FOCAL DUODENAL NECROSIS: CHARACTERIZATION OF THE EPIDEMIOLOGY, BACTERIOLOGY AND EVALUATION OF THE PATHOGENICITY OF *CLOSTRIDIUM PERFRINGENS* FIELD ISOLATES IN COMMERCIAL EGG-LAYING CHICKENS

by

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(Under the Direction of MONIQUE FRANCA)

ABSTRACT

Focal duodenal necrosis (FDN) is an intestinal disease observed in egg-laying chickens, characterized by multifocal mucosal erosions mainly observed in the duodenal loop. It has a worldwide distribution and it is considered one of the top 5 disease concerns of the table egg layer industry in the USA. Affected flocks exhibit lower egg case weights and sometimes a drop in egg production. Previous studies have associated this condition with different *Clostridium* species: *C. colinum* and *C. perfringens*. In order to determine the role of *C. perfringens* type A in the pathogenesis of FDN, *C. perfringens* isolates from affected FDN birds were characterized.

The main objective of this study was to better understand FDN by performing field visits, collecting samples and to provide a preliminary field data evaluation based on an electronic survey to gauge knowledge toward the management, diet and disease prevention and control of FDN affected flocks.

The second objective in this work, was to try to reproduce FDN by experimentally infecting egg laying chickens with different *Clostridium perfringens* isolates. The results of this work indicate that *C. perfringens* is capable of inducing mild lesions in the intestine of egg laying chickens, however, mucosal necrosis as seen in field conditions was not observed. The reproduction of FDN with characteristic lesions as seen in the field may require the presence of other infectious agents or predisposing factors that are still undetermined.

INDEX WORDS: Focal Duodenal Necrosis: FDN, *Clostridium perfringens* type A, oral challenge, cross-sectional profile, egg laying chickens

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Focal duodenal necrosis (FDN) is an intestinal disease of egg-laying chickens (3, 7) that results in economic losses in the table-egg industry (3). The economic impact of this condition is due to reductions in egg case weighs and drops in egg production (3, 8). FDN was first described by Dr. Patricia Dunn in 1996 (4) in a cage-free layer flock. Since then, it has been detected in all major genetic lines of laying chickens raised in different management systems; cage, cage-free and organic (13, 14). This disease has a worldwide distribution and it has been diagnosed in most of the states in the US, Canada and Europe (6, 8, 10, 12).

Despite the fact that the disease was first described in 1996, the etiological agent of FDN remains unknown (7). However, the disease has been associated with *Clostridial* species (1, 5, 11). A non-culture molecular profiling study of the duodenal microbiota showed some differences in the molecular microbial profiles of layers with and without duodenal lesions, where the affected group had a higher *C. colinum* prevalence (11). In one report in the Netherlands, NetB and beta2 producing *C. perfringens* were isolated from duodenal lesions present in different layer hen flocks with FDN (1). More recently, a study at the University of Georgia reported the presence of *Clostridium perfringens* and its toxins in FDN lesions (5).

Clostridium perfringens-associated enteritis are commonly multifactorial diseases that involve the combination of infection with virulent *Clostridium perfringens* strains and the presence of environmental, dietary and other pathogenic factors that may predispose to disease (9), (2).

In an effort to better understand the epidemiological and pathogenic characteristics of this disease the following studies were assessed in the present project:

1. A descriptive epidemiologic study was performed in FDN affected flocks in order to obtain reliable information regarding housing, rearing, management, nutrition, health status and methods used for disease prevention and control.
2. Sampling of farms for bacteriology, histopathology and immunohistochemistry was performed in order to evaluate clinical characteristics of flocks affected with FDN.
3. *In vivo* pathogenicity study with *Clostridium perfringens* strains isolated from affected flocks was performed to evaluate the virulence and ability of *Clostridium perfringens* to induce disease.

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CHAPTER 2

LITERATURE REVIEW

Focal Duodenal Necrosis (FDN) is an intestinal disease of layers. It is considered one of the top five disease concerns of the table egg layer industry. The economic impact of this condition is due to reductions in egg case weighs and drops in egg production in the laying hens. Affected birds may have subclinical disease or show unspecific symptoms such as pale combs and poor body weight. The lack of clinical symptoms can lead the diagnosis of FDN to be missed in many flocks.

HISTORY

Focal Duodenal Necrosis was first described by Dr. Patricia Dunn in 1996 (18). This enteric disease of table egg laying chickens was first described in a cage-free layer flock at the Animal Diagnostic Laboratory at Pennsylvania State University (27). Since then, the disease has been diagnosed in most of the states in the US and Europe (27).

In 2005, an enteritis in the proximal part of the small intestine, similar to Focal Duodenal necrosis, was described in layer hens in the Netherlands (34). In 2013, Dr. Rubinoff recognized FDN in the United Kingdom and Sweden (61). In 2014, the disease was first reported in Canada in the states of Ontario and Saskatchewan (29),(45).

EPIDEMIOLOGY

Focal Duodenal Necrosis has been described with a similar flock-level prevalence in Europe (12.3%), Canada (14%) and USA (13%) for more than 15 years (61),(22). In the UK, FDN

has been observed in pullets and layers, however, hens from 20 to 29 weeks of age have the highest prevalence (46%) (61).

Likewise, this disease has been diagnosed in pullets from 14 weeks of age and egg laying hens in most states around the USA (20). The most affected region is the Northeast with 16.1% disease prevalence, followed by the Central region with (14.8%) and the Southeast (7.5%) (78). Although FDN affects all major genetic lines of laying chickens raised in different management systems; cage, cage-free and organic (8), FDN can be observed with higher prevalence in organic farms (18%) comparative to non-organic (10.8%) (78).

ETIOLOGY

Despite the fact that the disease was first described in 1996, the nature of the infectious agent has not been identified (27). Previous studies, however, have associated FDN with *Clostridia* species (67),(19), (3). In one study conducted in the Netherlands, NetB and beta2 producing *C. perfringens* were isolated from multifocal duodenal necrosis lesions. The affected flocks presented abnormal egg production performance and had poor body condition (3). More recently, Franca et al., reported an association between *Clostridium perfringens* and FDN. In this study, *C. perfringens* was cultured from lesions and the presence of alpha and beta2 toxins were detected in duodenal samples from layers with FDN by immunohistochemistry (19). *Clostridium colinum* has also been associated with focal duodenal necrosis in chickens. Non-culture molecular profiling study of the duodenal microbiota showed some differences in the molecular microbial profiles of layers with and without duodenal lesions, where the affected group had a higher *C. colinum* prevalence (67).

- ***Clostridium perfringens***

Clostridium perfringens is the causative agent of clinical and subclinical necrotic enteritis in poultry (80). It belongs to the phylum *Firmicutes*, class *Clostridia*, family *Clostridiaceae* and genus *Clostridium(83)*. Based on the 16S rRNA sequence, the species *Clostridium perfringens* belongs to the phylogenetic group cluster 1 or *Clostridium sensu stricto* (57),(14).

The first *Clostridium perfringens* fully genetically characterized (strain 13) is composed by 3,031,430 bp and 2,660 predicted open reading frames (ORF). The chromosome consists 10 rRNA, 98 species of tRNA and 28% content of guanine-cytosine (G-C). The plasmid has a size of 54,310 bp containing 63 ORF and 25.5% content of G-C. The genome possesses a 27kb phage and 11 transposon genes (65). *Clostridium perfringens* genome encodes for numerous enzymes that are involved in the glycolysis and glycogen metabolic pathways and lacks genes for tricarboxylic acid cycles that control the respiratory chain. When compared to saccharolytic enzymes, few genes have been identified in the amino acid synthesis which reveal an important growth limitation for *C. perfringens* (65),(72). Virulence genes have been identified in the genome and are designated as hemolysins, enterotoxins, fibronectic-binding protein, proteinase genes, perfringolysin O, collagenase, phospholipase C, sialidase, protease and hemagglutinin (65) (42).

Clostridium perfringens is a moderate obligate anaerobic, Gram-positive rod-shaped bacterium with approximate size of 0.8-1.5 μm diameter and 2-4 μm by up to 20 μm long and rounded ends, cells are commonly seen single or in pairs (32, 40, 83). The size of the bacilli depends on the kind of carbohydrate present in the growth media, which varies between short rods in the presence of glucose or long bacilli in the presence of starch (82). Colonies are 2-5 mm in diameter, translucent, round, flat, raised, and gray to grayish in appearance (83), (32), (56). It produces alpha, delta and theta hemolysis, depending on the *Clostridium perfringens* type and the species of blood agar used.

Most of them produce a double zone of hemolysis composed by completed hemolysis due to theta toxin and an incomplete one due to the alpha toxin (83), (56), (11). Minimizing oxygen, a growth limiting factor, is critical for culturing *Clostridium perfringens.* Reducing agents, such as thioglycolate and L-cysteine are commonly used to help maintain a low oxidation-reduction potential (E_h). Ideal E_h limits are about -50mV and +500mV (40), (58). In Peptone Yeast Glucose (PYG) broth, *C. perfringens* cultures are turbid with a sediment at pH of 4.8 to 5.6 (83). Ideal growth temperatures depend on the *Clostridium perfringens* type; for types A, D and E it is 45°C and for B and C it is 37 to 45°C. In general, most of the strains grow at 20-50°C (83), (82). The optimum pH for growth is between 5.5 and 8 (83). Growth is stimulated by fermentable carbohydrates and inhibited with sodium chloride at a concentration of 5-6 %(83), (82). Due to the inability of *C. perfringens* to synthesize 13 essential amino acids, strains cannot grow in environments where the amino acid source is not suitable (65).

C. perfringens is spore-forming and non-motile with a polysaccharide capsule (83), (32). The capsule has an unknown function, but it is believed to be crucial in the prevention of phagocytosis (32). Endospores are large, oval, central or sub terminal and distend the cell, however, those are not commonly seen *in vivo* or *in vitro* (83). As a result of anaerobic metabolism fermentation, the following are produced: lactate, alcohol, acetate and butyrate; butyrate being the major fermentation product. *C. perfringens* is glycolytic and utilizes sugars such as amygdalin, cellobiose, fructose, galactose, glycogen, inositol, inulin, lactose, maltose, melibiose, raffinose, ribose, sorbitol, starch, sucrose and trehalose as a source of energy (83), (65). There are also peptolytic pathways that use serine and threonine (65). *Clostridium perfringens* is a catalase positive organism and catalase attacks hydrogen peroxide resulting in production of abundant gas. Most of the strains produce lecithinase. Other enzymes are produced, including

deoxyribonuclease, acid phosphatase, ribonuclease, elastase, hyaluronidase, hemagglutinin, exo-β-D-galactosidase, ferredoxin-linked nitrate reductase and superoxide dismutase (83).

Clostridium perfringens is classified into 5 different groups according to the toxins being produced (69). These toxins are responsible for the tissue and cell damage in the host, and are usually produced during the exponential growth phase (52) (75). Seventeen toxins have been recognized. Based on the major toxins produced, *Clostridium perfringens* is classified into five types (A-E) (69), (75), (79). The four major toxins are known as alpha (CPA or PLC), beta (CPB), epsilon (ETX) and iota (ITX). Other toxins are; enterotoxin (CPE), beta 2 toxin (CPB2), necrotic enteritis beta-like toxin (NetB), perfringolysin O (PFO), toxin *perfringens* large (TpeL) and perfrin. (79).

Clostridium perfringens type A is composed by all strains that are able to produce alpha toxin (69). CPA is produced by almost all *Clostridium perfringens* strains, it is a 43kDa protein composed of 370 amino acids (79), it is considered a zinc phospholipase C enzyme and it has phosphatidylcholinase, sphingomyelinase and hemolytic activities (70). When *Clostridium perfringens* is cultured in egg yolk agar, the action of alpha toxin is observed by an increase in the agar turbidity, this reaction is known as The Nagler reaction (77). CPA is composed of two external domains known as N-terminal and C-terminal. The phospholipase C active site is located in the N-terminal domain, and the C-terminal is responsible for the binding of the toxin to the phospholipids of the host cell. The toxin binding is a calcium dependent process and is facilitated by the C-terminal domain, which is known as the principal immunogen domain protein (48, 75).

Once the alpha toxin binds to the binding site, a subsequent C-domain insertion into the cell membrane occurs. Then the N-domain initiates the hydrolysis of phosphatidylcholine and sphingomyelin in liposomes, resulting in the production of diacylglycerol and ceramide, and the

disruption of the cell membrane. This cell membrane disruption causes a subsequent activation of the arachidonic cascade, release of cytokines and superoxide production (75), (77, 79),(63).

These processes are zinc and calcium dependent (77). According to a recent study, endocytosis of alpha toxin is required for phosphorylation signaling activation which is followed by lysosomal damage and cell death (48).

The gene encoding for alpha toxin (*cpa*) is located in the chromosome close to the origin of replication (77). Transcription of *cpa* is regulated by the VirR/VirS system and Agr quorum sensing system (7),(49). The VirS is a sensor histidine kinase, and VirR is a response regulator, when VirS receives the signal from the environment, autophosphorylation of VirS occurs with the subsequence activation of VirS which leads to gene expression (49). The CPA translation is regulated by a small regulatory RNA known as a VR-RNA(79) (49).

CPA has been associated with gas gangrene in humans and with several enteric diseases in mammals, such as yellow lamb disease in sheep, enteritis, abomasitis and/or enterotoxaemia in cattle, horses, goats, dogs and pigs (69),(79). In poultry, it has been largely recognized that *cpa* strains are related to avian necrotic enteritis, and it has been considered as the main virulence factor associated with this condition (2), (23). However, pathogenicity pathways have not been identified yet (71). A recent study has identified that alpha toxin mutants are able to reproduce necrotic enteritis experimentally and concluded that CPA is not the major toxin causing this particular disease (37) (38).

CPB is produced by *Clostridium perfringens* B and C types (79),(69). Type B is associated with fatal hemorrhagic dysenteric syndrome in sheep. Type C is associated with enteritis necroticans in humans and with necrotic enteritis and enterotoxaemia in most livestock species (79), (69). CPB is a pore-forming protein with approximate size of 35 kDa. It is highly trypsin and

pepsin sensitive (62), (69). CPB induces pore formation in target cell membranes, creating a cellular osmotic imbalance, leading to cell lysis of intestinal endothelial cells (75) (30). The *cpb* gene is located in a 65 to 110 kb plasmid. This plasmid possesses a conjugation locus known as *tcp,* which indicates that it can be easily transferred. The expression of *cpb* is regulated by the VirS/VirR and Agr quorum sensing system (75).

ETX is associated with Clostridial enterotoxaemia in sheep and goats and less frequently in cattle (79), (69). ETX toxin is secreted by *Clostridium perfringens* types B and D (69), (75), (79). It is a prototoxin that is activated by the proteolytic removal of 14 N- terminal amino acid (69) (79). It belongs to the family of aerolysin-like pore forming toxins with a 29kDa size (75, 79). ETX is a necrotizing and lethal toxin (LD_{50} of 100ng/kg) and it is considered the third most potent of all *Clostridial* toxins after *Clostridium botulinum* and *C. tetanus*. The binding to the unknown cell receptor is mediated by the domain I, and the insertion of the pore membrane is facilitated by the amphipathic loop present in the domain II of the protein (75). Pore formation creates an osmotic imbalance with a subsequent loss of intracellular K^+ and increase of Cl^- and Na^+, resulting in a lysis, swelling and membrane disruption of the cell (79).

ETX gene (*etx*) is located on a conjugative plasmid and possesses a transposon gene *tpa,* this finding could explain the diversity of *C. perfringens etx* plasmids (75, 79). The *etx* regulation is still unknown but Agr-like quorum system is believed to be involved (75).

Iota toxin (ITX) is known as a Clostridial binary toxin. It is composed of two enzymatic toxins, Ia and Ib. The two toxin components are trypsin activated by the removal of 20kDa N-terminal peptide and 9-11 N terminal residues from Ia and Ib, respectively (79). The active Ib is responsible for the binding to the lipolysis-stimulated lipoprotein (LSR) receptor and then forms a heptamer to internalize the cell by endocytosis (75, 79). Once the endocytic vesicles are in the

cytoplasm of the cell, the component Ia is released. The release of Ia causes the depolymerization of ADP-ribosylation of G actin at Arg-177, resulting in the disorganization of intercellular junctions, cell rounding, leukocyte activation, inhibition of smooth muscle contraction, increase of permeability and cell death (75, 79).

Genes that encode for Iota toxins are *iap* and *iab* and are localized in a conjugative plasmid with approximate size of 97 to 135 Kb (79). *Clostridium perfringens* type E secretes iota toxin and is usually associated with enterotoxaemia in calves, lambs and rabbits, with a rare occurrence (75).

CPE is a 35kDa peptide with 319 amino acids. It is composed by a N-terminal domain that is essential for pore formation and cytotoxicity and, the C-terminal domain that binds to the receptor (79),(75). CPE is a pore forming protein and a member of aerolysin family (75). CPE toxin is secreted by accumulation in sporulating bacteria cells and is activated by trypsin or chymotrypsin by the removal of 24 or 36 N-terminal amino acids (53).

The gene that codifies for the CPE protein can be found in plasmids or in the chromosome (79). The *cpe* gen is highly conservative and is present in all *Clostridium perfringens* types expect for type B (1). In 80% of foodborne *Clostridium perfringens* type A isolates, *cpe* is located in the chromosome, while it is found in plasmids in non-food borne disease in animal or humans (53), (75). The *cpe* plasmids are found in *Clostridium perfringens* type C, D and A types. Some *Clostridium perfringens* type A isolates can carry the plasmids for *cpe* and *cpb2* simultaneously. Transferring of *cpe* is possible due to the presence of *tpc* conjugation locus (79). The expression of *cpe* occurs only during sporulation and is controlled by the Spo0A master regulation of sporulation and alternative sigma factors Sig F, Sig E and Sig K (1) (53), (75).

The cytotoxicity starts by binding of the CPE to the claudin receptors which are components of tight junctions of epithelial and endothelial cells present in the gastrointestinal tract

in jejunum, ileum and colon (75),(79). Binding to the receptors forms small and large complexes by the insertion of the β-hairpin loop present in the N-terminal domain of the CPE protein with a subsequent formation of the active pore (53), (75). The pore formation produces a calcium influx that activates calpain, causing cell apoptosis following by villus blunting, necrosis, epithelial desquamation and fluid accumulation in the lumen (1) . At high CPE concentration, the massive calcium entry into cells induces cell necrosis.

CPE is responsible for food poisoning in humans, being the second most common bacteria food borne disease in the USA (53), (75). CPE is particularly associated with hospital antibiotic associated diarrhea, chronic non-food borne diarrhea, infant death syndrome in humans and diarrhea in foals and piglets (53).

NetB named as Necrotic Enteritis Toxin B-like due to its genetic similarity with beta toxin (38% amino acid sequence). NetB is a pore-forming toxin and is considered a key virulence factor; essential to the induction of necrotic enteritis. It belongs to the Leukocidin/hemolysin family of toxins and it has a molecular size of 33 kDa (37), (36),(59). NetB consists of three domains, the β-sandwich domain, the rim domain and the stem domain (59). It produces cytolysis of cells by disrupting the phospholipid membrane, which changes the influx of ions (Na^+,Ca^{2+},Cl^-) and forms unregulated ion channels (75),(60). The pore formation initiates with the binding of the toxin to the cell receptor with a subsequent oligomerization of NetB into a pre-pore, followed by the insertion of the pre-pore into the host cell (75). In LMH cells, NetB produces rounding, cell lysis and hemolysis of red blood cells (RBC) (75),(60). However, the cellular receptor, target cell and the mechanism that the toxin utilizes to initiate and cause disease is still unknown (81).

The *netB* gene is located on the 42 KB locus (NeLoc-1) which is carried on an 85kb plasmid and is regulated by the VirS-VirR and Agr-dependent quorum systems. This suggests that once

Clostridium perfringens reaches the exponential growth phase in the intestine, the production of NetB is activated (36). The plasmid can be transferred from *C. perfringens* exogenous to *C. perfringens* endogenous which amplifies its virulence potential and makes colonization an unnecessary step in the disease pathogenesis (59) .

