NECROTIC ENTERITIS IN BROILER CHICKENS: STUDIES IN DISEASE REPRODUCTION AND PATHOGENESIS

By

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Kerry Kevin Cooper
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DEDICATION

To

Margarethe Alexandra Cooper

My Wife

And

My Heart and Soul

And

My Parents:

Ray and Shirley Cooper

My Family:

Dan and Ida Stringham,

Daniel Stringham,

And

Dr. Albert and Barbara Koy
# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ 8

LIST OF TABLES ........................................................................................................... 9

ABSTRACT .................................................................................................................. 11

1.0 INTRODUCTION .................................................................................................... 12

1.1 Problem ................................................................................................................ 12

1.2 Overview ............................................................................................................. 12

1.3 Etiology of NE ................................................................................................... 14

1.4 Historical Aspects .............................................................................................. 15

1.5 Signs, Lesions, and Diagnosis ......................................................................... 20

1.6 Pathogenesis and Host Response .................................................................... 23

1.7 Epidemiology .................................................................................................... 29

1.8 Treatment and Prevention .............................................................................. 36

1.9 Reproduction of NE ......................................................................................... 41

1.10 Research contributions ................................................................................... 44

2.0 PRESENT STUDY ................................................................................................ 45

2.1 Methods ............................................................................................................. 45

2.2 Results ............................................................................................................... 48

2.3 Conclusions ....................................................................................................... 52
3.0 REFERENCES............................................................................................................55

APPENDIX A: NECROTIC ENTERITIS IN BROILER CHICKENS:
REPRODUCTION OF DISEASE AND ROLE OF *IN VIVO* PASSAGE ON
VIRULENCE OF *CLOSTRIDIUM PERFRINGENS* ISOLATES...............................69

APPENDIX B: VIRULENCE FOR CHICKENS OF *CLOSTRIDIUM PERFRINGENS*
ISOLATED FROM POULTRY AND OTHER SOURCES.............................................96

APPENDIX C: IMMUNIZATION WITH RECOMBINANT ALPHA TOXIN
PROTECTS BROILER CHICKS AGAINST EXPERIMENTAL CHALLENGE WITH
*CLOSTRIDIUM PERFRINGENS*...............................................................................119

APPENDIX D: SUPPLEMENTAL MATERIAL: THE ABILITY OF CRUDE TOXINS
FROM *CLOSTRIDIUM PERFRINGENS* TYPE A TO PRODUCE NECROTIC
ENTERITIS IN BROILER CHICKENS.......................................................................137
LIST OF FIGURES

Appendix A

Figure 1. Pulsed field gel electrophoresis of recovered JGS 4143 isolates from chicken intestinal samples.................................................................92

Figure 2. Gross lesions typical of necrotic enteritis from reproduction studies........93

Figure 3. Histopathology of the jejunum of challenged and unchallenged chickens......94

Appendix B

Figure 1. Gross examination of intestinal tract of birds challenged with various C. perfringens strains.................................................................................................114

Figure 2. Histopathology of intestinal tract of birds challenged with various C. perfringens strains.................................................................................................115

Figure 3. Alpha toxin assays of challenge inocula........................................................116

Figure 4. Pulsed field gel electrophoresis of recovered JGS 1473 isolates from chicken intestinal samples.................................................................117
LIST OF TABLES

Present Study

Table 1. Toxinotypes of Clostridium perfringens ..............................................................15
Table 2. Elimination of antimicrobial growth promoter (AGP) use in countries of the European Union (EU) ........................................................................................................19
Table 3. Eimeria spp associated with coccidiosis in poultry ..............................................34
Table 4. Lesion scoring systems for necrotic enteritis ...........................................................43
Table 5. Clostridium perfringens strains used in bacteriocin and pilA studies ................45
Table 6. PCR primers and reaction parameters ......................................................................47
Table 7. Zone of inhibition results of potential bacteriocin production ..........................49
Table 8. Presence of two pilA genes in non-avian strains and necrotic enteritis strains .................................................................51

Appendix A

Table 1. Major toxin production by Clostridium perfringens ...........................................88
Table 2. Strains used in these studies ..................................................................................89
Table 3. Effect of in vivo passage on virulence of Clostridium perfringens strain JGS 4143 ...................................................................................................................................90
Table 4. Response of birds to challenge with various necrotic enteritis strains ...........91

