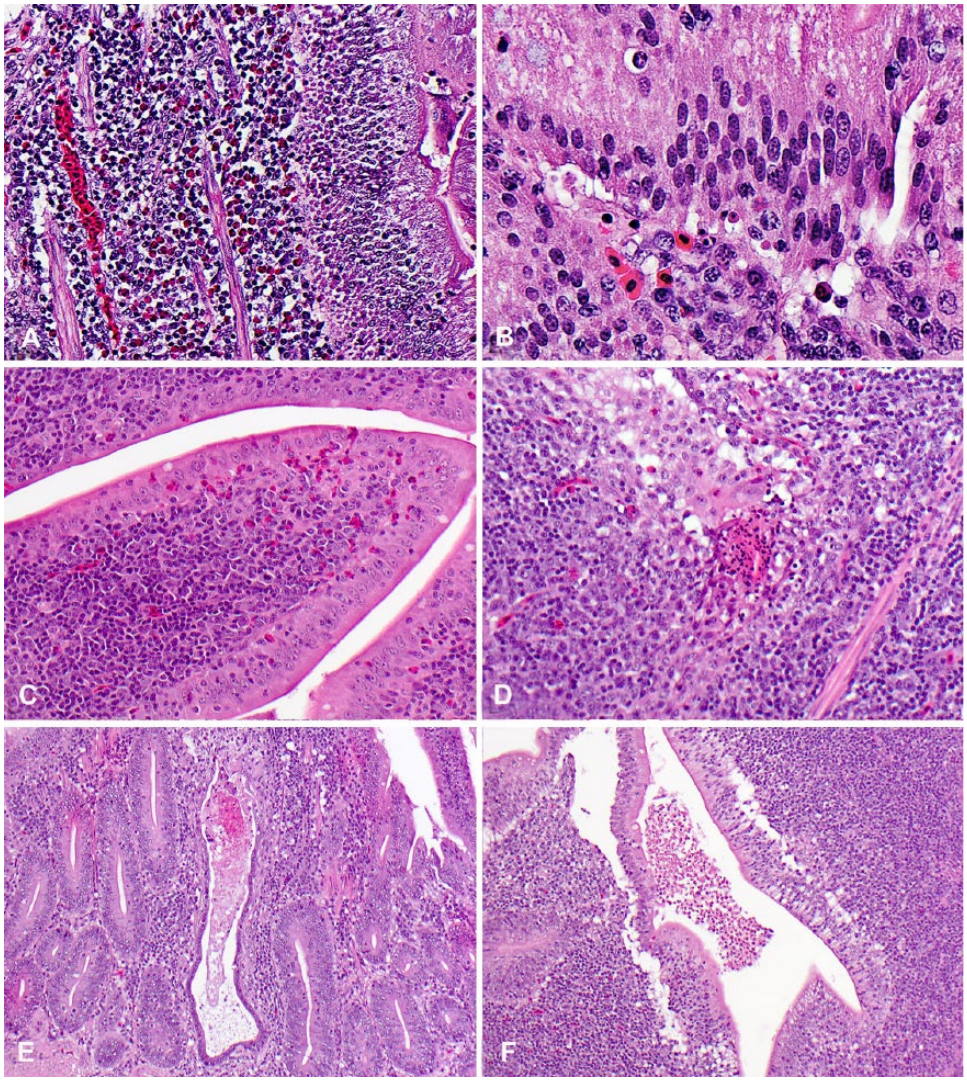


**Figure 2.** Duodenal lesions in chickens inoculated with *Clostridium perfringens*. H&E. **A.** Expansion of the lamina propria by heterophils and lymphocytes in a laying hen inoculated with *netB*-negative *C. perfringens* (experiment 1, group 2). **B.** Individual enterocyte necrosis and epithelial separation from the lamina propria in the duodenum of a laying hen inoculated with *netB*-negative *C. perfringens* (experiment 1, group 2). **C.** Expansion of the lamina propria by heterophils and lymphocytes in a laying hen inoculated with *C. perfringens* and duodenal homogenate (experiment 4, group 2). **D.** Small focus of epithelial necrosis at the base of the villus of a laying hen inoculated with *C. perfringens* and duodenal homogenate (experiment 4, group 4). **E.** Ectatic intestinal crypt containing necrotic cellular debris in a laying hen inoculated with *C. perfringens* and duodenal homogenate (experiment 5, group 3). **F.** Small numbers of heterophils and fibrin in a crypt of a laying hen inoculated with *C. perfringens* and duodenal homogenate (experiment 5, group 3).

content (2 of 10 birds). Erosions in the duodenal mucosa were not observed in sham-inoculated chickens.