NetB is commonly found in necrotic enteritis affected chickens with a range of 60-90% but can also be isolated from healthy birds (36), (81). However, *netB* mutants lacking this toxin are unable to cause disease under experimental conditions; compared to *netB* positive strains that can cause disease in 45% of the chickens tested (75),(81) (39). These findings suggest that NetB plays the most important virulence role in the NE pathogenicity (75), (37),(59).

Beta 2 toxin (CPB2) was characterized in 1997 from a piglet with necrotic enteritis (26). It has been associated with enterotoxaemia and enteritis in many species including cattle, horses, goats, bears, poultry and humans (31), (44), (6), (12), (17), (64), (10), (19), (3). Beta 2 toxin can be secreted by all *Clostridium perfringens* types (79). Despite the nomenclature, this toxin has no significant homology with beta toxin, however, it shares biological activities, and it is believed to be a pore forming protein (26). Mature CPB2 has a sequence size of 28kDa, and the cytopathic effect is characterized by rounding and membrane disruption of I407 and CHO cell lines at >20μg/ml concentration (26), (79). It has been suggested that the disruption and pore formation in the cellular membrane are the principal mechanisms behind the cell lysis (21). However, the mechanism of cytotoxicity remains unclear (75).

The gene encoding for CPB2 is located in a large plasmid with variable size of 45 to 90kb. This plasmid, is the same plasmid encoding for ETX (type B and D strains) and CPE (some type A isolates). The *tcp* conjugation locus is only present in some of the *cpb2* type E plasmids. In Necrotic enteritis type A strains, *cpb2* plasmid is located on a conjugative plasmid distinct from

netB plasmid (21). The *cpb2* gene locus is conserved (1). VirS/VirR and the Agr Quorum sensing systems control the production of CPB2 (49).

The relation between the presence of *Clostridium perfringens* carrying *cpb2* and disease is still ambiguous. In horses, the presence beta2 toxin in intestinal lesions has been detected, indicated a direct implication of *cpb2* in disease (6).

In poultry, *cpb2 Clostridium perfringens* isolates have been found in healthy and diseased animals suffering intestinal disease including chickens, layers, turkeys, quail and psittacines (68), (19), (24), (25), (13), (41, 50), (16). However, some studies have reported an important role of CPB2 in enteric diseases in poultry. One report in chickens in intestinal ligated loop model showed higher histological necrotic enteritis lesions with *cpb2* positive when compared with *cpb2* negative strains (51).

Studies with layers have shown association between CPB2 and Focal Duodenal Necrosis (3, 19). One study in the Netherlands reported that nineteen out seventy-six diseased birds carried *C. perfringens* strains harbored *cbp2*, these isolates were able to produced beta2 toxin *in vitro*. In contrast, *Clostridium perfringens* was isolated from only four out of 15 SPF birds where only one isolate was *cpb2* positive and able to produce beta2 toxin *in vitro (3)*.

In a study performed in Georgia, USA, four *C. perfringens* strains were isolated from intestinal samples with characteristic microscopic lesions of FDN, all isolates carried *cpb2* gene and the presence of CPB2 toxin in the lesions was confirmed by immunohistochemistry (19).

Perfringolysin O also known as a theta toxin is a member of the cholesterol-dependent cytolysin family of toxins (53, 79). Mature protein is composed of 472 amino acids with 4 domains (79). The C-terminal region contains the domain 4, that mediates the binding of the toxin to cholesterol membranes (53), domain 3 consists of β sheets and α helices which facilitates the

penetration of the pore into the cell membrane (75). Once the toxin binds to the cholesterol receptor, domains 1 and 3 catalyze the oligomerization of the transmembrane hairpins to form the pre-pore (53) . The insertion of the transmembrane hairpins into the cell membrane leads the formation of the active pore (75). The large pores with approximate size of 25 to 45 nm, induce disruption of the cell membrane by colloid osmotic mechanism resulting in cell lysis. PFO is more active at low pH which means that is able to act in the cell membrane surface of glycosylated proteins and phagosomes (53).

Perfringolysin is produced by all the *C. perfringens* strains (79). The *pfo* gene is found in the chromosome (53) and the expression is regulated by the VirS/VirR and Arg-like Quorum Sensing systems(75).

It has been largely recognized the synergism between perfringolysin and alpha toxin in gas gangrene. At high concentrations, PFO is cytotoxic for leukocytes and macrophages, whereas at low concentration, mediates the superoxide anion production, reduces the migration of PMN/macrophages and decrease the blood supply into affected tissue contributing to cell damage (53). Although the role of PFO in intestinal disease is still poorly described, it is believed that PFO has a synergistic activity by increasing the cytotoxicity of ETX during enterotoxaemia in sheep and goats (79).

Toxin *perfringens* large or TpeL, as the name implies, is the largest *C. perfringens* toxins with an approximate size of 206 kDa. It belongs to the family of large Clostridial glycosylating cytotoxins (75). The protein is composed by three domains; the C-terminal is responsible for mediating the binding of the protein with the receptor, the enzymatic domain, constituted by glucosyltransferase which is located in the central core and the cysteine proteinase domain, that is involved in the membrane interaction with the host cell (54),(75).

This family of toxins enter the cell by endocytosis. At low pH, there is a channel formation in cell membranes characterized by a conformational chance that facilitates the entry and release of the endosome to the cytosol (54). The cytopathic effect of this toxin is produced by the inactivation of Rho proteins, causing damage of intercellular junctions, increase of cell permeability and cellular apoptosis (54),(75).

TpeL is encoded by a gene located in a large plasmid that is present in *C. perfringens* type A, B and C strains. TpeL is secreted during sporulation and its expression is regulated by the master sporulation regulator Spo0A and the sigma factor SigE (54).

TpeL has been associated with Necrotic enteritis type A strains in chickens (15). Significant differences were observed between chickens orally challenged with *TpeL-positive* and *TpeL* negative *C. perfringens* strains. In a necrotic enteritis *in vivo* study, the severity of the lesions was higher in the group challenged with *TpeL*-positive strains as compared with the TpeL-negative and the control group (15). The higher intestinal gross lesions correlated with significantly higher microscopic lesions and mortality observed (15). This data suggested that TpeL can act synergetic with other virulence factors to increase the pathogenicity of disease (15),(66).

Perfrin is a bacteriocin-like molecule produced by *C. perfringens*. It has an inhibitory growth effect to themselves or to other bacteria. In general, bacteriocins kill species that are closely related or those that share the same ecological niche (35). This novel protein was recently found in *netB* positive strains. The purified protein has an approximate size of 16 kDa and is composed by four transmembrane helices. The C-terminal domain contains a transmembrane helix typically found in the spore forming toxin family indicates that perfrin protein may be able to form a pore to penetrate the cell and induce cell lysis. The N-terminal domain is believed to confer protection

against other inhibitory molecules (76).

The fact that this bacteriocin has only been found in strains carrying *netB* gene, may indicate a correlation between perfrin and necrotic enteritis in chickens (76).

- ***Clostridium colinum***

C. colinum is the causative agent of ulcerative enteritis in chickens, quail, turkeys, grouse, partridge and other game birds (69). *C. colinum* has also been associated with focal duodenal necrosis in chickens. Non-culture molecular profiling study of the duodenal microbiota by denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and polymerase chain reaction (PCR) showed some differences in the molecular microbial profiles of layers with and without duodenal lesions, where the affected group had a higher *Clostridium colinum* prevalence (67). In contrast, a study at The Pennsylvania State University showed non-*C. colinum* DNA detection on fixed tissue lesions by 16S rRNA PCR assays (18).

It is a Gram-positive, anaerobic, motile, spore-forming bacillus belonging to the class *Clostridia* and the subcluster XIV-b based on the 16s rRNA sequence classification. It is closely related to *C. piliforme* (73).

C. colinum can be observed single or in pairs and are straight or curved rods with rounded ends (73). It has an approximate size of 1 x 3-4 μm, and observed spores are oval and subterminal. Colonies are pinpoint, hemolytic and 0.5 mm diameter (56). This organism has a complex nutritional requirement. To be able to grow, tryptose-phosphate agar has to be enriched with 0.2% glucose, 0.5% yeast extract and 8% horse plasma. However, growth can be achieved in Columbia or Brucella blood agar (73),(56). Ideally, plates must be pre-reduced during 24 to 48 hours at 35 to 42°C (73).

Clostridium colinum ferments glucose, mannose, raffinose, sucrose, trehalose, fructose and maltose. Mannitol is fermented in some strains. The fermentation products are acetic and formic acids (73). It can hydrolyze esculin and less commonly starch (9).

Ulcerative enteritis has been observed worldwide (73). It has been detected in different avian species, the most susceptible host is the bobwhite quail. While chickens are more resistant to the disease but other concomitant diseases such as coccidiosis, infectious bursal disease and chicken infectious anemia can predispose to infection (55).

Birds get orally infected by ingestion of contaminated feed, water or litter. The endospores are resistant to disinfectants which contributes to the persistency of *C. colinum* in the environment (55), (73).

Infection of quail can produce severe disease with high mortality, with or without apparent clinical signs (69). In chickens, clinical signs include hemorrhagic diarrhea, depression and emaciation with a 2-10% flock mortality (55).

Gross lesions are mainly observed in the gastrointestinal tract and are characterized by hemorrhagic enteritis that may be covered with diphtheritic membranes in the duodenum. The hemorrhages in the duodenum may result in ulcers that can affect the entire length of the small intestine and result in cecal necrosis and ulceration (55), (69). Severe ulcers in the intestinal lumen may perforate the serosa resulting in peritonitis and polyserositis (55). Lesions in the liver are less commonly found and are the consequence of bacterial migration through the portal circulation (73). When observed, they are yellowish to greyish with pinpoint to multifocal areas of necrosis that are sometimes surrounded by a yellow halo in the liver surface and the parenchyma. The spleen is usually enlarged and may be necrotic (55),(73).

Microscopic lesions vary with the severity of the disease. In the acute stage, there are focal to multifocal areas of ulceration of the intestinal epithelium that may be covered with a pseudomembrane. Edema, congestion and heterophilic inflammatory infiltrate are observed in the lamina propria. The intestinal lumen usually contains desquamated epithelium, red blood cells, large clumps of Gram-positive bacteria and fibrin (73). As the infection progresses, the areas of necrosis and ulceration can extend to the submucosa and muscularis. Necrosis of epithelial cells, lymphocytes and granulocytic infiltration are also observed around the ulcers (55),(73). Multifocal areas of coagulative necrosis and Gram-positive bacteria are commonly seen in liver lesions (73).

PATHOGENESIS

Focal Duodenal Necrosis does not produce characteristic signs of disease. When observed, the clinical sign most commonly detected in affected birds is pale comb (27). Pale comb may result from decrease in duodenal iron absorption resulting in anemia (8). Decrease in egg case weight appears to be the first production performance indicator of FDN presence in a flock. Affected flocks can have a weight reduction of 2- 2.5 pounds per case which represents 2.4 to 4 grams per egg. Lower egg production ranging from 1 to 10%, abnormal body weight gains up to 32 weeks of age and hens out of production have also been observed in affected flocks. Regardless of the enteric presentation of this disease, diarrhea and mortality are not typically observed (27), (8).

Gross lesions are characterized by single to multiple reddened to greyish mucosal erosions that are commonly covered with a yellow pseudomembrane with approximate size of 3 to 15mm. These lesions are frequently located in the duodenal loop but can extend to the proximal jejunum, and sometimes the mucosal erosions can be observed through the serosa (20), (27).

Early gross FDN lesions result in unspecific changes in the duodenal mucosa, such as abnormal increase of gas and multifocal areas of hyperemia (84). Although the severity of

macroscopic lesion varies, characteristic microscopic changes can be observed in the initial stage of the disease. Microscopic changes are described as necrosis and loss of the enterocytes from villous tips and infiltration of lumen with fibrinoheterophilic inflammatory cells, mixed with clusters of long rod-shaped bacteria, sloughed degenerated cell and red blood cells. Lymphocytes, heterophils and plasma cells are also observed in the lamina propria of the affected and necrotic villi (20). Large clumps of Gram-positive and Gram-negative rod-shaped bacteria may also be found in the exudate or attached to the villi (20).

DIAGNOSIS

FDN is a disease of commercial layers that can cause significant effects in production performance. Reduction in egg case weight and egg production drops are the most significant effects observed. Young flocks can have an abnormal egg weight increase, whereas mature flocks exhibit a drop in egg case weights (84).

Diagnosis of FDN should involve a combination of production performance evaluation and the detection of characteristic gross lesions within the duodenum and proximal jejunum of freshly dead or euthanized birds (33).

Routine monitoring of 5 to 10 birds is recommended for disease surveillance (8). There is a higher prevalence of FDN in birds with pale combs, therefore, selection of these birds for postmortem examination is recommended. Due to rapid autolysis of the intestinal tissue, necropsy has to be performed within 10 minutes to avoid mistaken interpretation of lesions (20). Duodenal samples with gross lesions should be collected for histopathology examination. Immunohistochemistry can also be performed for *in situ* detection of toxins produced by *Clostridium perfringens* (19).

PREDISPOSING FACTORS

- **Environmental contamination**

Clostridium perfringens is widespread in the environment. It has been found in soil, dust, small and large intestine of humans and animals (82). The ability of *C. perfringens* to form spores contributes to the persistence of this organism in the environment. Sporulation is an evolutionary process that guarantees the survival in wide and extreme environments. When conditions have deteriorated, bacteria trigger signal transduction pathways for the vegetative cell to differentiate into an endospore. In the initial sporulation stage the vegetative cell is divided into a larger mother cell and a smaller cell or prespore. The mother cell engulfs the prespore, providing nutrients and enabling the maturation of the small cell to acquire full resistance properties. The final stage is the release of the mature spore and lysis of the mother cell (74). The vegetative form is susceptible to environmental conditions, whereas endospores are resistant to drying, heating, irradiation and disinfectants (32).

In chickens, *C. perfringens* strains have been widely detected in the entire poultry vertical integration chain, including bacterial detection from breeder feces, fluff egg shells, grow-out farms and carcass rinses in the processing plant (68). Although *C. perfringens* is ubiquitous in nature and a normal inhabitant of the intestine, birds can get orally infected with virulent exogenous strains present in the environment resulting in the displacement of endogenous *C. perfringens* strains present as normal flora in the intestinal tract (4).

- **Nutritional factors**

Many dietary factors such as non-starch polysaccharides, feed with high protein concentration and anti-nutritional ingredients, can provide favorable conditions that enhance the overgrowth of *C. perfringens* (4).

In order to induce disease, pathogenic strains that infect birds must be actively proliferating (47). Extracellular matrix adhesive proteins are able to strongly bind to virulent strains providing an advantage for them to grow.

The degradation of feed components with high levels of soluble non-starch polysaccharides (NSP) such as wheat, rye, barley and oats result in an increase in intestinal viscosity with a consequent reduction in feed passage rate. The presence of these components in the intestine, represents an ideal substrate for bacterial growth (4),(47). NSP also bind to intestinal enzymes inhibiting its catalytic function and reducing the activity of pancreatic enzymes like trypsin that is known to have an antibacterial effect against *C. perfringens* toxins (4).

In addition, NSP can complex with glycoproteins present in the intestinal epithelium and induce mucin production. *Clostridium perfringens* genome encodes for numerous enzymes that are involved in mucin glycolysis, thereby enhancing bacterial proliferation (66).

Diets composed of high crude protein concentration such as fish meal, predispose to *Clostridium perfringens*-associated disease, the presence of glycine and methionine in these ingredients constitute a bacterial substrate, stimulating *C. perfringens* overgrowth and toxin production (4),(66). Furthermore, proteins are degraded to ammonia and amines which increase the intestinal pH (47). It has been recognized that the ideal pH for an optimal growth and production of *C. perfringens* is between 6.5 and 7.5, which indicates that an increase in intestinal pH enhances colonization and growth of *C. perfringens* (4).

Dried distiller's grains with soluble (DDGS) are high in dietary fiber (42%). Higher concentration of insoluble fiber stimulates mucin production resulting in an ideal substrate for *C. perfringens*. It has been reported that high concentration DDGS in the feed produce significantly higher intestinal lesions in necrotic enteritis challenge models (43).

The cleavage of trypsin to alpha, beta and beta2 toxins produces the inactivation of the toxicity activity. Anti-trypsin factors present in some dietary proteins such as soybean meal and potato protein also contribute to maximize bacterial proliferation (4).

Other anti-nutritional components are: lectins, protease inhibitors and tannins. Lectins are present in soybeans. Tannins from beans and most cereals (maize, oat, rice, rye, sorghum and wheat). Together, they are responsible for epithelial damage in the intestine, resulting in microbiota alteration (46). Protease inhibitors are found in heat-treated soy beans that can reduce the protein digestibility by increasing the concentration of nitrogenous products in the intestinal tract. The damage of the epithelial tissue and the alteration of microbial population increase *C. perfringens* proliferation (46).

Mycotoxins such as fumonisins are able to alter the bacterial profile by reducing the population of *Candidatus savagella* and *Lactobacillus spp*. and by promoting the growth of *C. perfringens* population (5).

- **Concomitant infections**

In general, any perturbation or damage to the intestinal epithelium will increase disease susceptibility. Viruses can produce villous atrophy reducing the capacity of absorption in the intestinal lumen which make carbohydrates and proteins more available for *C. perfringens* (4). Other immunosuppressive viruses such as Marek's disease virus, infectious bursal disease and chicken infectious anemia can increase the severity of enteric diseases (4), (47).

Parasitic infection can increase mucus production by direct irritation of the mucosa which can increase the proliferation of *C. perfringens* and toxin production. It is also well known that *Eimeria spp*. can produce necrosis of the intestinal villi, inhibition of the epithelial regeneration and a shift in microbiota population (4). Although some researchers have detected high coccidia counts in

feces from FDN affected flocks (18), the presence of coccidia in FDN lesions analyzed by histopathology is minimal (19). Protease inhibitors present in roundworms of the genus *Ascaridia* reduce the activity of trypsin, encouraging the growth of *C. perfringens* (4). However, field observations have not shown a strong association between nematodes and cestodes with FDN (18),(20).

CONTROL

FDN is usually controlled by prevention and treatment measures. Flock monitoring by posting sessions every 4 weeks is central for successful prevention of this disease. Probiotics, prebiotics, organic acids, yeast culture products and essential oils, are preventive options for control of FDN (8, 20, 27, 28). Antibiotics against Gram-positive bacteria such as bacitracin, chlortetracycline and tylosin have been shown to be effective in reducing the impact of the disease. House treatment within the flock with antimicrobial is recommended when lower egg case weight and poor egg production is detected. The treatment is prolonged until productive parameters are back to normal (8). Despite the use of antibiotics, recurrent infection is common after six to eight weeks (28).

Bacitracin had proven to be efficacious for prevention and treatment of FDN. It is widely used at 25 g per ton as a preventive medication from housing to 40 weeks of age (8). Probiotics at 0.5 lbs. per ton can also aid in disease prevention in some flocks(84).

Cleaning and disinfection have shown no effect in minimizing the negative effects of the disease, and FDN has been observed in complexes with optimal biosecurity levels (84).

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CHAPTER 3

DESCRIPTIVE ANALYSIS OF EGG LAYER FLOCKS AFFECTED WITH FOCAL DUODENAL NECROSIS BASED ON A QUESTIONNAIRE SURVEY[1].

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Key words:

Focal Duodenal Necrosis, commercial layer farms, epidemiological profile, FDN affected flocks, online questionnaire.