Appendix B

Table 1. Major toxin production by types of Clostridium perfringens .............................110
Table 2. Clostridium perfringens strains used in studies ....................................................111
Table 3. Response of birds to challenge with non-avian strains of Clostridium perfringens ........................................................................................................................................112
Table 4. Response of birds to challenge with re-isolated non-avian strains.................113

Appendix C

Table 1. Response of vaccinated birds and controls to challenge with Clostridium perfringens..................................................................................................................134

Table 2. AntiCPA antibody response of birds following vaccination with recombinant CPA and challenge with C. perfringens.................................................................................................................135

Appendix D

Table 1: Response of birds to challenge with filtered culture supernatant fluid from Clostridium perfringens.................................................................................................................................144
ABSTRACT

Necrotic enteritis in poultry is caused by *Clostridium perfringens* type A, and is estimated to cost the worldwide poultry industry approximately $2 billion dollars a year, due to increased mortality and decreased feed conversion and weight gain. Very little is known about the pathogenesis of this disease due to the lack of a consistently reproducible experimental model. This dissertation outlines the development of an effective and consistent experimental model for necrotic enteritis in broiler chickens. It was also found that *in vivo* passage through the chicken’s intestinal tract let to increased virulence; we increased the proportion of birds developing disease from 34.6% to 81.4%. Researchers have proposed that alpha toxin (CPA) is believed to be the critical virulence factor of the disease. All type A isolates have the potential to produce CPA, thus we challenged birds with numerous type A isolates that are virulent in other animal hosts. However, we found that they did not produce necrotic enteritis in broiler chickens. In addition, challenge with culture supernatant alone failed to produce gross lesions in the birds, although challenging with washed whole cell cultures did do so. Vaccinating birds with HIS-tagged recombinant CPA provided partial protection against disease; there was a 42.0% decrease in lesion development. The conclusion of this doctoral research is that CPA does have a role in the pathogenesis of necrotic enteritis in broiler chickens, but there are apparently other critical virulence factors involved in the development of disease.
1.0 INTRODUCTION

1.1 Problem

Over the last few decades, necrotic enteritis (NE) in poultry has been controlled and treated by addition of antimicrobials to feed or water. However, due to rising consumer concerns, many countries are banning routine use of antimicrobials in feed. The European Union has already banned antimicrobials, including many used to prevent NE, and a complete ban on the use of antimicrobials in animal feed is planned. The ban has been accompanied by an increased number of European outbreaks of NE, and similar scenarios are anticipated elsewhere. Thus, alternative prevention methods, such as vaccines, are needed. However, little is known about the pathogenesis of NE, and a major hindrance has been lack of a reliable model.

1.2 Overview

The poultry industry has grown dramatically worldwide over the past 40 years, and with this growth have come new demands that have changed the industry’s infrastructure. The US broiler industry produced 1.11 billion pounds live weight in 1945, and was valued at $327 million. In less than 60 years, industry productivity rose to 44 billion pounds and value to $15.2 billion (181, 182). In 2003, the total value of poultry production in the US had reached $23.3 billion. The US poultry industry now produces 8.49 billion broiler chickens and 87.2 billion eggs annually (181). In Europe, poultry production has also increased dramatically (e.g., 23.8 million to > 65 million broilers annually over 30 years in Sweden and 113 million to >137 million broilers annually over
10 years in Denmark) (59, 60). Annual production worldwide is at 34 billion broilers, 4 billion layers, 800 million breeders, 600 million turkeys, and 800 million ducks. This growth results from increased poultry production in many countries, although production in India, Pakistan, and Bangladesh has massively increased (173).

Higher demands for quality meat, and improvements in housing, equipment, and genetics, have transformed poultry production into a highly specialized field where success requires significant economic investment (42, 51). Startup costs in the US have risen to ~ $ 80 million for a one million bird farm, while the past 50 years have seen the live weight price rise only from $ 0.36 per lb to $ 0.393 per lb (19). Genetic selection has resulted in faster growth, reductions in feed conversion, higher meat yields, and low mortality rates. For example, the average final live broiler weight in the US in 1945 was 3.03 pounds, but in 2001, the average final live weight was 5.06 pounds (182). In 1976, birds increased in weight to 2 kg in 64 days, whereas today that time is only 38 days (145). Faster growing birds with higher feed efficiency must be raised to offset the 65 - 70% of producers' total costs that are invested in feed (1, 142). Improvements in housing and equipment have led producers to dramatically increase the number of birds per house. In Denmark, for example, the average broiler house carried ~27,000 birds 10 years ago, but flocks are now 40,000 - 50,000 per house (51, 61). In toto, NE costs the worldwide poultry industry $2 billion annually (89, 131).
1.3 Etiology of NE

Disease prevention is a major concern in poultry production, due to resulting decreased growth and increased mortality (142). Several clostridia cause disease in poultry, including *C. botulinum* (botulism) and *C. colinum* (ulcerative enteritis) (142). *Clostridium perfringens* is the most important clostridial pathogen of poultry, causing avian malignant disease, gizzard erosions, and gangrenous dermatitis (32, 80, 92, 110, 120, 144, 151, 177). However, this organism is most important as an enteric pathogen. A common and severe example is NE, which some consider to be the most clinically-dramatic bacterial enteric disease of poultry (51, 116).