Histopathology revealed duodenal lesions in pullets challenged with *netB*-positive *C. perfringens* isolates. Lesions in these birds included mild-to-moderate lymphoplasmacytic and heterophilic inflammation in the lamina propria, mucosal hyperemia, and mild-to-moderate mucosal hemorrhage. Characteristic microscopic lesions of FDN (necrosis in villus tips with fibrinoheterophilic exudate in the lumen) were not observed in challenged birds. Statistically significant differences in microscopic lesion scores were only observed between group 3 (inoculated with isolate 5) and group 4 (control group; $p \leq 0.05$; Supplementary Fig. 3). Duodenal lesions from 2 of 10 birds in group 2 (inoculated with isolate 3) and from 3 of 10 birds in group 3 (inoculated with isolate 5) were culture-positive for *C. perfringens*. All isolates were confirmed as *C. perfringens* by RapID ANA II and PCR, positive for the *cpa* gene, but negative for the *netB* gene by PCR.

## Experimental trial 4

Egg layers challenged with a combination of *C. perfringens* and duodenal homogenate had a sharp drop in daily egg

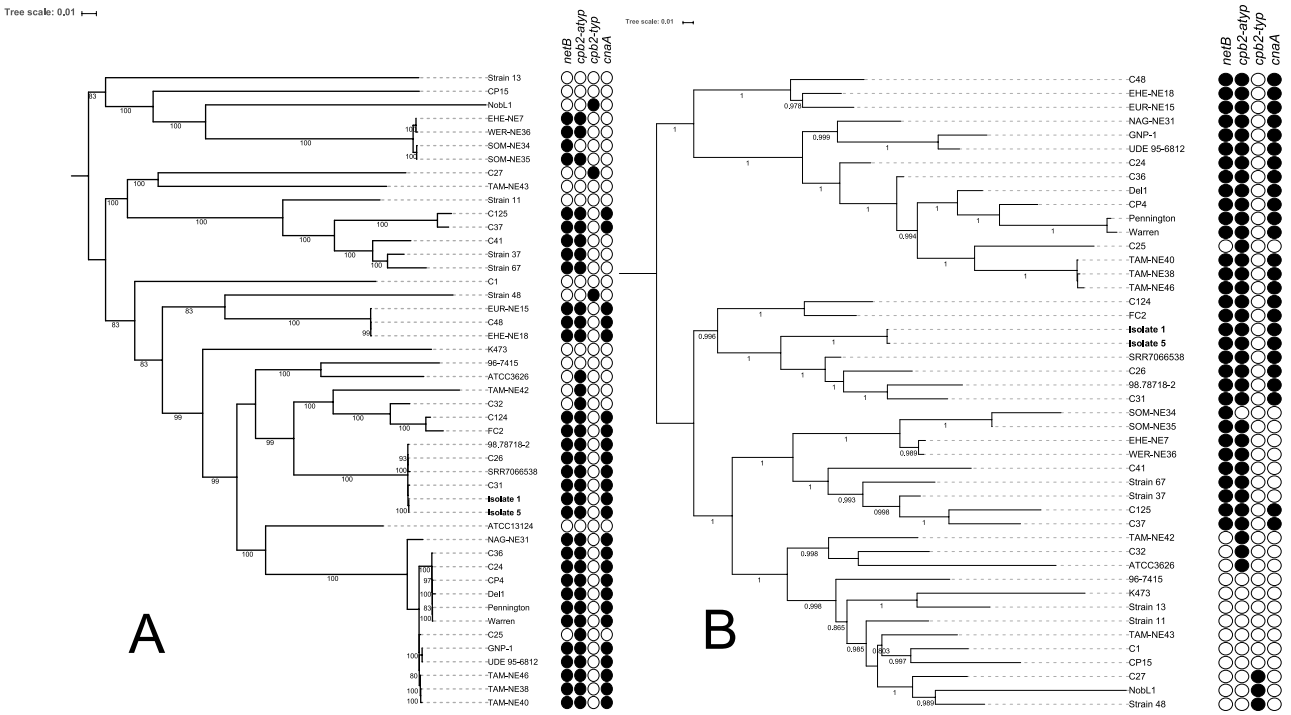


**Figure 3. A.** Maximum-likelihood tree of the core genome phylogeny of 47 isolates of *Clostridium perfringens*. The tree was inferred from 60,779 single nucleotide variants and built using IQ-TREE, using the general-time reversible model (GTR+f+G4) with 1,000 bootstrap 6 replicates. Scale bar shows the nucleotide divergence. Key accessory genome elements, including *netB* and *cnaA*, are shown (dot = present; open circle = absent). The core genome alignment was built using snippy against ATCC13124 as the reference genome. Isolates 1 and 5 share a monophyletic clade with isolates C31, C25, 98.787.18-2, and SRR7066538. **B.** Pan-genome tree showing the accessory genome relationships of 47 isolates of *C. perfringens* based on the presence and absence of accessory genes. Scale bar shows divergence. The presence of key accessory genome elements, including *netB* and *cnaA*, are shown (dot = present; open circle = absent). Isolates 1 and 5 form an individual monophyletic clade, with the nearest isolates C31, C25, 98.787.18-2, and SRR7066538 closely corresponding to the core genome maximum-likelihood tree. Isolates 1 and 5 are distinctly placed within other *netB*-positive strains isolated from chickens, suggestive of isolate 1 and isolate 5 being typical pathogenic chicken strains.