Abbreviations

FDN: Focal duodenal necrosis

ABSTRACT

Focal duodenal necrosis (FDN) is an intestinal disease of egg laying chickens, characterized by multifocal mucosal erosions in the duodenal loop and proximal jejunum. It is considered one of the top 5 disease concerns of the table egg industry in the US. Previous studies have associated this condition with *Clostridium* species. The purpose of this study was to investigate the epidemiological characteristics of table egg layer flocks affected with FDN. An online questionnaire was distributed to commercial layer operations in 7 different states in the USA. Layer farms that had diagnosed FDN within the past 12 months were surveyed. The questionnaire had 45 questions about management, nutrition, housing and methods for disease prevention and control. Thirty-seven surveys were sent, 21 were completed which represents a response rate of 56.7%. The survey results showed FDN in 5 egg layer strains of different ages. The pullets were cage-reared in all affected flocks and the majority of flocks in production were housed in traditional cages. Most of FDN affected flocks had more than 12 feed formulations from pre-lay to 60 weeks of age. Distiller's dried grains with solubles was a common ingredient added to the feed in the majority of affected flocks and all flocks were provided with limestone as a calcium source for egg production. The majority of surveys reported that coccidiosis and roundworm parasitism was not a problem in affected flocks in production. For disease prevention, bacitracin methylene disalicylate was the most common antibiotic used. These survey results provide a descriptive epidemiological profile of flocks affected with FDN and help to better understand this condition.

INTRODUCTION

Focal duodenal necrosis (FDN) is an intestinal disease that affects table egg layers (11) and represents one of the top five disease concerns for the table egg layer industry (27). FDN affects all major genetic lines of laying chickens raised in different management systems; cage, cage-free and organic (4). The economic impact of FDN is related to decrease in egg case weight and drop in egg production in affected flocks (4).

Despite the fact that the disease was first described in 1996, the causative agent of FDN has not been identified (11). However, *Clostridial* species have been associated with this condition (26), (8). A non-culture molecular profiling study of the duodenal microbiota showed some differences in the molecular microbial profiles of layers with and without duodenal lesions, where the affected group had a higher *C. colinum* prevalence (26). More recently, a study at the University of Georgia reported the presence of *Clostridium perfringens* and its toxins in FDN lesions (8). *Clostridium perfringens*-associated enteritis are commonly multifactorial diseases that involve the combination of infection with virulent *Clostridium perfringens* strains and the presence of environmental, dietary and other pathogenic factors that may predispose to disease (21), (1).

A web-based questionnaire was distributed among different layer operations in the US in order to determine the epidemiological characteristics of flocks diagnosed with focal duodenal necrosis. In order to obtain reliable information, surveys were sent to individuals most familiar with farm's management and operations.

The results of this survey serve as a preliminary evaluation of flocks affected with FDN in order to better understand this condition. Specifically, it helped determine the profile of affected flocks in terms of housing, rearing, management, nutrition, health status, and methods used for disease prevention and control.

MATERIALS AND METHODS

A preliminary survey was created, and a group of people representing academia, field veterinarians, nutritionists, statisticians, and production layer company representatives were assembled to refine the survey prior to distribution. Survey content was evaluated to consider whether each question was clearly stated and would provide accurate responses. After some modifications, the preliminary survey was tested online using Qualtrics software to identify any technical issues in the questionnaire and to estimate the average time to complete the survey.

The final web-based questionnaire consisted of 45 quantitative, qualitative and open questions that provided information about management, nutrition, prevention and control of focal duodenal necrosis.

Questions in the survey include the following topics:

- General questions: facility location, age at focal duodenal necrosis (FDN) outbreak and affected egg lines.
- Management: rearing conditions, pullet source, housing conditions and manure disposal type.
- Nutrition: type and levels of ingredients commonly added to diets in pullets and layers.
- Prevention and control: prevention program for FDN and concomitant diseases (parasites) and biosecurity (downtime, cleaning and disinfection).

In order to identify potential respondents, a data base was created. This data base consisted of 34 people including veterinarians, technical consultants and representatives from different layer companies.

The survey was electronically distributed via email; a brief message was added to thank participants for their collaboration and to explain the purpose of the research study. The email also summarized the instructions to take the survey and recognized the confidentiality of the respondents. A survey link, open to forwarding, was sent to different veterinarians and technical consultants working in the table egg layer industry. These people were asked to help get contacts or possible volunteers to participate in this study. A follow-up email and/or phone call was performed after the distribution in order to generate more responses. The original purpose of this research was to evaluate both affected and unaffected flocks in order to identify risk factors associated with Focal Duodenal Necrosis. A case control study was designed to obtain a farm-level comparison of case farms versus control farms. However, few control responses were obtained. The low response rate was attributed to the difficulties in the matching between case and control farms among the layer complexes. Most of the time these complexes had only case farms and not matching controls. Similarly, it was difficult to identify farms without the disease (control farms) because few unaffected farms routinely perform posting sessions to diagnose FDN. However, a descriptive analysis of affected flocks was performed. The population targeted consisted of layer farms that had diagnosed FDN within the past 12 months, flocks selected to be surveyed were monitored by posting sessions for FDN diagnosis. Respondents were individuals most familiar with the farm's management and operations.

RESULTS AND DISCUSION

A total of thirty-seven surveys were sent to farms that met the inclusion criteria and twenty-one were returned, with an overall response rate of 56.7%. The general characteristics of FDN flocks surveyed are summarized in table 3.1. Ten table-egg layer companies located in 7 different

states were represented from 21 surveys and the majority of responses were collected from Georgia and Nebraska with 5 responses each. Since only seven states in the U.S. were represented, the obtained responses may not characterize the profile of FDN-affected flocks in other states and countries.

The age of the flock at FDN outbreak ranged from 24 to 79 weeks of age and outbreaks were most frequently reported in flocks between 30 and 39 weeks of age (47.6%). This observation differs from field findings reported in Europe, where 45.7% of FDN outbreaks occurred between 20 and 29 weeks of age (14). Although FDN has been diagnosed in pullets in the US and Europe (4), FDN was not described in pullet flocks in this study.

Tables 3.2 and 3.3 summarize the characteristics of pullets and layers in flock affected with FDN, respectively. In this study, FDN was detected in 5 different egg layer lines and the highest percentage of FDN affected flocks housed Hy-line W-36 birds. This reflects the current situation of the table egg industry in the US, where the Hy-line W-36 is the dominant layer strain used in commercial egg production(24) . In the majority of FDN affected flocks, pullets were received from a different farm belonging to the same company. Birds were housed in traditional cage houses with stacked, "A" frame and enriched cage systems being represented. The downtime period ranged from less than seven to more than twenty-one days in pullet and layer houses. Nipple drinkers were used in the majority of pullet flocks and in all layer flocks. Fifty percent of the flocks were housed at farms with a capacity between 80,000 and 120,000 laying chickens. All flocks surveyed were maintained in cages during production, therefore, the question regarding the floor space provided in production was not applicable. This housing system also reflects the current situation in the US where the majority of table egg layers are raised in caged systems (2).

Manure handling methods included manure belts and deep pits in affected layer flocks

(Table 3.3). The frequency of manure removal from manure belts was every other day in about half of flocks and these manure belts were in a good condition in the majority of the flocks. Access to manure belts was described in the majority of FDN-affected flocks that used this method of manure handling. This may possibly expose the birds to *Clostridium* spp. present in fecal droppings (1). All flocks that used deep pits (high-rise houses) to handle manure, had a frequency of manure disposal from the pits after each flock was removed or more frequently.

The severity of problems with parasites and pest conditions in pullets and layers are described in table 3.4. Parasitism caused by roundworms and tapeworms was reported as not a problem in pullets and layers in the majority of affected flocks. Similarly, field observations have not shown a strong association between nematodes and cestodes with FDN (7),(9). Additionally, results from this survey do not show an association of coccidiosis with FDN since most flocks did not report coccidiosis as a problem in birds in production (Table 3.4). These results are also supported by our previous study which reported rare occurrence of coccidiosis in FDN lesions analyzed by histopathology (8). Nevertheless, a previous study reported high coccidia counts in feces from FDN affected flocks (7).

Fly infestation was described as a problem in layer flocks with low to moderate severity. It is known that necrotic enteritis outbreaks have been associated with vectors, specifically, one study in cage reared layers detected the presence of *Clostridium perfringens* in flies. Birds can get infected with *C. perfringens* by the ingestion of maggots or flies (6). Furthermore, it has been recognized that the *Musca domestica* can fly long distances (1-3 miles) which can contribute to the horizontal spreading of disease when these flies are infected (12). Moreover, rats and mice were described as problems with low to high severity in layer flocks affected with FDN. *Clostridial* species, specifically, *C. piliforme, C. difficile* and *C. perfringens* can cause spontaneous

gastrointestinal infections in rats and mice (25), (17). The mode of transmission of these *Clostridial* diseases is fecal-oral by the ingestion of spores from environment contaminated with feces. Since these *Clostridia* can affect avian species (10), rodents may possibly serve as carriers of these bacteria and may contribute to the transmission of *Clostridial* diseases in layer houses.

The nutritional characteristics of flocks affected with FDN are summarized in table 3.5. Distiller's dried grains (DDGS) were used in pre-lay and layer diets in the majority of affected flocks, with level of inclusion ranging from 1 to more than 10% (Table 3.5). Since DDGS can enhance intestinal proliferation of *Clostridium perfringens* and increase susceptibility to necrotic enteritis (16), the inclusion of DDGs in the diet may possibly be a predisposing factor for Focal Duodenal Necrosis. Frequent changes in feed formulation can also disturb the intestinal microbiota and predispose to Clostridiosis (21). Since more than 12 diets were provided to the hens during the production cycle in affected flocks surveyed, FDN-affected flocks may perhaps be more prone to develop *Clostridial*-associated enteritis. The survey results also showed that mycotoxins in diet are not a problem in half of FDN-affected flocks; however, since the mycotoxin status of the feed was unknown in 35% of affected flocks, the possible role of mycotoxicosis in the pathogenesis of FDN is still uncertain. Mycotoxins such as fumonisins can alter the intestinal microbiota by reducing the population of *Candidatus savagella* and *Lactobacillus spp.* and can promote the growth of *C. perfringens* (3).

All FDN-affected flocks were provided with limestone as a source of calcium during production. In layers, the requirement of calcium is calculated based on the flock age and egg production. Normally, hens with superior percentage of egg production require a higher content of calcium in the diet (13). It has been reported that the cytotoxic activity of *Clostridium perfringens* requires calcium for binding the toxin to the cellular receptor (22) and that high levels of calcium

in the diet produced higher mortality in a natural necrotic enteritis outbreak in broilers (23). As previously described, the FDN outbreaks were most frequently reported in flocks between 30 and 39 weeks of age, which is the period of peak and post-peak of egg productions. The fact that during this time, feed and calcium intake are highest, it is possible that high calcium intake might be a predisposing factor for FDN.

The methods used to prevent recurrent diseases and coccidiosis in FDN-affected flocks are described in table 3.6. It is believed that FDN can be prevented or controlled by in-feed administration of antibiotics against Gram-positive bacteria (9). Bacitracin is normally used for the prevention and treatment of FDN and it is widely used as a preventive medication from housing to 40 weeks of age in layer operations (4). Alternatives to antibiotics such as probiotics and enzymes can be also used to prevent the colonization, proliferation and persistency of *C. perfringens* (5). In this present study, the majority of FDN affected flocks used bacitracin as well as enzymes and probiotics for the control of recurrent diseases. Probiotics are live microbial food supplements that can benefit the host in terms of improving the intestinal immunity, producing antimicrobial molecules (bacteriocins) and competing against pathogenic bacteria (5). There are different probiotics commercially available that have proved to be efficient against *C. perfringens* and necrotic enteritis (15). In addition, the supplementation of enzymes in poultry diets facilitate the digestion of soluble non-starch polysaccharides making the nutrients more available for the birds in the intestinal tract (19).

However, the fact that affected flocks received the combined use of antibiotics, probiotics and enzymes suggest that these methods of disease prevention and control were not effective in terms of controlling FDN. Therefore, a better understanding of the relationship between management, nutrition, housing and biosecurity methods in affected layer flocks and the

improvement of these methods might be necessary to control the disease.

Table 3.7 describes the cleaning and disinfection procedures performed during downtime. The majority of flocks in this study disinfected feeders, cages, walls and ceilings after each flock, but, a comparable percentage of flocks never washed this equipment before disinfection. Disinfection without cleaning is not enough for the control of pathogens in a biosecurity program and cleaning prior to disinfection is recommended (20). By definition, pre-cleaning is the removal of organic material and pathogens without the addition of water. Emptying feeders can be considered as a pre-cleaning procedure and it was performed after two or more flocks in the majority of flocks. Washing of equipment was never performed in the majority of flocks, but dry cleaning was the alternative option used in almost all the flocks.

Less than fifty percent of the flocks washed and disinfected the water tanks after each flock, flushing of water lines was performed in most of the affected flocks and less than 60% of flocks disinfected those lines. When birds are drinking water from nipple drinkers, the water column moves, creating a movement of air in the water line which can introduce contaminated dust and pathogens in the water system (18). Likewise, cleaning and disinfection of water lines is extremely important to prevent intestinal diseases and to provide water free of pathogens to the new flock (18). For the reasons mentioned, water disinfection is essential in a poultry production facility.

Since birds can get orally infected with *Clostridial* species by ingestion of contaminated feed and water and *Clostridium spp*. can form endospores that are resistant to disinfectants, the procedures performed in affected flocks during the downtime may not be sufficient to prevent the persistency and transmission of the causative agent of FDN.

In conclusion, these survey results serve as an indicator of management, nutritional, sanitation and prophylactic characteristics that may possibly be associated with FDN. The management and health characteristics of FDN-affected flocks identified in this study require further evaluation to determine if these characteristics represent risk factors for this disease.

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Table 3.1. General characteristic of FDN affected flocks surveyed.

Characteristic	Total number of responses	Response	Number of responses (%)	
Farm location	21	Georgia	5	(23.8)
		Pennsylvania	4	(19)
(State)		Indiana	2	(9.5)
		Nebraska	5	(23.8)
		New Jersey	3	(14.3)
		California	1	(4.8)
		Minnesota	1	(4.8)
Age at Outbreak	21	Between 20-29 WOA	5	(23.8)
		Between 30-39 WOA	10	(47.6)
(Weeks)		Between 40-49 WOA	2	(9.5)
		Between 50-59 WOA	1	(4.8)
		Between 60-69 WOA	0	(0)
		Between 70-79 WOA	3	(14.3)

Table 3.2. Characteristics of Pullets in affected FDN flocks surveyed.

Characteristic	Total number of responses	Response	Number of responses (%)	
Source of the replacement pullets	21	Obtained from a different farm site, same company	17	(81)
		Purchased from a different company	3	(14.3)
		Raised on the farm site	1	(4.7)
Type of rearing	21	Cage reared	21	(100)
		Floor reared	0	(0)
Cage area (In^2/bird)	14	Less than 40	1	(7.1)
		Between 40 and 43	3	(21.4)
		Between 44 and 46	6	(42.9)
		Between 47 and 49	1	(7.1)
		More than 50	3	(21.4)
Cage system	20	Stacked cage	10	(50)
		A frame	10	(50)
Type of drinker	21	Nipple drinker	20	(95.2)
		Cup drinker	1	(4.76)
Downtime (Days)	21	Less than 7	4	(19)
		Between 7 and 14	4	(19)
		Between 15 and 21	4	(19)
		More than 21	7	(33.3)
		Do not know	2	(9.5)

Table 3.3. Characteristics of layers in FDN affected flocks surveyed.

Characteristic	Total number of responses	Response	Number of responses (%)	
Type of egg line	21	Hy-Line W36	9	(42.9)
		Shaver White	3	(14.3)
		Lohmann LSL-Lite	5	(23.8)
		Bovans White	3	(14.3)
		Isa White	1	(4.8)
Farm capacity	20	Less than 80,000	6	(30)
		Between 80,000 and 120,000	10	(50)
		More than 120,000	4	(20)
Housing type	20	Traditional Cage housing	19	(95)
		Enriched cage housing	1	(5)
Cage system	21	Stacked caged	9	(42.8)
		A frame	10	(47.6)
		Enriched cage housing	2	(9.5)
Cage area (In^2/bird)	16	67	12	(75)
		68	3	(18.7)
		More than 70	1	(6.3)
Manure handling method	21	Deep pit (high-rise houses)	12	(57.1)
		Manure belt	9	(42.9)
Frequency of manure removal from the belts	9	Daily	2	(22.2)
		Every other day	5	(55.5)
		Three times a week	1	(11.1)
		Every five days	1	(11.1)
Access of hens to the belts	9	Yes	8	(89.9)
		No	1	(11.1)
Conditions of the belts	9	Excellent	1	(11.1)
		Good	7	(77.8)
		Moderate	1	(11.1)
Frequency of manure disposal from the pits	12	After each flock removed or more frequently	12	(100)
Type of drinker	21	Nipple drinker	21	(100)
Downtime (Days)	21	Less than 7	9	(42.9)
		Between 7 and 14	6	(28.6)
		Between 15 and 21	2	(9.5)
		More than 21	4	(19.0)
Treatment in drinking water	21	None	10	(47.6)
		Acidifiers	8	(38.1)
		Chlorination	3	(14.3)

Table 3.4. Severity of problems with parasites and pest conditions in pullets and layers.

	Characteristic	Total Number of responses	Not a problem (%)	Low (%)	Moderate (%)	High (%)	Do not know (%)
Pullets	Mice	21		17 (81.0)	2 (9.5)		2 (9.5)
	Rats	21		13 (61.9)	4 (19.0)		4 (19.0)
	Flies	21		16 (76.2)	4 (19)		1 (4.8)
	Darkling beetles	21		14 (66.6)	1 (4.7)		6 (28.5)
	Roundworms (Ascarids, Capillaria)	20	15 (75)				5 (25)
	Tapeworms	21	12 (57.1)	3 (14.3)			6 (28.6)
	Coccidiosis	20	6 (30)	10 (50)			4 (20)
Layers	Mice	21		11 (52.4)	5 (23.8)	2 (9.5)	3 (14.3)
	Rats	21		10 (47.6)	4 (19)	2 (9.5)	5 (23.8)
	Flies	21		10 (47.6)	9 (42.9)		2 (9.5)
	Darkling beetles	20		13 (65)		1 (5)	6 (30)
	Roundworms (Ascarids, Capillaria)	21	17 (81.0)	1 (4.8)			3 (14.3)
	Tapeworms	21	9 (42.9)	6 (28.6)	1 (4.8)		5 (23.8)
	Coccidiosis	21	11 (52.4)	4 (19)			6 (28.6)

Table 3.5. Nutritional characteristics of FDN affected flocks surveyed.

Characteristic	Total number of responses	Response	Number of responses (%)	
Feed form	21	Mash	21	(100)
Ingredients used	21	DDGS	17	(81)
		Bakery Products	8	(38.1)
		Wheat	1	(4.8)
Level of inclusion of DDGS (percent)	21	1-5 %	10	(47.6)
		6-10%	4	(19)
		More than 10%	7	(33.3)
Animal protein sources	20	None	5	(25)
		Poultry meat and bone meal	7	(35)
		Bovine meat and bone meal	7	(35)
		Porcine meat and bone meal	3	(15)
		Feather meal	1	(5)
Vegetable protein sources	21	Soybean meal	21	(100)
		Canola meal	1	(4.8)
Supplements	21	Vitamins	19	(90.5)
		Enzymes	17	(81.0)
		Probiotic	14	(66.7)
		Saccharomyces cerevisiae	8	(38.1)
		Organic acids	5	(23.8)
		Mannan-oligosaccharide	3	(14.3)
		Prebiotics	2	(9.5)
		Essential oils	1	(4.8)
Diet for specialty eggs	21	No	18	(85.7)
		Eggland's best diet	1	(4.8)
		Antibiotic free	1	(4.8)
		Omega 3 enriched diet	1	(4.8)
Number of diet formulations	21	2-4	3	(14.3)
		5-8	1	(4.8)
		9-12	4	(19)
		More than 12	13	(61.9)
Mycotoxins in diet	20	None	10	(50)
		Do not know	7	(35)
		Aflatoxin	2	(10)
		T-2 toxin	2	(10)
		Deoxynivalenol	2	(10)
Calcium source	21	Limestone	21	(100)
		Egg shell	5	(23.8)

Table 3.6. Coccidiosis and recurrent diseases prevention methods.