*Clostridium perfringens* is an anaerobic, spore-forming, non-motile, large Gram-positive rod (36, 69, 83, 137, 142, 155, 161, 166), which is commonly isolated from any environment which may have been exposed to fecal contamination (69, 83, 92, 142, 161). It is a commensal in the intestinal tract of vertebrates, but is considered the most widely-occurring pathogenic bacterium (92, 161); it is responsible for a wide range of diseases in humans and animals (137, 199). *Clostridium perfringens* lacks the ability to produce 13 amino acids, and often obtains these from hosts via the action of exotoxins or degradative enzymes (36, 69, 155). It produces up to 17 exotoxins (36, 137, 161), and is classified into five toxigenic types (A to E) based on production of four major toxins (alpha, beta, epsilon, and iota) (85, 92, 137, 161, 166, 199) (Table 1). NE is most commonly caused by *C. perfringens* type A (5, 10, 11, 14, 54, 55, 58, 68, 75, 84, 85, 92, 101, 102, 118, 120, 129, 142, 156, 161, 177, 196) and, in rare cases, type C (68, 73, 101, 102, 109, 130, 144)
Table 1: Toxinotypes of *Clostridium perfringens*

<table>
<thead>
<tr>
<th>Toxinotype</th>
<th>Major Toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alpha (CPA)</td>
</tr>
<tr>
<td>A</td>
<td>X</td>
</tr>
<tr>
<td>B</td>
<td>X</td>
</tr>
<tr>
<td>C</td>
<td>X</td>
</tr>
<tr>
<td>D</td>
<td>X</td>
</tr>
<tr>
<td>E</td>
<td>X</td>
</tr>
</tbody>
</table>

1.4 Historical aspects

In 1930, Bennetts isolated *Bacillus welchii* (later renamed *Clostridium welchii* and then *C. perfringens*) from intestinal lesions in a Black Orpington pullet, and attributed the bird’s death to this organism (119, 136). In the 1940's, Mann described "six-day disease," in which he observed *C. perfringens* invading the intestinal walls of chicks (97). Later, McGaughey reproduced this condition in day-old chicks by feeding them cultures of *C. perfringens* (97, 194). In 1961, Parish described an intestinal disorder in a flock of six to seven week-old cockerels, naming the disease NE and isolating *C. perfringens* from the intestinal tract of affected birds (6, 27, 45, 76, 87, 115-117). Extracts from intestinal contents of diseased birds were lethal for chickens and mice, and isolates were originally classified as type F (115, 136). However, toxicity was neutralized by type B and type C antitoxin, and these isolates are now believed to have been type C (115). Parish also successfully reproduced the disease by *per os* administration of *C. perfringens*. 
perfringens, with opium to slow peristalsis (24, 68, 115, 136). Since Parish's description of NE in England, the disease has been reported worldwide (68, 84, 96, 116).

NE was first diagnosed in western Australia in 1961, and within three years had been reported by 10 producers in that state (73, 136). Between 1968 and 1971, more than 1000 cases of NE were reported by veterinary diagnostic laboratories in Canada (25, 115). A recent survey by Kaldhusdal and Skjerve revealed that, between 1969 and 1989, the incidence of NE in Norway varied from 0% to 34% in chicken populations (103). As the number of outbreaks of the disease increased around the world, researchers began to investigate methods for treatment and prevention.