production (from 100% to 40% in group 2, and from 95% to 44% in group 4, at 7 d post-inoculation [dpi]), regardless of diet provided. Egg production started to drop at 4 dpi. Sham-inoculated control layers in groups 1 and 3 had 100% egg production at 7 dpi. Other clinical signs were not observed in control and treatment birds. Red foci in the mucosa of the duodenal loop were observed in some challenged chickens from groups 2 (4 of 10 birds; Fig. 1C) and 4 (2 of 9 birds) on autopsy day 8. Other intestinal lesions in challenged birds included thickening of the intestinal wall and small intestinal watery and frothy contents (Fig. 1D). There were no significant gross lesions in the intestines of sham-inoculated control birds from groups 1 and 3.

Histopathology revealed duodenal lesions in groups challenged with a combination of *C. perfringens* and duodenal homogenate (groups 2 and 4). Lesions observed in these birds included mild-to-moderate lymphoplasmacytic inflammation in the lamina propria with variable numbers of heterophils (Fig. 2C), as well as hyperemia, mild hemorrhages, and necrosis of individual enterocytes in some birds. Cystic crypts and crypt necrosis were observed in chickens from groups 2 (7 of 10 birds) and 4 (7 of 9 birds) on autopsy day 8. A focus of epithelial necrosis at the base of the villus was observed in 1 bird from group 4 on autopsy day 4 (Fig. 2D). Challenged birds had higher microscopic lesion scores on autopsy day 8. Treatment groups had significantly higher microscopic lesion scores than sham-inoculated groups. Statistically significant differences in microscopic lesion scores between groups 1 and 2 and between groups 3 and 4 were observed on autopsy days 4 and 8 ($p \leq 0.01$; Supplementary Figs. 4, 5). There were no significant differences ($p \leq 0.05$) in microscopic lesion scores between groups 1 and 3 and between groups 2 and 4.

Duodenal lesions from 5 of 20 birds in group 2 and from 3 of 19 birds in group 4 were culture-positive for *C. perfringens*. The isolates were confirmed as *C. perfringens* by RapID ANA II, and the *cpa* and *netB* genes were detected by PCR. Selected isolates recovered from lesions were identical, as determined by PFGE, to the *netB*-positive *C. perfringens* isolate 5 used in the inoculum. Genome sequence analysis revealed *C. perfringens* isolate 5 as a pathogenic clade 1 strain, closely related to *C. perfringens* isolate 1 (Fig. 3), even though these isolates were cultured from chickens in different states. Like *C. perfringens* isolate 1,

isolate 5 encodes a typical *netB*-encoding plasmid, a *cpb2*-tetracycline combination plasmid, as well as a novel pCP-13 –like plasmid with a highly divergent sequence of unknown function. Isolate 5 also encodes *pfoA*, an atypical *cpb2* gene, the *VR-10B* adhesion locus, and *cnaA*.