Characteristic	Total number of responses	Response	Number of responses (%)
Coccidiosis Prevention/control method	19	None	2 (10.5)
		Amprolium	9 (47.4)
		Ionophores	5 (26.3)
		Vaccination	3 (15.8)
Coccidiosis vaccine	3	Coccivac	2 (66.6)
Coccidiosis application place	3	Hatchery	2 (66.6)
Ionophores	5	Salinomycin	3 (60)
		Monensin	2 (40)
Antibiotics for prevention of recurrent diseases	14	None	1 (7.1)
		Bacitracin	12 (85.7)
		Tylosin	2 (14.2)
		Chlortetracycline	1 (7.1)

Table 3.7. Procedures performed during the downtime.

Characteristic	Number of responses	Between each flock (%)	After two or more flocks (%)	Never (%)
Empty feeders	21		20 (95.2)	1 (4.8)
Wash feeders	21	2 (9.5)	7 (33.3)	12 (57.1)
Disinfect feeders	21	9 (42.9)	6 (28.6)	6 (28.6)
Wash water tanks	11	5 (45.5)	2 (18.2)	4 (36.4)
Disinfect water tanks	12	5 (41.7)	2 (16.6)	5 (41.7)
Flush water lines	21	17 (81)	3 (14.3)	1 (4.8)
Disinfect water lines	21	12 (57.1)	0	9 (42.9)
Dry clean (Blow down) cages, walls, ceilings	21	20 (95.2)	1 (4.8)	0
Wash cages, walls, ceilings	21	7 (33.3)	3 (14.3)	11 (52.4)
Disinfect cages, walls, ceilings	21	15 (71.4)	1 (4.8)	5 (23.8)
Fumigate cages, walls, ceilings	18	7 (38.9)	5 (27.8)	6 (33.3)
Scrape manure belts	9	8 (88.9)		1 (11.1)
Wash manure belts	9	5 (55.5)	1 (11.1)	3 (33.3)
Disinfect manure belts	9	6 (66.7)	2 (22.2)	1 (11.1)

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CHAPTER 4

HISTOPATHOLOGY AND BACTERIOLOGY CHARACTERIZATION OF COMERCIAL LAYER FLOCKS AFFECTED WITH FOCAL DUODENAL NECROSIS

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ABSTRACT

Focal duodenal necrosis (FDN) is an enteric disease of egg-laying chickens that results in economic losses in table egg layer's operation. The economic impact of this condition is due to reductions in egg case weighs and drops in egg production. FDN is characterized by multifocal mucosal erosions mainly observed in the duodenal loop. This disease has a worldwide distribution and it has been diagnosed in most of the states in the US, Canada and Europe. Previous studies have associated this condition with different *Clostridium* species: *C. colinum* and *C. perfringens*. The objective of this study was to investigate the bacteriological and histopathological findings in duodenal samples with FDN. Nine different table egg companies were visited for sampling. A total of 182 duodenal samples were collected from 34 flocks representing the states of Georgia, Indiana and Pennsylvania. Duodenal samples with the presence of FDN lesions were tested for the presence of *Clostridium spp.* by anaerobic bacteriology. Histopathology was also performed to confirm the presence of characteristic FDN microscopic lesions in the samples. Microscopic FDN lesions were observed in 7 out of 9 farms and rare occurrence of coccidiosis and cestodes were observed in samples with FDN lesions. *C. perfringens*-antibody positive bacteria were detected in all samples with characteristic FDN lesions. *Clostridium spp*. were isolated from 5 out 9 farms. A total of 37 *Clostridium* spp. strains were isolated and identified by RapID ANA II system; 17/37 *C. perfringens*, 12/37 *C. innocuum*, 2/37 *C. sporogenes*, 1/37 *C. bifermentans*, 1/37 *C. clostridioforme*, 1/37 *C. difficile*, 1/37 *C. limosum*, 1/37 *C. novyi*, 1/37 *C. paraputrificum.* Toxinotyping of *C. perfringens* strains by Polymerase Chain Reaction (PCR) was performed. Results showed that all *C. perfringens* isolates were positive for alpha toxin gene (*cpa*), 3/17 *C. perfringens* isolates were detected positive for the necrotic enteritis B-like toxin (*netB*) and beta2 toxin (*cpb2*) genes and 10/17 strains carried the *cpb2* gene. This research provides detailed

information about the bacteriological and histopathological findings associated with FDN and help us to better understand this disease.

Key words:

Focal Duodenal Necrosis, commercial layer farms, Clostridium perfringens, toxinotyping, netB, cpb2.

Abbreviations

FDN: Focal duodenal necrosis

cpa: Clostridium perfringens alpha toxin

netB: necrotic enteritis B like toxin

cpb2: Clostridium perfringens beta2 toxin

INTRODUCTION

Focal duodenal necrosis (FDN) is an enteric disease of egg-laying chickens that results in economic losses in table egg layer's operation (15),(5). The economic impact of this condition is due to reductions in egg case weighs and drops in egg production (5). FDN was first described by Dr. Patricia Dunn in 1996 (9) in a cage-free layer flock. Since then, it has been detected in pullets and layers from different genetic lines raised in different types of management systems; cage, cage-free and organic eggs (12). This disease has a worldwide distribution and it has been diagnosed in most of the states in the US, Canada and Europe (10), (32).

Although the etiological agent of FDN remains unknown, the disease has been associated with *Clostridial* species (29), (3), (11). One previous study described higher prevalence of *C. colinum* molecular markers in FDN affected birds (29). *Clostridium perfringens* has also been associated with FDN (3), (11). In one report in the Netherlands, NetB and beta2 producing *C. perfringens* were isolated from duodenal lesions present in different layer hen flocks with FDN (3). More recently, the presence of *C. perfringens* positive for alpha and beta2 toxins in duodenal samples from layers with FDN was detected by bacteriology, PCR and immunohistochemistry (11).

Affected birds may have subclinical disease or show unspecific symptoms such as pale combs and be underweight (15),(5). The lack of clinical symptoms can lead the diagnosis of FDN to be missed in many flocks (5). FDN diagnosis consisted of the detection of characteristic gross lesions within the duodenum and proximal jejunum of freshly dead or euthanized birds (18). Gross lesions are characterized by single to multiple reddened to greyish mucosal erosions that are commonly covered with a yellow pseudomembrane with approximate size of 3 to 15mm (15). Early gross FDN lesions result in unspecific changes in the duodenal mucosa, such as an abnormal

amount of gas and multifocal areas of hyperemia (35).

Flock routine monitoring of 5 to 10 birds is recommended for disease surveillance every 4 weeks (5). There is a higher prevalence of FDN in birds with pale combs, therefore, selection of these birds for postmortem examination is suggested. Due to rapid autolysis of the intestinal tissue, necropsy has to be performed within 10 minutes to avoid mistaken interpretation of lesions (12). Duodenal samples with gross lesions should be collected for histopathology examination. Immunohistochemistry can also be performed for *in situ* detection of toxins produced by *Clostridium perfringens* (11). Treatment of affected flocks with antibiotics against Gram-positive bacteria such as bacitracin, chlortetracycline and tylosin have been shown to be effective in reducing the impact of the disease (5). However, recurrent infection within the affected flock after treatment is common (5). Antibiotic alternatives including probiotics, prebiotics, organic acids, yeast culture products and essential oils are preventive option that have been also used (5, 12, 15, 16).

In this study, we evaluate the bacteriology and histopathology findings in duodenal samples of FDN to better understand the disease. Duodenal samples with the presence of FDN lesions were tested for the presence of *Clostridium spp*. by anaerobic bacteriology where all *C. perfringens* strains isolated from these lesions were molecularly characterized to evaluate virulence factors Histopathology was also performed to confirm the presence of characteristic FDN microscopic lesions in the samples.

MATERIALS AND METHODS

Sampling of farms

A total of 182 samples were collected from layer hens from 34 flocks in 9 layer farms located in Georgia, Indiana and Pennsylvania. Sampling was conducted between October 10th 2015 and November 9th 2016. The majority of samples were collected from flocks that exhibit lower egg case weights. Laying chickens with pale combs were euthanized for postmortem examination. Samples from unaffected birds from 6 farms were also collected for comparison.

Histopathology

Forty-six duodenal samples from FDN affected (focal to multifocal erosion in the duodenum) and 14 from unaffected birds were collected for histopathology. Collected samples were placed in 10% buffered formalin, embedded in paraffin and sectioned at 4 μm. Samples were stained with hematoxylin and eosin and examined by bright microscope.

Immunohistochemistry

Twenty-six paraffin-embedded tissue sections from samples with characteristic microscopic FDN (lymphoplasmacytic enteritis with heterophilic infiltration, loss of enterocytes and necrosis at villus tips and/or presence of fibrinonecrotic luminal exudate) were examined for detection of *Clostridium perfringens* cells as previously described by Franca et al. (11).

Bacteriology

Samples from duodenal segments with presence of gross lesions (focal to multifocal areas of mucosal erosion in the duodenum) and from unaffected birds were aseptically collected in sterile Whirl Pak bags and immediately placed in Gas Pak anaerobe pouches. (BD GasPak^{TM}, Sparks, Maryland, USA). Duodenal contents were anaerobically incubated at 37°C for 48 hours in pre-reduced trypticase soy agar (TSA) with 5% sheep red blood cells (Remel®, Lenexa, Kansas,

USA). Affected tissue was also anaerobically incubated at 37°C for 24 hours in thioglycolate broth media and plated into blood and PEA agars as previously described (11).

Hemolytic colonies were selected and plated in selective PEA media for aerobic and anaerobic incubations. Hemolytic strains obtained from the first four farms visited were further identified by RapID system. For the next farms visits, bacteria identification was assessed by focusing on *Clostridium perfringens* strains. Gram positive-anaerobic colonies resembling *C. perfringens* were identified by RapID^{TM} Ana II System (Thermo Scientific, Carlsbad, California, USA). Lecithinase activity was tested by cultivation on egg yolk agar.

Nineteen samples from unaffected birds were collected from farm 1 visit 2, farm 2 visit 2 and farm 3 for comparison (table 4.3). Since, hemolytic colonies were not isolated, further identification of non-hemolytic colonies was not performed.

Toxinotyping

All *Clostridium perfringens* isolates obtained from anaerobic bacteriology were toxin typed by Polymerase Chain Reaction (PCR). Two reference isolates (ATCC 3626 and ATCC 27324) were obtained from American Type Culture Collection (ATCC) and used as positive controls to determine the presence of genes coding for the toxins; epsilon (*etx*), iota (*iA*), enterotoxin (*cpe*), beta (*cpb*), and beta2 (*cpb2*) from *Clostridium perfringens.* Two field isolates were used for the detection of *netB* (CP#6) (17) and *tpeL*.

DNA Bacterial Extraction

Colonies were subcultured and anaerobically incubated at 37°C for 48 hours in pre-reduced trypticase soy agar (TSA) with 5% sheep red blood cells (Remel®, Lenexa, Kansas, USA). Three colonies were suspended in 500 µL of nuclease free water (Ambion® Nuclease-Free water,

Thermo Scientific, Carlsbad, California, USA). Bacterial DNA was extracted using QIAamp DNA Stool Mini Kit according to the manufacturer's recommendations.

Polymerase Chain Reaction

The genes *netB, tpeL, cpb, etx, iA* were amplified using primers described by Bailey et al. (4). Previously published primers by Bano et al. (24) were utilized for *cpa* detection (Table 4.1).

Multiplex PCR

Multiplex PCR to detect 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 2x GeneAmp® Fast PCR Master mix (Applied Biosystems™, Foster city, California, USA), 0.75µL of each pair of primers with final concentration of 0.25µM (*cpa, netB*), 10 µL of water and 2 µL of DNA template. PCR reaction was amplified using MyCycler™ Thermal Cycler (Applied Biosystems, Foster City, California, USA) 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 10s.

Single PCR

Single PCR for *tpeL* was performed under same conditions described before. The presence of *etx, iA* and *cpb* genes was detected by individual PCR using the same reagent concentration for each reaction with an annealing temperature of 61°C.

For *cpe* amplification, 25 µL of 2X FailSafe™ PCR premix (Epicentre, Madison, Wisconsin, USA), 1 µL of 0.25µM of each primer, 1 µL of FailSafe™ PCR enzyme mix (2.5U/ mL), 17 µL of nuclease free water and 5 µL of DNA sample. PCR was conducted with the following conditions; heating 95 °C for 10 s, 40 cycles of denaturation at 95°C for 5 min, annealing

at 55 for 30 s and polymerization at 72°C for 30 s with a final elongation of 6 min at 72°C with a MyCycler™ Thermal Cycler (Applied Biosystems, Foster City, California, USA).

Detection of *cpb2* was done using 2X Promega Master Mix, with 0.8 μM concentration of the primers and 2 μL of DNA template, with a final volume of 30 μL. PCR cycle parameters consisted of; 95°C of heating for 15 min, 35 cycles with; initial denaturing at 94 °C for 30 s, annealing at 55 °C for 90 s, elongation at 72°C for 90 s and final elongation at 72°C for 10 min.

PCR product was visualized by electrophoresis (100V constant voltage) in a 1.5% agarose gel with ethidium bromide and 1kb plus DNA ladder was included as a molecular marker (Thermo Scientific, Carlsbad, California, USA).

RESULTS

Sampling of farms

A total of 332 birds were necropsied, with 182 birds sampled. Table 4.2 summarizes the general characteristics of flocks sampled. Thirty-four flocks from 9 farms were represented within the sampled birds. The age of the flocks ranged from 19 to 104 weeks of age with nearly 50 percent between 30 and 49 weeks of age. Birds were cage raised in 28 of the flocks, 3 were cage-free organic, 2 cage-free Omega-3 antibiotic free and 1 cage-free antibiotic free. Figures 4.1 and 4.2 are examples of the egg productive performance of a FDN affected flock.

Bacteriology

Table 4.3 summarizes the bacteriology findings from egg layer flocks with gross lesions of FDN. A total of 37 *Clostridium* species were isolated and identified: 17/37 *C. perfringens,* 12/37 *C. innocuum,* 2/37 *C. sporogenes,* 1/37 *C. bifermentans*, 1/37 *C. clostridioforme*, 1/37 *C. difficile,* 1/37 *C. limosum*, 1/37 *C. novyi,* 1/37 *C. paraputrificum*. All the *C. perfringens* strains presented double zone of hemolysis when streaked on blood agar. Increased turbidity consistent with phospholipase C activity was detected in egg yolk agars in all *Clostridium perfringens* strains

(Figure 4.3). Gram staining showed uniform to variable Gram-positive long rod-shaped bacteria with square ends.

Ten isolates were unable to identify by RapID ANA II System. 4/37 *Clostridial* strains isolated had multiple species identified in which isolates were listed with one choice; (C. *clostridioforme or Bifidobacterium sp.), (Clostridium paraputrificum or Clostridium perfringens), (C. novyi or C. tetani),* or more than two other possible choices; *(C. bifermentans, C. subterminale, C. sordelli or C. difficile).*

Twenty-four additional commensal or non-pathogenic Gram-negative and Gram-positive bacteria were isolated from samples in field visits.

Histopathology

Eighty-three (25%) out of 332 birds necropsied had gross lesions consistent with FDN (focal-multifocal reddened to brownish-gray areas of mucosal erosions).

Sixty slide sections were microscopically examined, 46/60 sections were from affected birds, with 25/60 (41.7%) sections having characteristic or classic microscopic lesions of FDN. These lesions were characterized by lymphoplasmacytic enteritis with heterophilic infiltration, loss of enterocytes and necrosis at the villus tips. Presence of fibrinonecrotic exudate with long rods in the lumen were observed in all samples with classic FDN lesions (Figure 4.4). In addition, 12/60 (20%) sections examined from duodenal samples had early FDN lesions, characterized by lymphoplasmacytic enteritis with mild to moderate heterophilic infiltration, enterocyte vacuolation and single enterocyte necrosis in the intestinal villus with enterocyte detachment from the lamina propria (Figure 4.5). Microscopic FDN lesions were not seen in 9/46 (19.5%) sections from affected birds. However, lymphoplasmacytic enteritis was observed, as well as in the 14 sections from the unaffected birds.

Few to moderate cestodes were observed in 2/37 (5.4%) sections with FDN lesions. Similarly, few *Isospora*-like cysts were seen in 2/37 (5.4%) samples. Coccidiosis ranging from minimal to moderate was only observed in two affected sections. Other findings included luminal hemorrhages and crypt hyperplasia. All samples with characteristics microscopic lesions of FDN had Gram-positive as well as long Gram-negative bacteria within the lesions.

Immunohistochemistry

Immunohistochemistry was performed on all sections with classic FDN lesions. The presence of *Clostridium perfringens*-antibody positive bacteria was detected in 25/25 (100%) samples. *Clostridium perfringens* was found in the luminal exudate in 13/25 sections (52%), and it was seen attached to the enterocytes in the lesions of 12/25 (48%) samples (Figure 4.6).

Toxinotyping

The detection of the genes; *cpa, netB, tpeL, cpb, etx, ia, cpb2* and *etx* was standardized by Polymerase Chain Reaction (Figure 4.7).

The table 4.4 summarizes the *Clostridium perfringens* toxinotyping results in strains isolated from focal duodenal necrosis lesions. The presence of *netB and cpb2* genes were detected in three out of 17 *Clostridium perfringens* strains. Ten out of 17 *C. perfringens* strains only carried the gene *cpb2.* All *Clostridium perfringens* strains were negative for *tpeL, cpb, etx, ia* and *etx.*

DISCUSSION

In this paper, we report the bacteriological and histopathological findings in duodenal samples with FDN collected from 34 flocks in 3 states. Affected FDN flocks are characterized by having egg production drops, decreased egg size or abnormal egg weight increases with unspecific clinical signs (5). Therefore, the majority of samples were collected from flocks that exhibit

decreased egg production and lower egg case weights. In fact, one flock evaluated with the characteristic gross and microscopic lesions showed an egg production drop below normal for 10 weeks (ranging from 0.2-10% below standard) and decreased egg case weights, lower than standard, for 19 weeks and having the lowest drop at week 28 (1.68lb.). These findings reflect previous reports by the table egg industry in the U.S. (decreased egg production ranging from 1 to 10% and approximately 2.5 lb. lowered egg case weight in flocks with FDN) (5).

All samples were plated onto blood agar, PEA selective agar plate and thioglycollate broth medium under anaerobic conditions for the detection of *Clostridial* species. In this study, the majority of identified bacteria isolated from duodenal samples corresponded to Gram-positive bacteria. Previous studies have shown that Gram-positive bacteria are usually the most cultured and abundant in the chicken intestinal microflora (21). Although in this study the intestinal microbiome was not fully investigated, 80% of identified bacteria were Gram-positive which corresponded to what was previously reported by Lu et al. (21).

RapID ANA II System was the biochemical test used for the identification of isolated bacteria in the present study. However, this method was designed for the detection of anaerobic bacteria isolated from human clinical samples (2). It is possible that the 10 isolates that were unable to be identified are unknown bacteria that are not available in the RapID ANA II System database. Similarly, 4/37 (10.8 %) *Clostridial* strains isolated had multiple species identified in which isolates were listed with one or more than two other possible choices. A previous study reported that one of the limitations of this probability method is the fact that it cannot recognize a strain outside of its existing database (2). Therefore, it is possible that an unknown strain can be misclassified and assigned to the most closely related taxon present in the data base (2). Although in this study all tests were consistently performed and read by only one person, the reactivity of

some organisms varies, making the interpretation of the color reactions difficult, resulting in a misidentification.