One method for prevention was addition of antibiotic growth promoters (AGPs) to poultry feed. In 1948, it was found that increased growth rates and decreased mortality followed inclusion of dried fungal mycelia in poultry feed (59, 74, 82, 98). The growth-promoting component was later found to be an antibiotic (98), and by 1951, the U.S. Food and Drug Administration (FDA) had approved use of antibiotics in animal feeds (98). Such use has grown dramatically worldwide, and it is estimated that 50% of all antibiotics used in the European Union are given to animals (65). In The Netherlands in 1990, an estimated 80,000 kg of antibiotics were used in humans, as compared to 300,000 kg in animals (183). The broiler industry accounted for 26% of those veterinary antibiotics, translating to 430 mg per kg of meat per year (183). A 1999 survey by The Animal Health Institute found that 20.42 million pounds of antibiotics were used on animals annually in the US (98).
AGPs are hypothesized to contribute to animal health by suppressing microbial growth which may induce subclinical disease, reducing microbial destruction of essential nutrients, and increasing synthesis of vitamins or growth factors (82, 106). Research has now shown that AGPs mainly work by inhibiting or killing intestinal microbial flora, particularly Gram-positive bacteria (82, 105), thus making nutrients more available to the animal by eliminating competition (63, 106). By the early 1970s it was common practice for Danish poultry producers to include AGPs in poultry feed. In the US, 32 antimicrobial compounds are currently approved for use in broiler feeds without veterinary prescription (98). These dramatic increases in use of AGPs have resulted in rising concerns by consumers about the overall effect of these products on human health (74, 120).

The largest concerns are the development of antimicrobial resistance by bacterial pathogens of animals, but there is also concern about possible effects on consumers of drug residues in foods (174). There is concern that animal pathogens will transfer antimicrobial resistance to human pathogens, and that humans will acquire these resistant pathogens by consumption of animal-derived food products or exposure to the animals (82). Of major concern is the ability of some AGPs to instill cross-resistance to antimicrobials of classes used in human therapy (61). Cross-resistance is instilled between avoparcin and vancomycin (79), virginiamycin and quinopristin/dalfopristin, tylosin/spiramycin and erythromycin, and avilamycin and evernimicins (60, 61). Studies in The Netherlands found that erythromycin resistance was much higher in broiler populations than in humans, perhaps due to heavy use of tylosin in poultry production (183). Among the most important cross-resistant bacteria are vancomycin-resistant
enterococci (VRE), which have become a major concern as a nosocomial infection in Europe and the US. Use of avoparcin as an AGP may have created a reservoir of VRE in animal products, leading to emergence and spread of VRE in humans in Europe (59, 79, 126). Pulsed-field profiles of human VRE isolates were identical or closely related to those of poultry isolates (126). VRE were found in poultry only where avoparcin had been used to prevent NE (126). Enterococci resistant to pristinamycin, a new antimicrobial for treatment of VRE infections, have been isolated from humans and domestic animals. Pristinamycin belongs to the same antimicrobial class as virginiamycin, another major AGP, also inferring the role of AGPs in generating antimicrobial resistant human pathogens (200). Clindamycin is not approved for use in animal feeds in Japan, and high level resistance of poultry enterococci to this antimicrobial may be due to cross-resistance to lincomycin, which has long been used by Japanese poultry producers (200). In consequence to these concerns, many countries in Europe have banned the use of AGPs.

Beginning in the 1970s, countries of what is now the European Union began eliminating the use of AGPs (Table 2) (38, 59-61, 79, 185). The positive results of the AGP ban in Europe have been that the prevalence of VRE in animals has decreased dramatically, although these findings have not been uniform (e.g., in Norway) (60, 126). The rate of VRE isolation rate from poultry in The Netherlands decreased by nearly 50% (61). Prevalence of isolates of *E. faecium* resistant to avoparcin, avilamycin, vancomycin, virginiamycin, and erythromycin in Denmark decreased significantly. The Netherlands,
Germany, and Italy reported decreased prevalence of VRE from poultry and humans since the avoparcin ban (60).

Table 2: Elimination of antimicrobial growth promoter (AGP) use in countries of the European Union (EU)

<table>
<thead>
<tr>
<th>Year</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1970s</td>
<td>EU eliminated use of human therapeutic antimicrobials as AGPs.</td>
</tr>
<tr>
<td>1986</td>
<td>Swedish ban of AGPs</td>
</tr>
<tr>
<td>1995</td>
<td>Ban of AGP use of avoparcin in Denmark and Norway</td>
</tr>
<tr>
<td>1997</td>
<td>EU ban of avoparcin as AGP</td>
</tr>
<tr>
<td>1998</td>
<td>Danish ban on virginiamycin use, then all AGPs</td>
</tr>
<tr>
<td>1999</td>
<td>British poultry industry ban of AGPs, EU ban of spiramycin, tylosin, bacitracin, and virginiamycin as AGPs</td>
</tr>
</tbody>
</table>