### Experimental trial 5

No clinical signs were observed in control and challenged birds. Small red mucosal erosions in the duodenal loop were observed in 1 of 10 birds from treatment groups 1–3. Mucosal erosions were not seen in sham-inoculated chickens. Other lesions observed included mucosal hyperemia in birds from group 1 (1 of 10 birds), group 2 (4 of 10), and group 3 (4 of 10). Intestinal contents were watery and frothy in challenged chickens from group 1 (4 of 10 birds), group 2 (6 of 10), and group 3 (3 of 10). Some sham-inoculated control birds had mild mucosal hyperemia (1 of 10 birds) and small amounts of watery intestinal content (2 of 10 birds). A few sham-inoculated control birds (group 4) had mild mucosal hyperemia and small amounts of watery or frothy intestinal content.

Histologic lesions were present in all challenged birds and included mild-to-moderate lymphoplasmacytic and heterophilic inflammation in the lamina propria, mucosal hyperemia, mild mucosal hemorrhage, and individual enterocyte necrosis. Cystic crypts and crypt necrosis were observed in challenged birds from group 2 (5 of 10 birds) and group 3 (7 of 10 birds; Fig. 2E). Chickens challenged with *C. perfringens* alone (group 1) did not have intestinal crypt lesions. One chicken from group 3 had small amounts of heterophils and fibrin (Fig. 2F). Statistically significant differences in microscopic lesion scores were observed between control and treatment groups (Supplementary Fig. 6). Microscopic lesion scores among the challenge groups were not statistically different ($p > 0.05$). Numerically higher microscopic lesion scores were observed in the group inoculated with a combination of *netB*-positive *C. perfringens* and duodenal homogenate (group 3). Duodenal samples in experiment 5 were negative for *C. perfringens* by bacteriology.

## Discussion

Although mild lesions were observed in the intestine of most chickens from all of our experimental trials, none of the characteristic microscopic lesions seen in field cases of FDN[4] were seen in any of our birds—namely no significant necrosis, loss of enterocytes from villus tips, or fibrinoheterophilic exudate in the intestinal lumen. The cause of the failure to reproduce full-blown FDN remains undetermined.

Intralesional adherence of antibody-positive *C. perfringens* bacteria to the duodenal mucosa is a common characteristic finding in field cases of FDN,[4] and it was previously suggested that this may be necessary for upregulation of toxin production and induction of more significant intestinal lesions.[13,17] We did not observe bacterial adherence to intestinal mucosa in duodenal lesions culture-positive for *C. perfringens*, and it is likely that this lack of adherence was at least in part responsible for the lack of significant lesions in our study. The mechanism of intestinal adherence of *C. perfringens* is not fully understood, but the *VR-10B* adhesion locus has been identified as important for adherence of this microorganism to intestinal cells and extracellular matrix proteins.[8,17] Two of our isolates encoded the *VR-10B* adhesion locus and it is therefore puzzling that no adhesion was observed. It is possible that other yet unknown factors, in addition to this locus, are required for adhesion.

Differences in the presentation and severity of duodenal lesions between our study and spontaneous cases of FDN might be explained, at least in part, by the absence of a specific predisposing factor, as well as differences in housing conditions, diet, and management practices in our challenge studies that may not have reproduced exactly the field conditions in flocks affected with FDN.

Another explanation for our limited success in inducing typical FDN microscopic lesions is that another infectious agent may be involved in the pathogenesis of FDN. In support of this possibility, we observed a drop in egg production and an increase in frequency and severity of duodenal lesions in chickens co-inoculated with duodenal homogenates. This suggests that a yet unidentified agent present in those homogenates may have acted together with *C. perfringens* in the production of those clinical signs and lesions. Furthermore, microscopic and ultrastructural evaluation of FDN lesions have revealed the presence of filamentous gram-negative, rod-shaped bacteria in samples with characteristic lesions,[4] and this bacterium seems to be capable of invading the intestinal epithelium (Franca M, et al. Histopathology and ultrastructural pathology of focal duodenal necrosis. Proc Am Assoc Avian Pathol Conf; 2018 July 14–17; Denver, CO). Identification of this gram-negative bacillus might help in determining its role in the pathogenesis of FDN.

Although our study showed an association between *C. perfringens* and the development of some FDN lesions in chickens, full-blown disease was not achieved, suggesting that other factors in addition to *C. perfringens* are needed for the production of this disease. The development of a challenge model for FDN may require a modification of the experimental design, coinfection with another infectious agent, or a predisposing factor that still needs to be determined.

#### Declaration of conflicting interests

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be interpreted as a potential conflict of interest.

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## Supplementary material

Supplementary material for this article is available online.

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