Forty-eight percent of the total isolates were *Clostridial* species. *Clostridium perfringens* was the most abundant culture-isolated (17/37) bacteria. However, from 11 field visits that showed histological FDN lesions, *C. perfringens* was cultured from samples in 6 visits. The low culture detection of *Clostridium perfringens* from lesions may possibly be explained by antibiotic treatment, overgrowth of other intestinal bacteria during culture and low or absence of *Clostridium perfringens* in the samples collected.

Detection of phospholipase C activity was evaluated by plating isolates on egg yolk agar. The reaction was considered positive if an increase in the turbidity of the agar was observed. All *C. perfringens* isolates (17/17) were positive for this reaction. However, other *Clostridial* species different to *C. perfringens* such as *C. absonum, C. baratti, C. bifermentans, C. novyi, C haemolyticum and C. sordelli* can also present phospholipase C activity (31). Therefore, the identification of all isolated *C. perfringens* was confirmed by PCR.

The presence of *netB* and *cpb2* toxin genes was detected in some *Clostridium perfringens* isolates. The *netB* gene is commonly found in isolates from chickens with necrotic enteritis with a prevalence ranging from 60-90% but can also be isolated from healthy birds (19), (34). However, *netB* mutants lacking this toxin are unable to cause disease under experimental conditions; compared to *netB* positive strains that can cause disease in 45% of the chickens tested (33),(34) (20). In poultry, *Clostridium perfringens* isolates harboring the *cpb2* gene have been found in healthy and diseased animals suffering intestinal disease including chickens, layers, turkeys, quail, psittacines (30), (11), (13), (14), (6), (22, (26), (7). However, no significant differences have been found in the prevalence of *cpb2* gene between healthy and sick animals and none of the studies

have successfully fulfilled Koch's Postulates (33). Nevertheless, some studies have reported an important role of beta2 toxin in enteric diseases in poultry. One report of experimental infection of chickens using an intestinal ligated loop model showed higher histological necrotic enteritis lesions in birds inoculated with *cpb2* positive when compared with *cpb2* negative strains (27).

Previous studies with layers have also shown an association between *cpb2* gene and FDN (3, 11). One study in the Netherlands reported that nineteen out of seventy-six diseased birds carried *C. perfringens* strains harboring *cbp2*, and these isolates were able to produce beta2 toxin *in vitro*. In contrast, *Clostridium perfringens* was isolated from only four out 15 SPF birds, where only one isolate was *cpb2* positive and able to produce beta2 toxin *in vitro* (3). In a study performed in Georgia, USA, four *C. perfringens* strains were isolated from intestinal samples with characteristic microscopic lesions of FDN, all isolates carried *cpb2* gene and the presence of beta2 toxin in the lesions was confirmed by immunohistochemistry (11). In agreement with these previous studies, we detected the presence of both *netB* and *cpb2* in 3/17 isolates, 10/17 *C. perfringens* isolates carried the *cpb2* gene. These results, further support previous findings and suggest that *C. perfringens* may play a role in the development of FDN. Other pathogenic species isolated include *C. difficile*, this pathogen affects numerous mammalian species. In humans, it is considered the major nosocomial pathogen in the USA causing pseudomembranous colitis and toxin megacolon. Although *C. difficile* has been isolated from the gastrointestinal tract and retail food of poultry it is not associated with disease (8). *Clostridium novyi* was also isolated from 1 sample and this bacterium can cause histotoxic infections in humans and animals. In cattle, *Clostridium novyi* has been associated with gas gangrene or blackleg (1).

Microscopic lesions of FDN were seen in 37/60 (61.7%) duodenal samples. From these affected samples, 25/37 (67.5%) had lesions of lymphoplasmacytic enteritis with heterophilic

infiltration, loss of enterocytes and necrosis at villus tips with the presence of fibrinonecrotic exudate and long rod-shaped bacteria. These lesions were previously described as characteristic FDN by Franca et al. (10). Similarly, 12/37 (32.4%) samples had lymphoplasmacytic enteritis with mild to moderate heterophilic infiltration with enterocyte vacuolation, single enterocyte necrosis in the intestinal villus, and enterocyte detachment from the lamina propria. Those lesions likely represent the acute stage of FDN. Additionally, low prevalence of cestodes and coccidiosis was seen in FDN samples 2/37 (5.4%). These results are also supported by our previous study which reported rare occurrence of coccidiosis in FDN lesions analyzed by histopathology (11).

Immunohistochemistry demonstrated the presence of *C. perfringens* antibody-positive bacteria in samples with classic FDN lesions. *Clostridium perfringens* was found in the luminal exudate in 13/25 (52%) sections and attached to the enterocytes in lesions from 12/25 (48%) samples, which means that the bacteria colonized the intestinal epithelium. The high content of glycoproteins in the mucus and inflammatory infiltrate secreted by intestinal cells may possibly serve as a substrate for the overgrowth of *C. perfringens*. It is also well known that mucin glycoproteins have attachment sites necessary for the adhesion of *C. perfringens* to the intestinal epithelium (28).

In addition, abundant long Gram-negative rod-shaped bacteria were commonly seen mixed within the lesions, confirming previous findings documented by Franca et al. (11). It is possible that this is not an incidental factor and a co-infection of unknown etiology may be involved in the pathogenesis of FDN. It is important to further investigate and characterize the nature of this pathogen.

In this study, *Clostridium perfringens* was the most frequently detected bacterium in flocks with clinical characteristics of FDN (egg production drops and decreased egg case weights), as well as in birds with typical gross lesions. These lesions were confirmed by histopathologic examination. *C. perfringens* was molecularly characterized and a few isolates harbored the *netB* gene. *In vivo* pathogenicity studies are needed to evaluate the virulence and ability of different *Clostridium perfringens* isolates to induce FDN.

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Table 4.1. Sequence of primers used for the detection of *Clostridium perfringens* toxin genes

Toxin gene		Sequence	Product length (bp)
cpa	Forward	TGC ATG AGC TTC AAT TAG GT	400
	Reverse	TTA GTT TTG CAA CCT GCT GT	
netB	Forward	CGC TTC ACA TAA AGG TTG GAA GGC	316
	Reverse	TCC AGC ACC AGC AGT TTT TCC T	
tpeL	Forward	ATA TAG AGT CAA GCA GTG GAG	466
	Reverse	GGA ATA CCA CTT GAT ATA CCT G	
etx	Forward	CGC ATC AGC GGT GAT ATC CAT	680
	Reverse	TCT CTC CCC ATT CAC TTC CAC TT	
iA	Forward	CGA TGA AAA GCC TAC ACC ACT ACT T	319
	Reverse	TGC GGT ATA TCC TCC ACG CA	
cpb	Forward	ACG GAT GCC TAT TAT CAC CAA CTT T	868
	Reverse	TGT CCT ACC CAG TTA GCA CCA T	
cpe	Forward	GGC GTT CTT CTA ACT CAT ACC CT	169
	Reverse	ACT CCA TCA CCT AAG GAC TGT T	
cpb2	Forward	AAA TAT GAT CCT AAC CAA MAA	525
	Reverse	CCA AAT ACT YTA ATY GAT GC	

Table 4.2. General characteristics of flocks sampled

Farm Number	Type of production	Field visit date	Number of flocks evaluated	Flock age
1 Visit 1	Cage	10.20.15	2	26,45
2	Cage	10.2215	1	84
3	Cage	10.27.15	4	26,59,79,94
1 Visit 2	Cage	10.27.15	1	22
4 Visit 1	Cage	10.29.15	2	39
5 visit 1	Cage	2.5.16	2	35,90
6 visit 1	Cage	3.7.16	2	35,57
7	Cage	4.21.16	1	33
8	Cage, cage-free organic, Cage-free ABF	5.10.16	8	19,24,31,40,42,51,56,107
9	Cage, Cage-free Omega-3 ABF	5.20.16	4	31,48,50,60
6 Visit 2	Cage	7.7.16	1	30,40
5 Farm 2 visit 2	Cage	8.23.16	1	104
6 Visit 2	Cage	10.6.16	2	24,40
4 Visit 2	Cage	11.9.16	2	36,46

Table 4.3. Summary of diagnostic findings from Egg layers sampled

Farm No	Number of birds necropsied	Number of samples taken	Intestinal gross FDN lesions (No of birds with gross lesions/ Total birds necropsied)	Histopathology FDN lesions (No of sections with FDN lesions/ Total sections evaluated)	Bacteriology findings
1 Visit 1	9	9	3/9	3/8 Classic FDN	*Eikenella corrodens (1), Streptococcus gordonii (1) Xanthomonas campestris (1)*
2 Visit 1	9	8	6/9	2/7 Early FDN 3/7 Classic FDN	(0)
3	37	5	0	0	(0)
1 Visit 2	4	4	0	0	(0)
4 Visit 1	30	17	17	2/5 Early FDN 3/5 Classic FDN	*Bacillus cereus (2), Chromobacterium violaceum (1) Enterococcus raffinosus (1), Streptococcus gordonii (2) Streptococcus anginosus (1), Bacteroides ovatus (1) Lactobacillus spp. (1), Bifidobacterium spp. (1) Prop. Granulosum (1), Atopobium minutum (1) Staphylococcus epidermidis (1), Collinsella aerofaciens (2) Cellulosimicrobium cellulans (1), Eikenella corrodens (1) Clostridium limosum (1), Clostridium perfringens (1) Clostridium sporogenes (1), Clostridium innocuum (3) C. novyi+C. tetani (1)*
5 visit 1	15	4	2	2/2 Classic FDN	*Clostridium innocuum (2), Collinsella aerofaciens (4) Clostridium perfringens (2), Bifidobacterium spp. (1) Unable to identify (3)*
6 visit 1	20	13	5	1/4 Classic FDN	(0)
7	20	11	5	2/6 Early FDN 4/6 Classic FDN	(0)
8	29	29	2	2/4 Classic FDN	*Clostridium innocuum (4), Clostridium perfringens (3) C. clostridioforme + Bifidobacterium sp. (1)*

9	41	37	26	1/6 Early FDN 5/6 Classic FDN	*Collinsella aerofaciens (2), Clostridium innocuum (2)* *Clostridium perfringens (5), Actinomyces israelli (1)* *C. bifermentans + C. subterminale + C. sordelli + C. difficile (1)* *Unable to identify (7)*
6 Visit 2	20	5	5	2/4 Early FDN 1/4 Classic FDN	*Clostridium perfringens (5),* *Clostridium innocuum (1)*
2 visit 2	100	10	2	0	(0)
5 Visit 2	20	20	3	3/7 Early FDN 2/7 Classic FDN	*Gallibacterium anatis (2)*
4 Visit 2	20	7	7	1/2 Classic FDN	*Clostridium perfringens (1), Clostridium difficile (1)* *Clostridium sporogenes (1)* *Clostridium paraputrificum + Clostridium perfringens (1)*

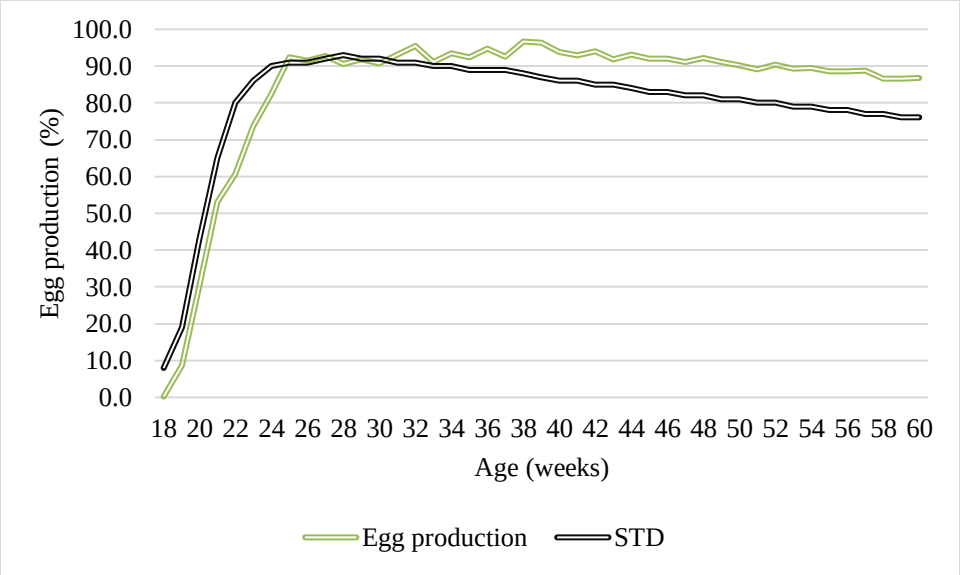

Figure 4.1. Example of egg production drop in a FDN affected flock. Egg production was below normal for 10 weeks ranging from 0.2% to 10% below standard (STD).

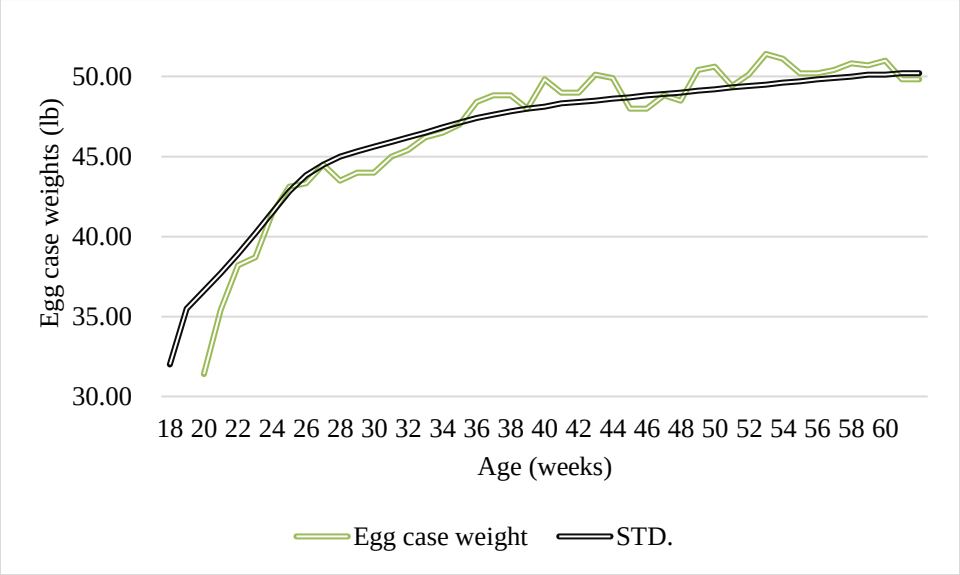

Figure 4.2. Example of decreased egg case weight in a FDN affected flock. Egg case weight was lower than standard for 19 weeks. The lowest egg case weight was observed during week 28 (1.68 lb. per case).

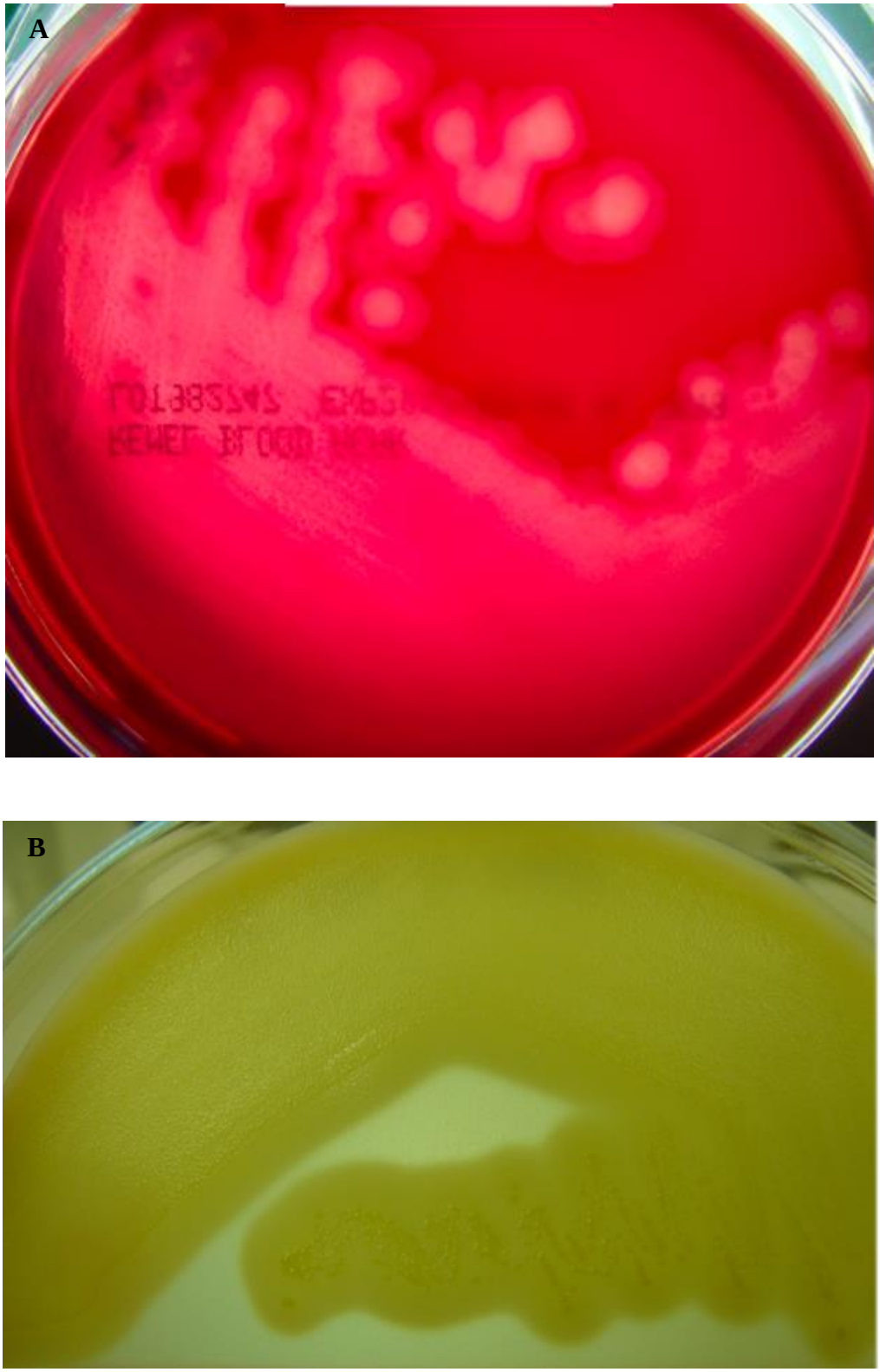

Figure 4. 3. *Clostridium perfringens* on blood (A) and egg yolk agars (B).