The negative effect of withdrawal of AGPs from poultry feed has been an increased number of outbreaks of NE and other clostridial diseases, especially in western Europe (44, 185, 198). In France, incidence of NE rose from 4% in 1995 to 12.4% in 1999 (38, 53). In Norway, Sweden, Denmark, and elsewhere, an immediate increase in the number of NE cases was followed by a decline to pre-ban levels with the introduction of the coccidiostats narasin and salinomycin (63, 79, 119). Most countries outside Europe still control NE with combinations of AGPs and ionophores, but in Europe, particularly the Nordic countries, NE is controlled by use of ionophores, good hygienic practices, and modified diets (63, 79).
### 1.5 Signs, Lesions, and Diagnosis

Typical clinical signs of NE in chickens include depression (4, 6, 31, 32, 68, 87, 100, 115, 131, 136, 142, 185), reluctance to move (68, 87), diarrhea (68, 87, 131, 142, 161, 185), ruffled feathers (4, 68, 87, 115, 142, 185), somnolence (4, 185), decreased appetite/anorexia (31, 32, 68, 131, 142, 161, 185), dehydration (116), and huddling (115, 142). However, the clinical course is often very short, with birds dying within 1 or 2 h (31, 32, 68, 136). Similar clinical signs are seen in other birds (e.g., ostriches or turkeys) with NE (55, 57, 110). Mortality rates range from 0.1% to 50% (49, 73, 84, 87, 115, 156, 185, 194).

Gross lesions are typically restricted to small intestine (25, 115, 142). They are most common in jejunum and ileum (68, 110, 116, 136, 138, 142, 185), but can occur in duodenum and ceca (4, 68, 109, 116, 136, 138, 142, 156, 185). Long et al (116) suggested that cecal lesions occur when necrotic debris with associated \textit{C. perfringens} is shed from jejunum and passes into the ceca. Small intestine is usually distended with gas (33, 52, 68, 70, 87, 109, 110, 115, 142, 156, 185), and contains a foul-smelling dark brown, bile-stained fluid (4, 68, 87, 100, 115, 156). From the serosal side, small intestine can be gray, brown, or yellow-green (4, 87, 136, 148), and the intestinal wall is usually thin and extremely friable (4, 29, 68, 100, 109, 110, 115, 116, 138, 142, 185). The mucosal surface is typically covered by a focal-to-confluent (30, 32, 45, 116, 142, 156, 186), yellow-green to yellow-brown, loosely to tightly adhered diphtheritic membrane (25, 32, 33, 68, 87, 100, 109, 110, 116, 138, 142, 156, 185, 186). Hemorrhage is not
typically seen in field cases (68). In subclinical NE, lesions are focal in small intestine. Typically, they are circular depressions with peripheral hyperemia, usually 1 to 2 mm in diameter and covered with adherent yellow material (100).

Gross lesions of NE in turkeys are very similar (11, 55, 75, 151), but the duodenum is more frequently affected than in chickens (75) and lesions can extend into colon (57). Gross lesions in small intestine of ostriches were diffuse and fibrinonecrotic, and in the intestine, dilated with gas and yellowish fibrinohemorrhagic fluid (110).

Intestinal damage may allow \textit{C. perfringens} access to portal circulation and biliary ducts, resulting in cholangiohepatitis (118). Liver lesions associated with NE usually manifest as cholangiohepatitis, characterized by a pattern of small, pale, focal lesions (68, 94, 119, 151, 185, 186), with discolored and/or thickened bile ducts and gall bladder or as hepatitis (94, 119), with multi-focal to confluent parenchymatous nodules which are light yellow, grey, green, or red (94, 120). The liver is typically enlarged, firm, and pale, with multiple scattered yellow necrotic foci (68, 102, 118, 119, 151, 156, 185, 186). There is marked congestion, and in certain cases the gall bladder is distended with bile (156).

Microscopic examination of tissues from field cases in chickens reveals extensive necrosis of intestinal villi (33, 70, 109, 116, 136). In some cases, cellular degeneration may reach the submucosa (68, 136) or even the muscularis mucosa (109, 136). Lesions typically develop at villous apices, with sloughing of epithelial cells accompanied by coagulation necrosis (68, 138, 156). Heterophils are the dominant inflammatory cell infiltrating lamina propria, but mononuclear cell infiltration is found in more chronic lesions. Indeed, the sharp line of demarcation between necrotic and viable tissue (4, 115,
116, 136, 142) is due to accumulation of mononuclear cells at the outer limit of the latter
(33, 109, 115, 116). In the intestinal lumen, shed necrotic villi or degenerated epithelial
cells and inflammatory cells trapped in fibrin (4, 68, 87, 109, 115, 116, 138, 142)
comprise the diphtheritic membrane seen grossly (87, 109, 116, 138). Large, Gram-
positive rods are commonly associated with areas of necrosis (4, 33, 52, 68, 87, 109, 115,
116, 138, 142), but electron micrographs reveal that these bacteria do not invade the cells
(142). Lesions in species other than chickens are extremely similar (11, 55, 75, 110).