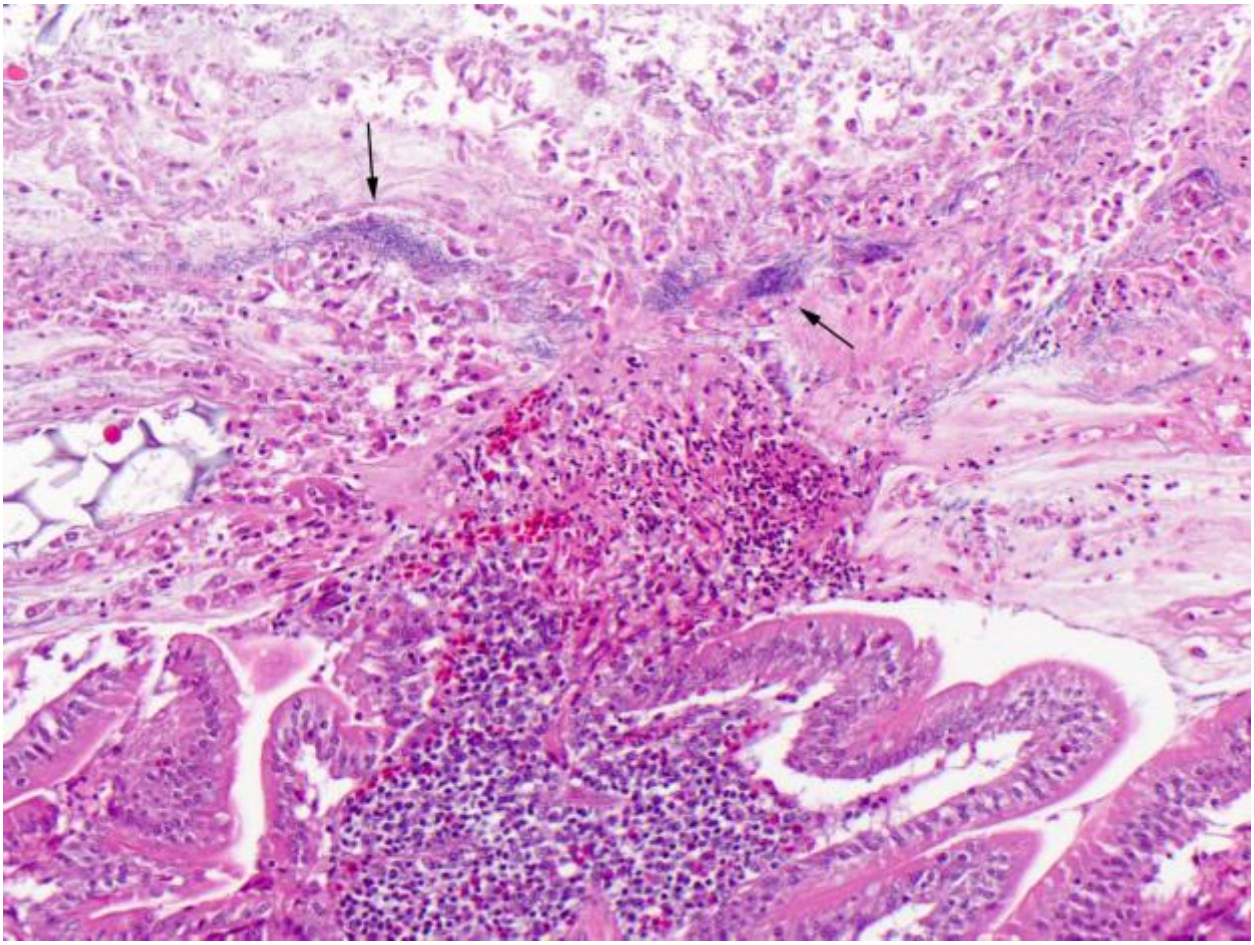

Figure 4.4. Photomicrograph of the duodenum, characteristic FDN lesion. Focal dissociation and loss of enterocytes at the villus tips with moderate fibrinoheterophilic infiltrate exuding from the lesions and in the lumen. Clusters of long rod-shaped bacteria mixed with the fibrinonecrotic exudate (arrows).

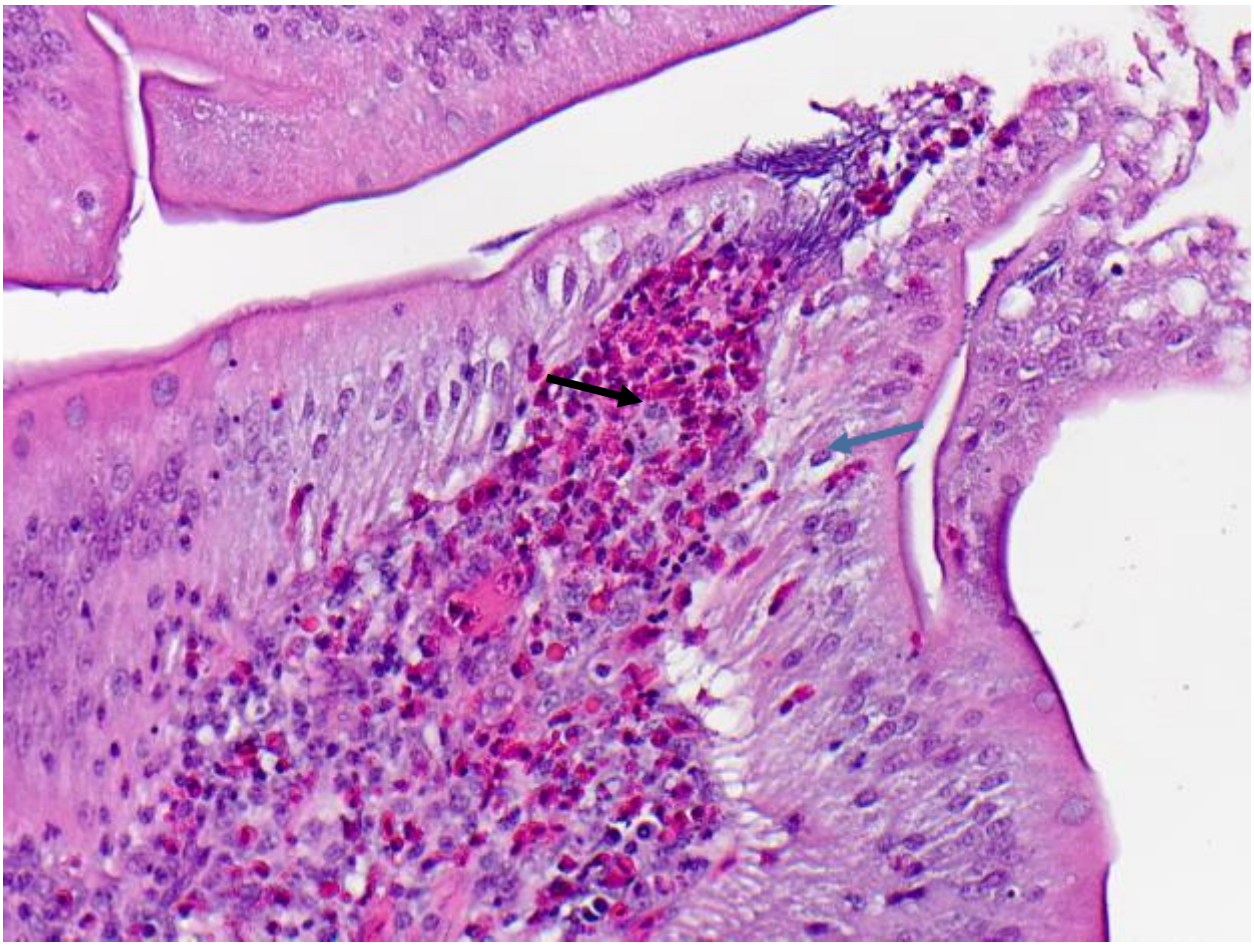

Figure 4.5. Photomicrograph of the duodenum, acute stage of FDN. The lamina propria is expanded with inflammatory infiltrate composed by heterophils and lymphocytes. Enterocyte vacuolation, single cell necrosis in the intestinal villus with enterocyte detachment (blue arrow) with long rods in lumen and attached to the epithelium in crisscross pattern (black arrow).

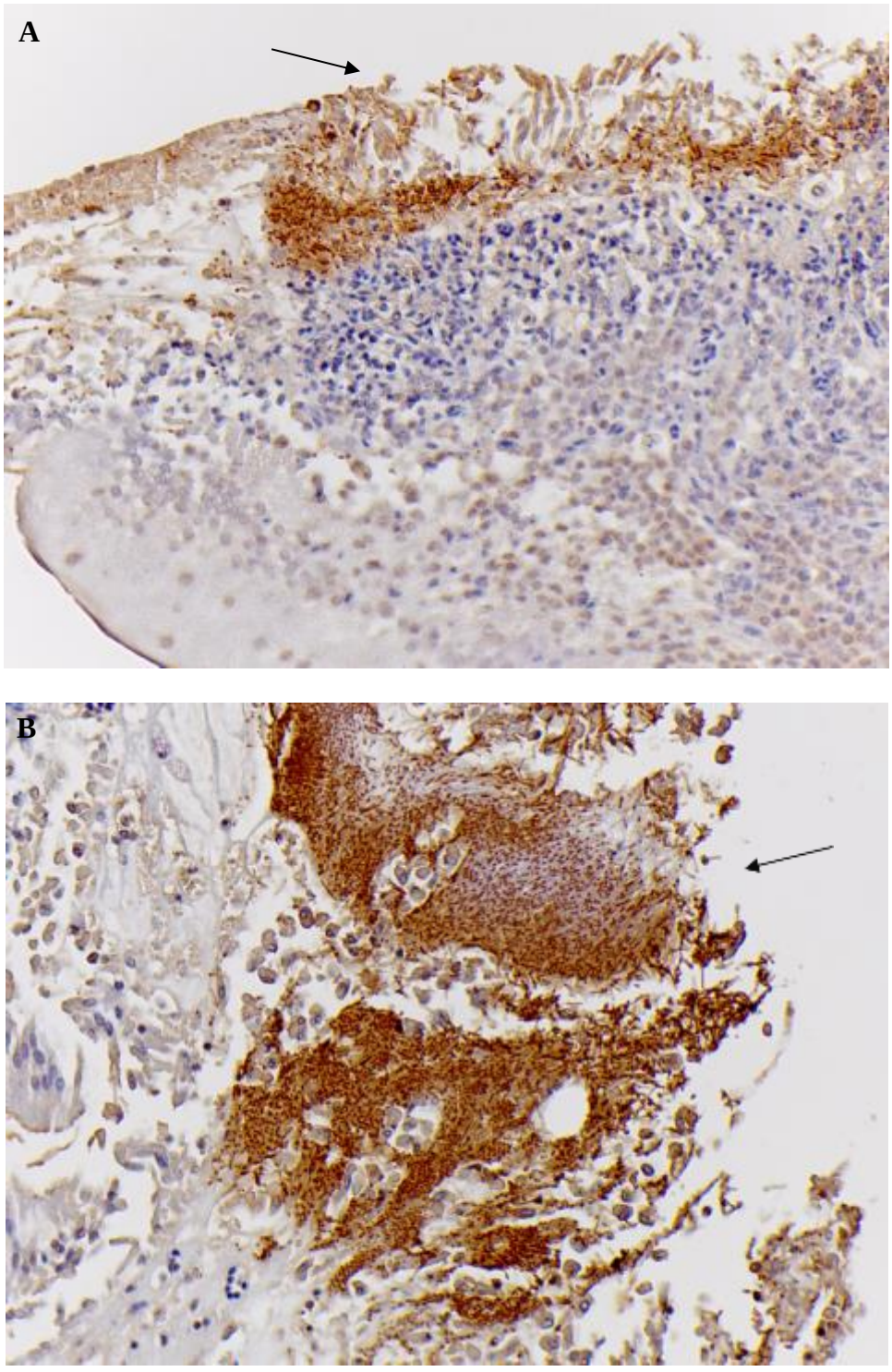

Figure 4.6. Immunohistochemistry of duodenum; A, Clusters of *C. perfringens* attached to enterocytes and in the lumen, B, Clusters of *C. perfringens* within the luminal exudate.

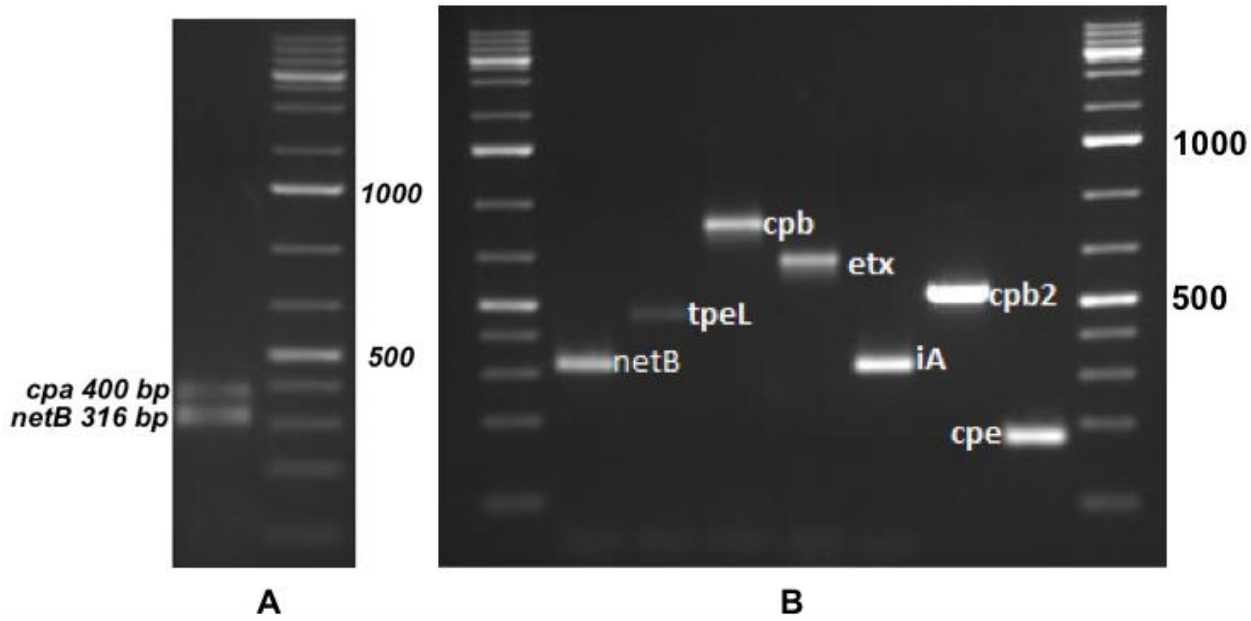

Figure 4.7. Multiplex and single PCR for the detection of *Clostridium perfringens* toxin genes. A: Multiplex PCR for *netB* and *cpa* toxin genes. PCR product size; *cpa* (400 bp), *netB* (316bp). B: Single PCR for toxin genes, *netB* (316bp), *tpeL* (466bp), *cpb* (860bp), *etx* (680 bp), *iA* (319 bp), *cpb2* (525bp) and *cpe* (169 bp).

Table 4.4. Toxinotyping of *Clostridium perfringens* strains isolated from FDN lesions

Farm no.	**Chicken No**	***cpa***	***netB***	***etx***	***tpeL***	***cpb2***	***cpb***	***cpe***	***iA***
Farm 4 visit 1	Bird 11	+	-	-	-	+	-	-	-
Farm 4 visit 2	Bird 7	+	-	-	-	-	-	-	-
Farm 5 visit 1	Bird 7	+	+	-	-	+	-	-	-
Farm 5 visit 1	Bird 7	+	-	-	-	+	-	-	-
Farm 6 visit 2	Bird 1	+	-	-	-	+	-	-	-
Farm 6 visit 2	Bird 2	+	-	-	-	+	-	-	-
Farm 6 visit 2	Bird 3	+	-	-	-	+	-	-	-
Farm 6 visit 2	Bird 4	+	-	-	-	+	-	-	-
Farm 6 visit 2	Bird 5	+	-	-	-	+	-	-	-
Farm 8	Bird 11	+	+	-	-	+	-	-	-
Farm 8	Bird 22	+	-	-	-	-	-	-	-
Farm 8	Bird 25	+	-	-	-	-	-	-	-
Farm 9	Bird 4	+	-	-	-	-	-	-	-
Farm 9	Bird 17	+	+	-	-	+	-	-	-
Farm 9	Bird 21	+	-	-	-	-	-	-	-
Farm 9	Bird 26	+	-	-	-	-	-	-	-
Farm 9	Bird 27	+	-	-	-	-	-	-	-

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CHAPTER 5

EXPERIMENTAL INFECTION OF EGG LAYING CHICKENS WITH *CLOSTRIDIUM PERFRINGENS* STRAINS ISOLATED FROM FOCAL DUODENAL NECROSIS LESIONS[1].

[1]Ana M. Villegas, Lisa Stabler, Charles Hofacre, Francisco Uzal, Margie Lee and Monique Franca**.** To be submitted to *Avian Diseases*

ABSTRACT

Focal duodenal necrosis (FDN) is an intestinal disease observed in egg laying chickens, characterized by multifocal mucosal erosions observed in the duodenal loop and proximal jejunum. It has a worldwide distribution and it is considered one of the top 5 disease concerns of the table egg industry in the USA. Affected flocks exhibit lower egg case weights. Previous studies have associated this condition with different *Clostridium* species; *C. colinum* and *C. perfringens*. The objective of this study was to try to reproduce FDN by experimentally infecting egg laying chickens with different *Clostridium perfringens* isolates. An experimental trial was conducted using commercial chickens. Challenge groups received *Clostridium perfringens* at approximate dose between 1x 10^8 to 10^9 CFU/ml daily for 8 days via oral gavage. Birds were fed with a diet containing dried distiller's grains with solubles at a concentration of 8.9%. Birds were euthanized and necropsied following inoculations. Gross lesions were evaluated, lesions were scored and samples were collected for histopathology and bacteriology. Mild gross lesions characterized by mucosal hyperemia, erosions, fibrinous exudate in the lumen and frothy duodenal contents were seen in birds from challenged groups. Histopathology revealed heterophilic inflammation, mucosal hemorrhages, epithelial cell sloughing and rare enterocyte necrosis in duodenal samples from some challenged birds. *Clostridium perfringens* was recovered from one sample of the *netB-cpb2* positive inoculated group that showed gross lesions. These results show that inoculation with *Clostridium perfringens* isolated from FDN samples may cause mild gross and microscopic duodenal lesions in experimentally infected chickens. Additional studies need to be performed to reproduce the characteristic gross and microscopic lesions seen in field cases of FDN.

INTRODUCTION

Focal duodenal necrosis (FDN) was first described by Dr. Dunn (1996) in a cage-free layer flock as an enteric disease of table egg laying chickens (4). Since then, the disease has been detected in most of the states in the US and Europe (7) and it is considered one of the top 5 disease concerns of the table egg industry in the USA (16). FDN has been diagnosed in chickens older than 14 weeks of age and in different genetic lines raised in different types of management systems; cage, cage-free and organic eggs (6). Clinical signs, when observed, are pale combs and poor body weight. Affected flocks exhibit low egg case weights and might have lower egg production performance (7).

Gross lesions are characterized by single to multiple reddened to greyish mucosal erosions that are commonly covered with a yellow pseudomembrane with approximate size of 3 to 15mm (7). These lesions are frequently located in the duodenal loop but can extend to the proximal jejunum and may be seen through the serosa (6). Early gross FDN lesions result in unspecific changes in the duodenal mucosa, such as an abnormal amount of gas and multifocal areas of hyperemia (17).

FDN is usually controlled by prevention and treatment measures. Flock monitoring by posting sessions every 4 weeks is necessary for diagnosis of this disease. Probiotics, prebiotics, organic acids, yeast culture products and essential oils, are preventive options that have been used in the prevention and control of FDN (3, 6-8). Antibiotics against Gram-positive bacteria such as bacitracin, chlortetracycline and tylosin have been shown to be effective in reducing the impact of the disease (3).

Previous studies have associated this condition with different *Clostridial* species; a non-culture molecular profiling study of the duodenal microbiota showed a higher prevalence of *C. colinum* in affected birds (14). In one study conducted in the Netherlands, NetB and beta2 producing *C. perfringens* were isolated from multifocal duodenal necrosis lesions between layer hen flocks presenting abnormal egg production performance and poor body condition (1). More recently, Franca et al. reported the presence of *C. perfringens* positive for alpha and beta2 toxins in duodenal samples from layers with FDN by bacteriology, PCR and immunohistochemistry (5).

In an effort to establish a causal role of *C. perfringens* in the development of FDN, an experimental infection of commercial egg laying chickens with *Clostridium perfringens* isolates harboring the *NetB* and *cpb2* genes was performed. Dried distiller's grains with soluble (DDGS) was added to the diet as a predisposing factor for disease induction. DDGS are high in dietary fiber (42%), which stimulates mucin production, an ideal substrate for *C. perfringens* (11). In commercial layers, DDGS is a common ingredient added to the diet. Results from a survey performed showed that 80% of affected FDN flocks used this ingredient in the pre-lay and layer diets (A. M. Villegas and M. Franca, unpublished data).

The current study was performed in an attempt to examine the capacity of the combination between virulence strains and predisposing factors to induce focal duodenal necrosis in mature commercial layers. We report that *Clostridium perfringens* strains with and without *netB* gene can cause mild duodenal lesions suggestive of early FDN under experimental conditions.

MATERIALS AND METHODS

Bacterial strains

The two challenge strains used in this study were identified as *Clostridium perfringens* type A and were originally isolated from clinical Focal Duodenal Necrosis (FDN) field cases. Toxinotyping of *C. perfringens* isolates was performed by polymerase chain reaction (PCR).

One of the *C. perfringens* isolates contained the genes that codified for NetB and beta2 toxins, being designated as *netB-cpb2* positive. The remaining isolate carried the *cpb2* gene and it was negative for *netB*. Sequencing of this strain was done by Dr. Moore (RMIT University, Australia). The molecular characterization performed showed that the gene was more closely related to atypical *cpb2* gene, however, it was significantly different from the atypical sequence and consensus sequences of *cpb2*. This strain was referred to as *netB* negative- *cpb2* positive.

Inoculum preparation and titration

Challenge strains were streaked onto trypticase soy agar (TSA) with 5% sheep red blood cells (Remel®, Lenexa, Kansas, USA) and incubated anaerobically overnight at 37° C. The strains were harvested using 1 ml sterile thioglycollate broth and suspended in equal volume of sterile glycerol and kept frozen at -80°C until use. A volume of 0.5 ml of this culture was inoculated onto 500 ml thioglycollate media containing 2% beef extract and incubated at 37°C for 18 hours for inoculum preparation. Titration of the inoculum was performed and the number of colony-forming units per ml was determined. Ten-fold dilution of bacterial broth was performed to a dilution factor of 10^8. A volume of 100 µL from the last 3 dilutions was plated into trypticase soy agar (TSA) with 5% sheep red blood cells and incubated anaerobically for 24 hours. Following incubation, colonies were counted and the colony-forming units per ml was calculated.

Experimental Design

Fifty-two laying chickens (Hy-line white W-36) obtained from a commercial flock at 22 weeks of age were distributed between two isolator units at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, University of Georgia (Athens, GA, US). They were randomly divided into 2 treatment groups and one control group. Each bird was placed in an individual battery cage. Birds were reared in battery cages during two weeks. At 24 weeks of age, treatment groups (18 birds per group) received serial oral inoculation at approximately $1x10^8$ to $1x10^9$ CFU/ml of *Clostridium perfringens netB-cpb2* positive or *netB* negative- *cpb2* positive during 8 days. Control group (16 birds) was inoculated with 1 ml of sterile thioglycollate broth. Clinical signs and mortality were monitored twice a day. One day after the last inoculation, all birds were euthanized by carbon dioxide and cervical dislocation. Bird care was provided according to an animal use protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Diet

A commercial diet for layer pullets was used for all treatment groups. The diet contained 50% large particle calcium, approximately 77% corn and distillers dried grain with solubles (DDGS) were added to the feed at a concentration of 8.9% (Table 5.1). All groups were fed with this diet for 2 weeks before challenge. Birds were provided with water, feed *ad libitum* and 15 hours of light during this challenge study.

Bacteriology

Samples from duodenal segments with presence of gross lesions were aseptically collected in sterile Whirl Pak bags and immediately placed in Gas Pak anaerobe pouches. (BD GasPak™, Sparks, Maryland, USA). Duodenal contents were plated in pre-reduced trypticase soy agar (TSA)

with 5% sheep red blood cells (Remel®, Lenexa, Kansas, USA) and anaerobically incubated at 37°C for 48 hours. Affected tissues were also anaerobically incubated at 37°C for 24 hours in thioglycollate broth media and plated onto blood agar media (5).

Colonies with a zone of hemolysis were selected and plated in selective phenylethyl alcohol blood agar media (PEA) for aerobic and anaerobic incubation. Gram positive-anaerobic colonies resembling with *C. perfringens* were identified by RapID^{TM} Ana II System (Thermo Scientific, Carlsbad, California, USA). Lecithinase activity was further confirmed by plating these colonies in egg yolk agar.

Scoring of Lesions

Duodenal loop and the proximal section of the jejunum were evaluated and scored for gross lesions. Intestinal tracts were removed and lesions were scored as previously described by Keyburn et al. (10). Scores included: 0, no apparent gross lesions; 1, no presence of erosions but removable fibrin deposit; 2, isolated focal necrosis or erosions (1 to 5 foci); 3, focal necrosis or erosions (6 to 15 foci); 4, focal necrosis or erosions (16 or more foci); 5, extensive areas of necrosis (2 to 3 centimeters long; 6, diffuse necrosis as seen in field cases.

Histopathology

Segments of 1-2 centimeters of duodenum were collected for histopathology examination. Duodenal samples were fixed in 10% neutral buffered formalin, trimmed, sectioned at 4mm and stained with hematoxylin and eosin (H&E).

DNA Bacterial Extraction

Colonies were subcultured and anaerobically incubated at 37°C for 48 hours in pre-reduced trypticase soy agar (TSA) with 5% sheep red blood cells. Three colonies were suspended in 500 µL of nuclease free water (Ambion® Nuclease-Free water, Thermo Scientific, Carlsbad,

California, USA). Bacterial DNA was extracted using QIAamp DNA Stool Mini Kit according to manufacturer's recommendations.

Multiplex PCR

Multiplex PCR for the presence of genes *cpa* and *netB* coding for alpha and NetB toxins was performed.

Final reaction volume was 28 µL, consisting of 15 µL of 2x GeneAmp® Fast PCR Master mix (Applied Biosystems™, Foster city, California, USA), 0.75uL 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 using MyCycler™ Thermal Cycler (Applied Biosystems, Foster City, California, USA) 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 10s.

PCR product was visualized by electrophoresis (100V constant voltage) in a 1.5% agarose gel with ethidium bromide, 1kb plus DNA ladder was included as a molecular marker (Thermo Scientific, Carlsbad, California, USA).

Statistical Analysis

Statistical analysis of intestinal gross lesion scores was performed by Kruskal-Wallis test using Prism 7.0 software (GraphPad Software Inc., La Jolla, CA). Differences were reported at the 5% level of significance.

RESULTS

Clinical signs

During this experiment, no overt clinical signs were observed in control and challenged birds. Likewise, none of the groups displayed mortality.

Necropsy findings

Duodenal lesions were observed in both challenged groups; "*netB*-negative-*cpb2* positive" and "*netB-cpb2* positive". Observed lesions consisted of focal areas of mucosal erosion, excess of gas, epithelial sloughing, and multifocal areas of hemorrhages in the duodenal loop (Table 5.2), (Figure 5.1 and 5.2). Although epithelial sloughing and excess of gas were seen in the control group, only 25% of birds in this group exhibited such lesions (Figure 5.3).

Scoring of FDN lesions

FDN gross lesions were evaluated in all groups during necropsy. Lesions scores 1 and 2 were seen in the treatment groups. In the *netB-cpb2* positive challenged group, score 1 representing removable fibrin, was present in 15 out of 18 chickens. Similarly, *netB* negative - *cpb2* positive group presented removable fibrin in 15 out 18 inoculated birds. Isolated focal necrosis and ulceration (1 to 5 foci) or score 2, was observed in 3 out 18 chickens in each bacterial challenge group, whereas the control group presented no apparent gross lesions in 10 out 16 birds. The remaining birds in the control group presented lesions score 1 characterized by mucus/epithelial sloughing resembling removable fibrin. The severity of the lesions was statistically different in both treatment groups "*netB-cpb2* positive" and "*netb* negative-*cpb2* positive" compared to the control group (p values <0.001, figure 5.4)

Histopathology

Histological evaluation of formalin fixed duodenal segments was performed to confirm the gross lesions observed. Histologically, mild duodenal lesions were present in both challenged groups.

For the *netb* negative-*cpb2* positive treatment group, inflammatory infiltration occurred in the lamina propria of the villus tips and was characterized by mild to moderate aggregation of

heterophils (7/18 birds) and lymphocytes with hyperemia (18/18 birds) and mild hemorrhages in the lamina propria and/or lumen (8/18 birds). Single enterocyte necrosis appeared as cells with vacuolated cytoplasm and karyorrhectic nuclei and this lesion was seen in 3/18 birds. Enterocyte detachment from the basal lamina propria was also observed in the villus tips in these birds (Figure 5.5). Similarly, duodenal findings in the *netB-cpb2* positive group included enterocyte detachment in the villus tips (10/18 birds), hyperemia (17/18 birds), mild heterophilic infiltration (3/18 birds) and mild to moderate hemorrhages in the lamina propria and/or intestinal lumen (9/18 birds) (Figure 5.6). These microscopic lesions resemble lesions of acute FDN. Such findings (enterocyte detachment, single cell necrosis and hemorrhages) were not present in the control group. Histopathological findings for this group included; lymphocytic infiltrate, mild hyperemia in the lamina propria (4/10 birds examined) and mild heterophilic infiltration (1/10 birds) (Figure 5.7).

Bacteriology

Clostridium perfringens was recovered from one sample of the *netB-cpb2* positive inoculated group that showed gross lesions. The isolate was confirmed by RapID Ana II and the presence of *alpha* and *netB* were detected by PCR. *C. perfringens* were not isolated in duodenal samples from the *netb* negative-*cpb2* positive and sham-inoculated control groups.

DISCUSION

In this study, mild duodenal lesions were observed when egg-laying chickens were experimentally infected with different *Clostridium perfringens* isolates from FDN lesions when using DDGS as a dietary predisposing factor. The development of the disease was evaluated by assessing gross lesions, histopathology examination and bacteriology. To our knowledge, this is the first attempt to try to reproduce FDN under experimental conditions.

Necropsy findings characterized by focal mucosal erosions, excess of gas, epithelial sloughing, and multifocal areas of hemorrhages with presence of fibrin in the duodenal loop were observed in *netB*-*cpb2*-positive and *netB*-negative-*cpb2*-positive treatment groups. Such gross lesions were statistically different in both treatment groups compared to the control group. Likewise, microscopic lesions in the duodenum suggestive of early FDN were seen in some birds from both challenged groups. These lesions were characterized by mild heterophilic inflammatory infiltration, hemorrhages in the lamina propria and lumen, separation of loss of enterocytes from basement membrane and rarely single cell necrosis. The observation of macroscopic and microscopic lesions in the duodenum of challenged birds indicates that NetB and beta 2-positive C. *perfringens* type A isolated from FDN lesions can cause mild duodenal lesions resembling the acute form of FDN.

Our toxinotyping of *C. perfringens* strains isolated from FDN lesions in field outbreaks revealed the presence of *netB and cpb2* genes in 3 out of 17 *Clostridium perfringens* strains, while eight out of 19 *C. perfringens* strains only carried the gene *cpb2*. We believe that the use of virulence genes from FDN outbreaks may enhance the ability of this pathogen to induce disease under experimental conditions. NetB is a pore forming toxin considered an essential virulence factor to induce necrotic enteritis in chickens (9). In experimental models of necrotic enteritis, more severe lesions have been observed in *netB* positive isolates. Birds orally inoculated with *Clostridium perfringens* containing the *netB* gene presented with a greater prevalence of lesions and approximately 50% of challenged birds developed necrotic enteritis (13). Similar to what was observed with *netB* gene, histological necrotic lesions were found when beta2-positive *C. perfringens* positive strains were inoculated in an intestinal ligated loop model (12). In addition, *C. perfringens* strains positive for *netB* and/or *cpb2* gene have been detected in focal duodenal

necrosis lesions (1), (5). One study in the Netherlands reported that 19 out of 76 *C. perfringens* strains isolated from diseased birds carried and produced beta2 toxin, whereas it was only found in 4 out of 15 isolates from SPF birds (1). In another study performed at The University of Georgia, 4 *C. perfringens* strains isolated from FDN lesions carried the *cpb2* gene and the presence of CPB2 toxin in the lesions was confirmed by immunohistochemistry (5).

In agreement with these previous studies, the severity of lesions was higher in inoculated birds when compared with the control group. However, the presentation of lesions was not as severe as seen in field conditions. One explanation for the limited success at inducing more severe lesions of FDN in this experiment is that there might be other infectious agents involved in the pathogenesis of FDN. Similar to necrotic enteritis, where a coinfection with coccidiosis is commonly observed, we speculate that an interaction between FDN and other disease causing organisms is possible. The histological evaluation of duodenal samples that had gross lesions suggestive of FDN have shown the presence of long Gram-negative rods located in the lesions (5). Although the nature of this bacterium is still unknown, it would be important to further investigate and characterize this pathogen in order to establish causal association with FDN.

In our study, *Clostridium perfringens* was isolated from only one sample. This sample showed gross lesions and belonged to the *netB-cpb2* positive inoculated group. Toxinotyping by PCR revealed that this isolate was positive for *plc* and *netB.* The low culture detection of *Clostridium perfringens* from lesions may possibly be explained by low-level colonization and proliferation of *C. perfringens* in our challenge model. In addition, since birds used in this experiment were 22 weeks of age and commercially raised, they probably had an established microbiota that did not allow *C. perfringens* to colonize and proliferate (2). The fact that there were low numbers of *Clostridium perfringens* to significantly induce disease may be associated

with the acute presentation of lesions or perhaps, the conditions in the duodenum of infected birds were not optimal for development of rapid bacterial growth. Moreover, lesions observed might have been caused by toxins present in the inoculum supernatant which would explain the presence of lesions even though *C. perfringens* was not detected.

In the present study, we used DDGS as a dietary predisposing factor to induce disease. DDGS are high in dietary fiber (42%) which can stimulate mucin production and may result in an ideal substrate for *C. perfringens* (11). DDGS are also high in non-starch polysaccharide which can increase the intestinal viscosity and reduce the digestibility (15). In commercial layers, DDGS is a common ingredient added to the diet. A survey performed in different layer operations showed that 80% of affected FDN flocks used this ingredient in the pre-lay and layer diets (A. M. Villegas and M. Franca, unpublished data). Although this study demonstrates that *netB*-positive and *netB*-negative *Clostridium perfringens* isolates are capable of producing mild gross and microscopic lesions using DDGS as a dietary factor, the results also suggest that FDN might be a multifactorial disease and the development of an experimental model of FDN with characteristic lesions might require other predisposing factors or a combination of infectious agents.

The gross and microscopic lesions produced in the duodenum by experimentally infecting egg laying chickens with *netB* and *beta2*- positive *Clostridium perfringens* isolates suggest that this bacterium may contribute to the development of FDN. Nevertheless, the reproduction of FDN with characteristic lesions as seen in the field may require the presence of other infectious agents or predisposing factors that are still undetermined.

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recommendations expressed in this material are those of the author(s) and not necessarily reflect the views of the Egg Industry Center or Iowa State University.

Table 5.1. Ingredients of developer pullet diet used in this challenge.

Ingredient	%
Corn	76.883
Soybean Meal	11.769
DDGS	8.854
Phosphate	1.412
Calcium carbonate	0.781
NaCl	0.111
Trace Mineral	0.075
Vitamin premix	0.065
Fat, Vegetable	0.05

Table 5.2. Gross lesions observed in treatment groups.

Treatment Group	Subclinical NE score	Focal areas of mucosal erosions	Excess of gas	Epithelial sloughing	Mucosal Hemorrhages	Thickened mucosa
netB-*cpb2* positive	1 (15/18) 2 (3/18)	3/18	2/18	15/18	15/18	17/18
netB-negative-*cpb2*-positive	1 (15/18) 2 (3/18)	3/18	5/18	15/18	18/18	15/18
Control	0 (10/16) 1 (4/16)	0/16	4/16	4/16	0/16	4/16

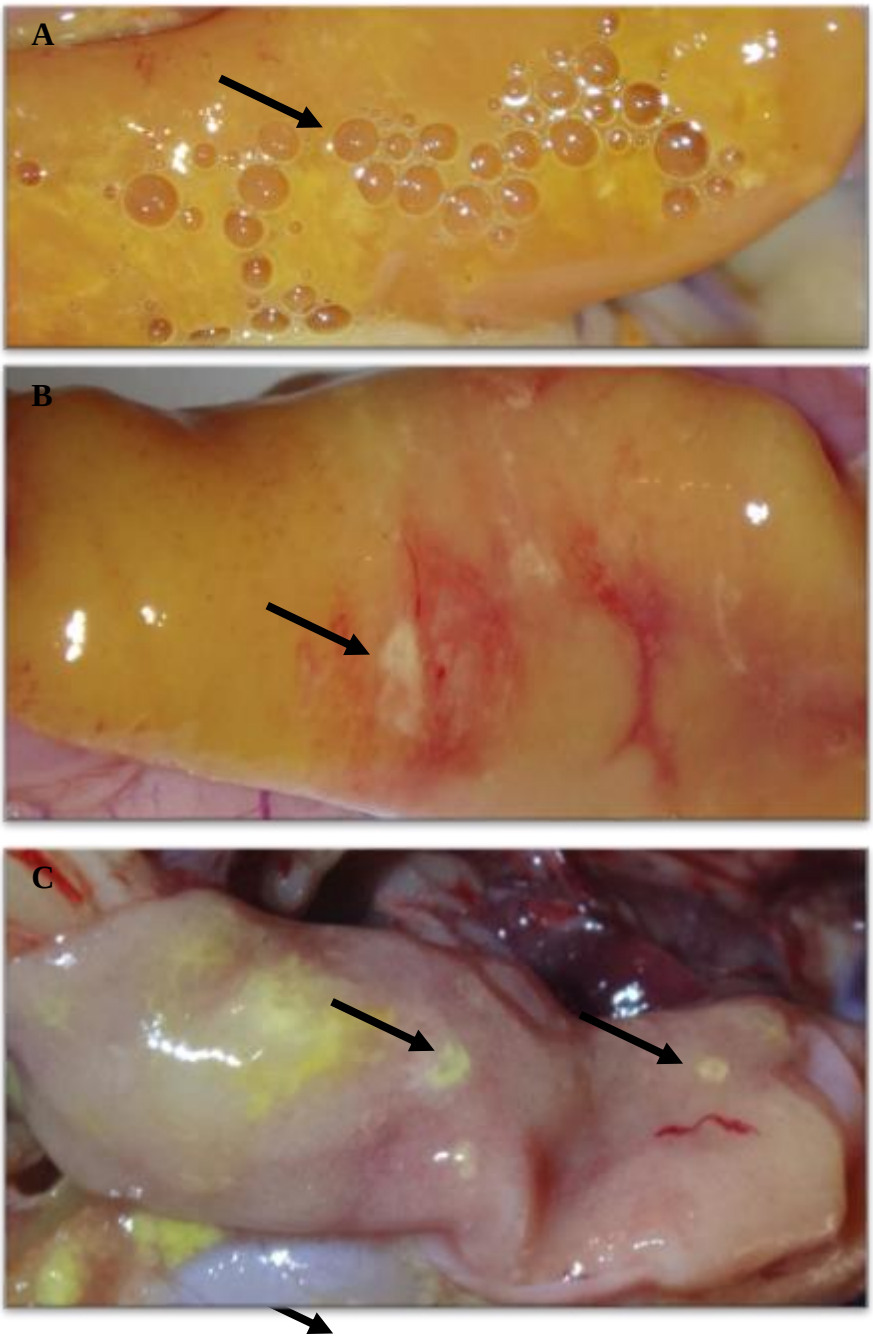

Figure 5.1. Necropsy findings, *netB* negative - *cpb2* positive group; **A**, excess of gas accompanied by presence of intestinal epithelial sloughing and mucus in the lumen, **B**, Multifocal areas of mucosal erosion with hyperemic areas and fibrinous exudate, **C**, multifocal mucosal erosions in the duodenal loop.

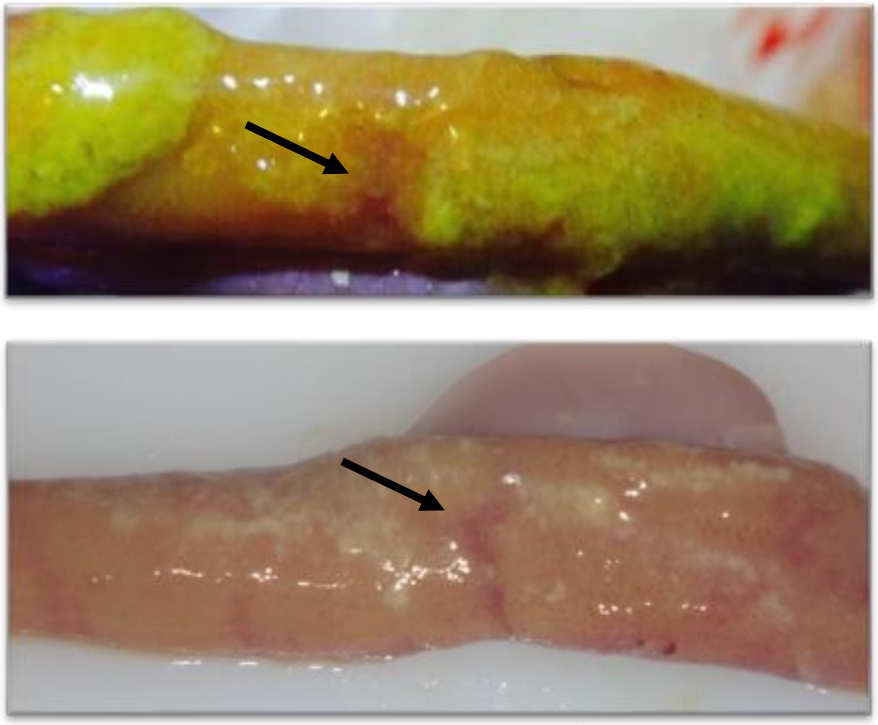

Figure 5.2. Necropsy findings, netB-cpb2-positive group; **A**, focal area of mucosal erosion, **B**, Multifocal hyperemic areas with presence of removable fibrin in the duodenal loop.

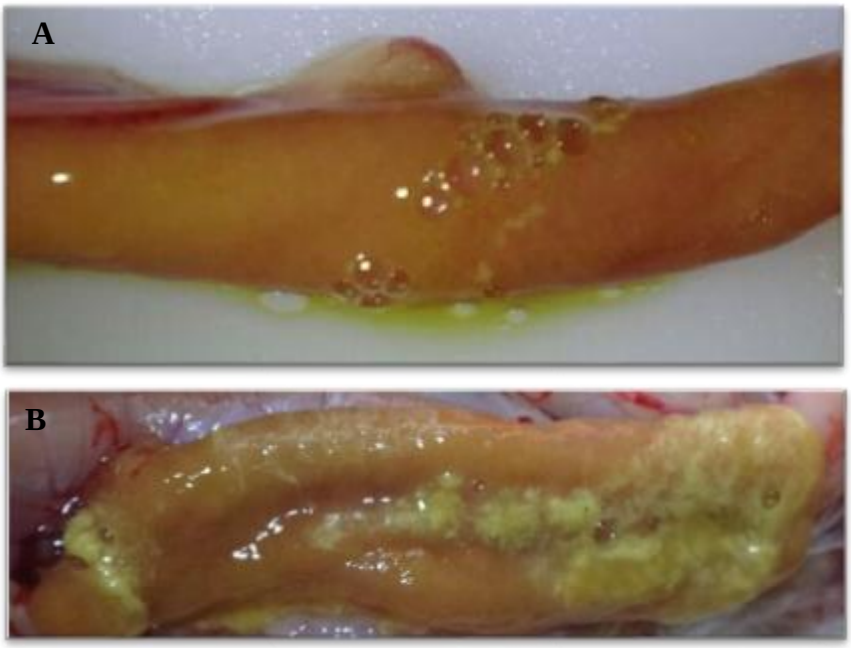

Figure 5.3. Necropsy findings, control group; **A**, normal intestinal tone, **B**, Intestinal epithelial sloughing/mucus. Absence of mucosal erosions.

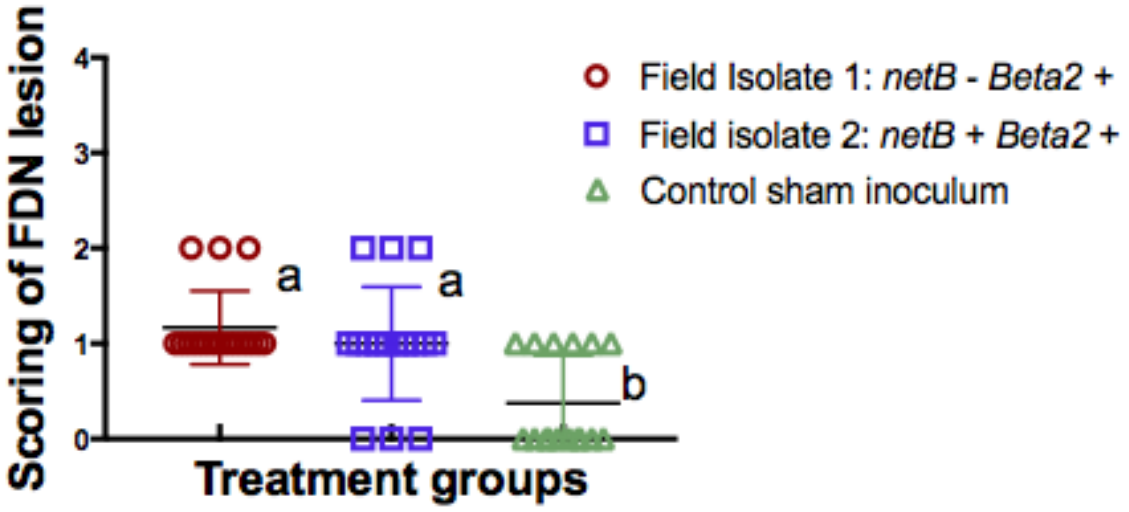

Figure 5.4. Scoring of FDN gross lesions in groups challenged with 2 different *C. perfringens* isolates and control group.

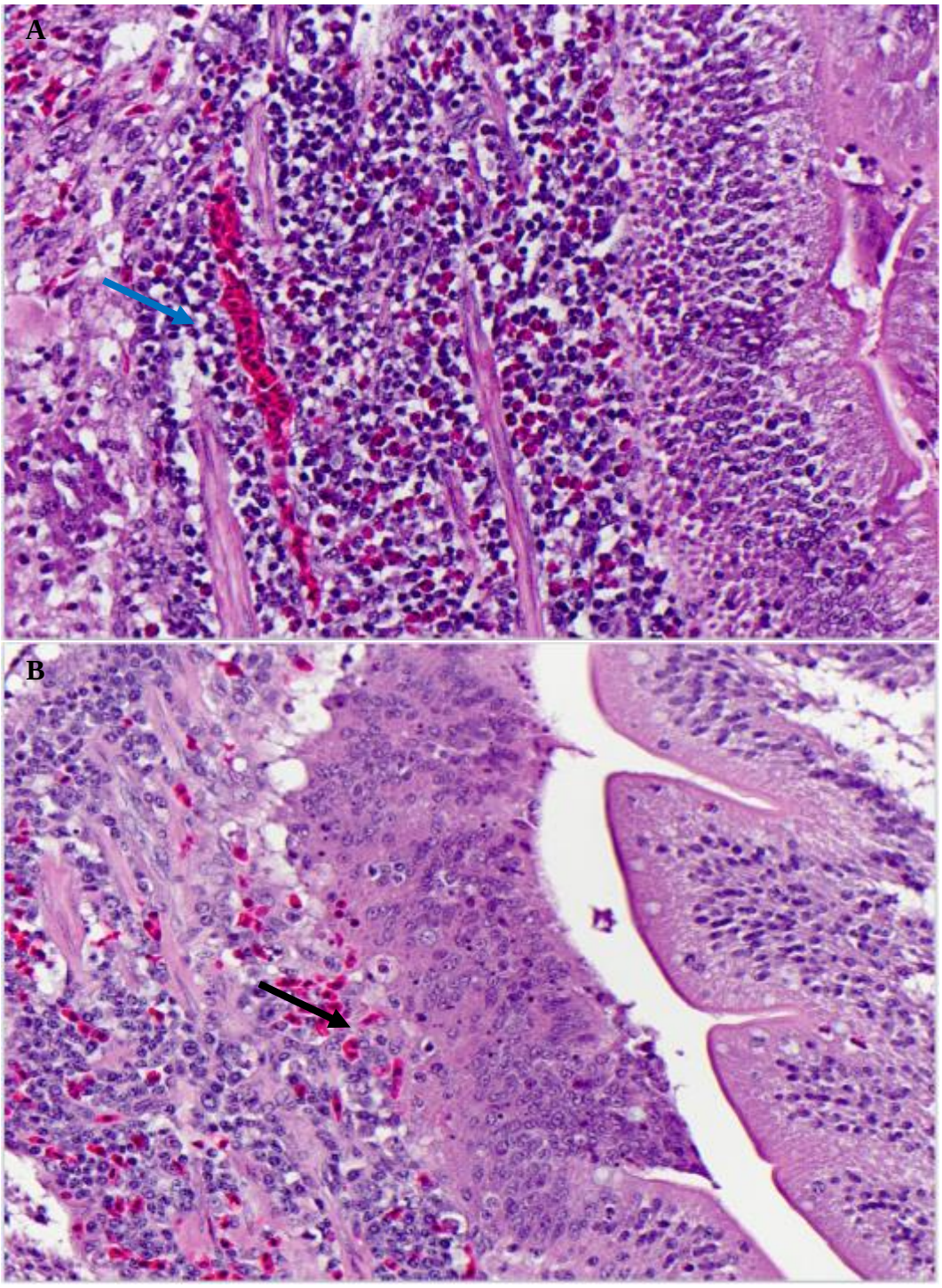

Figure 5.5. Photomicrograph of the duodenum, netB negative - cpb2 positive group; A, the lamina propria is expanded with inflammatory infiltrate composed by heterophils and lymphocytes. Hyperemia is also observed (blue arrow), B, single enterocyte necrosis in the intestinal villus with enterocyte detachment and mild hemorrhages in the lamina propria (black arrow).

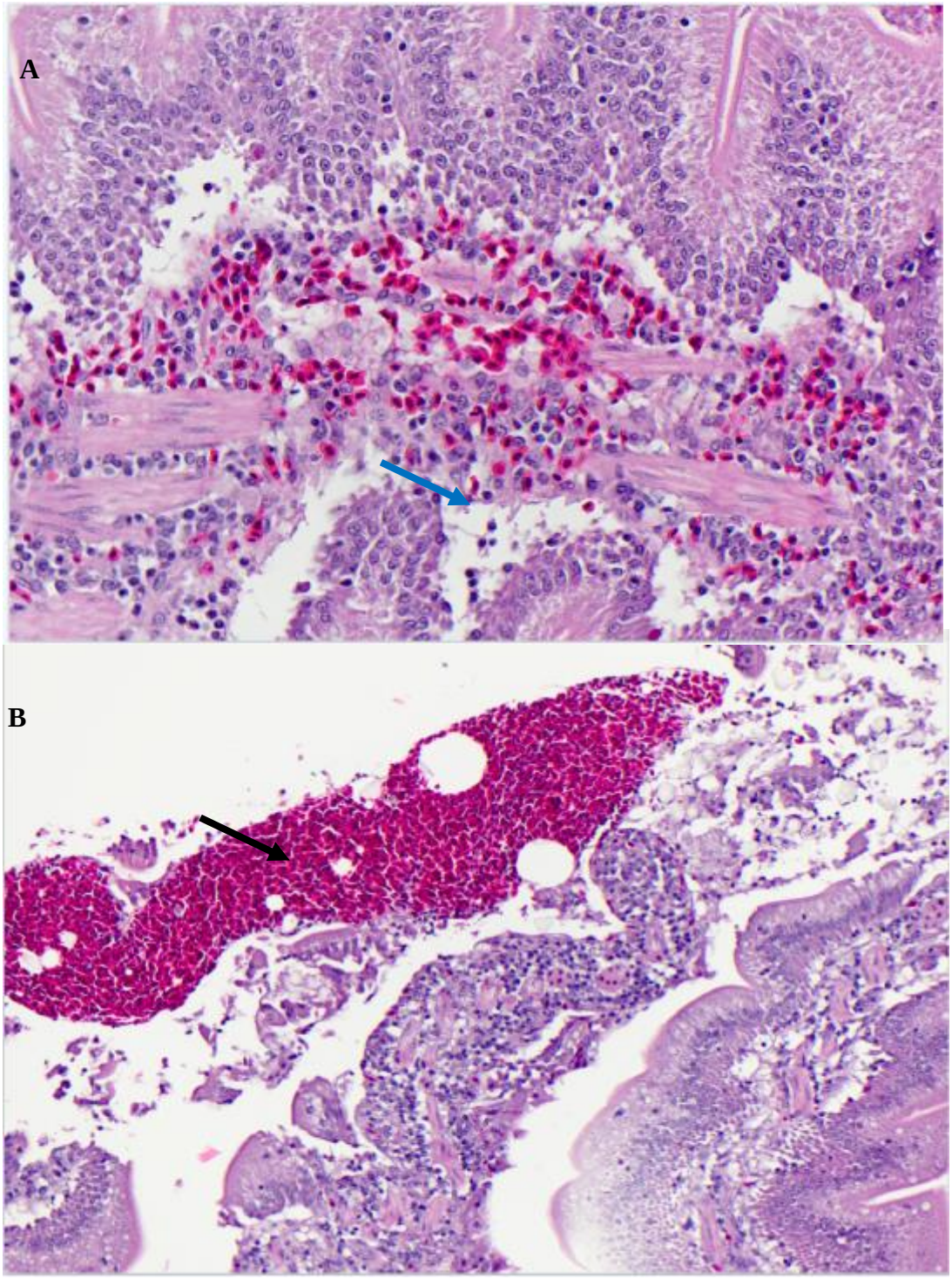

Figure 5.6. Photomicrograph of the duodenum, netB-cpb2 positive group; A, enterocyte detachment in the villus tips with mild hemorrhages in the lamina propria (blue arrow), B, hemorrhages in the lumen of the intestine (black arrow).

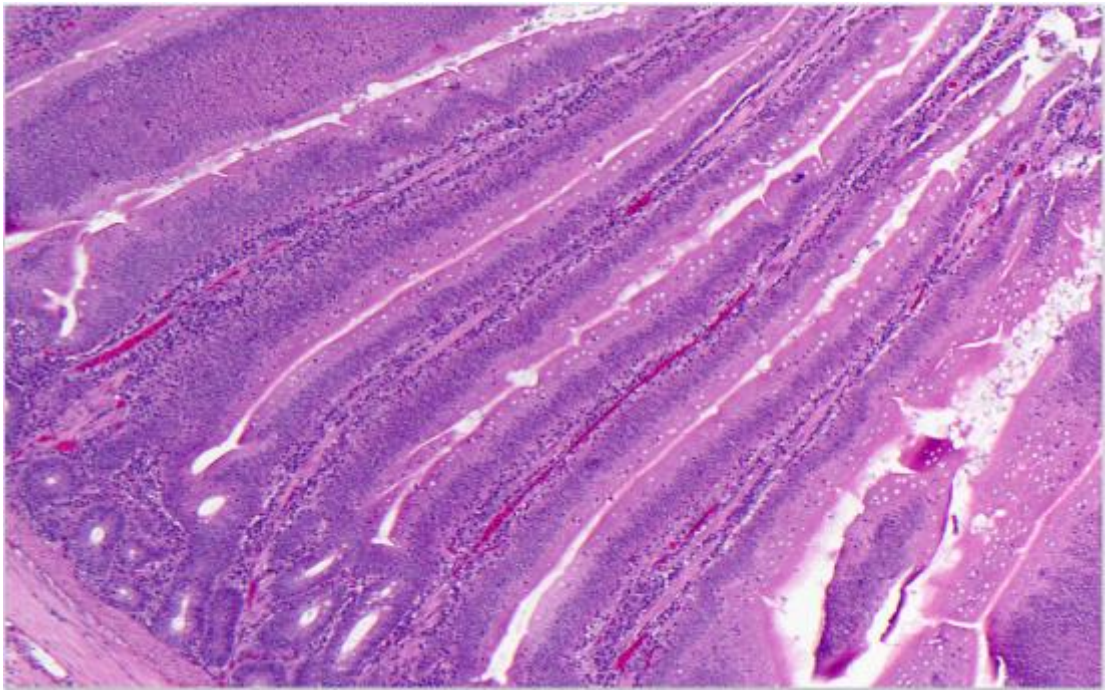

Figure 5.7. Photomicrograph of the duodenum, control group; normal villus with minimal lymphocytic infiltrates.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Focal duodenal necrosis (FDN) is a widespread enteric disease of table egg layers. Since FDN was first described in 1996, FDN has increasingly gained importance around the U.S. table egg industry. The decrease of egg case weights, coupled with the cost of treatment has resulted in important economic losses. Furthermore, the infection agent of FDN remains unclear which makes prevention and control of this disease difficult. Enhanced methods of disease surveillance including bacteriology and histopathology examination of affected birds are needed to better understand the disease.

One of the objectives of this study was to describe the epidemiology of layer flocks with FDN. FDN cases were defined as flocks that were diagnosed with FDN in the last year. This study utilized an online questionnaire as an indicator of management, nutrition, sanitation and prophylactic characteristics that may possibly be associated with FDN. Because the majority of affected flocks reported that coccidiosis was not a problem, we believe that infection by *Eimeria spp*. might not be an important factor for the development of FDN. Although, mycotoxins were not a problem in half of the flocks, the mycotoxin status of the feed was unknown in 35% of affected flocks. Therefore, the possible role of mycotoxin in the pathogenesis of FDN is still uncertain. Nutritional factors such as Distiller's dried grains (DDGS) and calcium that are known to be factors to predispose to Clostridiosis, were used in most of FDN-positive flocks. In addition, pests (rats and mice) that are known to be potential sources of *Clostridial* species were described as problems with low to high severity in layer flocks with FDN. However, since this study does

not fully elucidate risk factors associated with FDN, further research needs to be conducted. Another interesting observation in this study was that the majority of affected flocks surveyed received the combined use of antibiotics, probiotics and enzymes leading us to hypothesize that these methods of disease prevention and control were not effective in terms of controlling FDN. Moreover, while *Clostridium* species are spore forming organisms and the spores are highly resistant to disinfectants, the procedures performed in affected flocks during the downtime may not be sufficient to prevent the persistency and transmission of *Clostridial* diseases.

The second objective was to characterize the bacteriology and pathology of FDN. Sampling and collection of samples was performed in several farms for bacteriology, histopathology and immunohistochemistry. In our study, *Clostridium perfringens* was the most frequently detected bacterium in flocks with clinical characteristics of FDN (egg production drops and decreased egg case weights), as well as in birds with typical gross lesions. *C. perfringens* isolated were molecularly toxinotyping by PCR. Strains characterized carried the genes *netB* and *cpb2* which is consistent with previous FDN reports. In addition, immunohistochemistry analysis demonstrated the in-situ detection of *C. perfringens* in all samples with classic FDN lesions. Histopathology evaluation also revealed differences in the presentation of the microscopic lesions, with acute lesions presenting enterocyte detachment and single cell necrosis with mild inflammation and long rod-shaped bacteria, while characteristic lesions had necrosis of villus tips and fibrinoheterophilic inflammation associated with bacteria. Additionally, low prevalence of cestodes and coccidiosis was seen in FDN samples.

Finally, the ability of two *C. perfringens* strains isolated from FDN-affected flocks to cause disease was investigated in an in-vivo pathogenicity study in egg-laying chickens using DDGS as a dietary predisposing factor. Mild duodenal lesions were observed suggesting that *NetB* and

beta2- positive *Clostridium perfringens* may contribute to the development of FDN. Nevertheless, the reproduction of FDN with characteristic lesions as seen in the field may require the presence of other infectious agents or predisposing factors that are still undetermined.