Regeneration of the intestinal tract in field cases is characterized by proliferation
of epithelial cells at the crypts, production of a connective tissue network at the
inflammatory zone, decreased numbers of goblet and columnar epithelial cells, and
increased numbers of cubodial cells. Overall, regeneration leaves the intestinal tract with
short, flat villi that have a reduced absorptive surface (68, 116).

Microscopic lesions of hepatitis or cholangiohepatitis include extensive multi-
focal coagulative necrosis or liver (136, 151) and bile ducts (102). Gall bladder and extra-
hepatic bile ducts are thickened and distended with yellow inspissated material (152), and
hyperplasia and occasional granulomatous inflammation occurs in the bile ductules (118,
139, 185). Gram-positive bacilli are found in necrotic areas (102, 139, 151). Vissiennon
et al found mitochondrial lesions in hepatocytes, and cytoplasmic swelling of capillary
endothelial cells in liver (186).

The standard for diagnosing NE in poultry includes necropsy and bacteriologic
culture, with genotyping of isolates (67, 68, 142).
1.6 Pathogenesis and Host Response

Intestinal bacterial flora populations have a major impact on poultry growth and health, and diet influences both the activities and the composition of chicken microflora (27, 105, 184). When birds are fed poorly-digestible products (e.g., wheat and animal proteins), undigested proteins and fermentable carbohydrates reaching the hindgut lead to increased microbial activity (147). Birds on maize-based diets have lower numbers of Enterobacteriaceae and enterococci than birds on wheat- or rye-based diets (93). Duodenum and ileum of 14 day old chicks fed a corn-based diet were dominated by facultatively-anaerobic bacteria (i.e., *Streptococcus*, *Lactobacillus*, and *E. coli*), with only 9 to 39% of isolates being strictly anaerobic (*Clostridium*, *Fusobacterium*, and *Eubacterium*). Strict anaerobes (*Clostridium*, *Bacteroides*, *Gemminger*, and *Fusobacterium*) made up most of the microflora of ceca (150). Changes in the basic feed formula can alter composition of the intestinal microflora, allowing *C. perfringens* to either colonize or undergo anarchic multiplication (131).

Changes in intestinal flora are also presumably influenced by changes in diet throughout the feeding period. Overall, the small intestine microflora of chickens take about two weeks to establish, but cecal microflora can take up to 30 days to become completely established (9). Results from essentially all studies in this area are heavily influenced by culture techniques, so caution should be used when comparing study-to-study (198). It appears that flora of newly-hatched chicks is dominated by facultative anaerobes (e.g., *E. coli* and various streptococci), but over a few weeks becomes
predominantly obligate anaerobes (132). Numbers of streptococci decrease from ~ 30% to ~ 9% of the total population by 6 weeks of age (21). As the chick matures, lactobacilli increasingly dominate population in small intestine (184), while *Bacteriodes* spp., *C. perfringens*, and bifidobacteria play a similar role in ceca (26, 178). Highest numbers of bacteria are found in ceca (~ 10^{11} CFU per g of content), from which 38 anaerobic bacteria have been isolated (21, 122, 150, 178, 201).

*Clostridium perfringens* is a common member of the chicken intestinal flora (e.g., 75 - 95% in broiler chickens) (14, 49, 53, 105, 157, 170, 171, 185). Data on numbers of *C. perfringens* at various ages and in various locations in the gut are quite variable. Stutz and Lawton found that older chicks carried 10^2 - 10^3 CFU per g in diverse parts of small intestine (171), while others found 10^8 CFU per g (105). Results of one study suggest that numbers of *C. perfringens* in ceca are relatively low (10^3 CFU per g of ingesta) (171), while another study found much higher numbers (~ 10^9 CFU per g) (170). It seems likely that there are confounding factors among studies to make firm numbers unavailable and useful comparisons impossible. It does seem certain, however, that 10^6 to 10^8 CFU of *C. perfringens* are found per g of intestinal mucosa in birds with naturally-occurring NE (14, 116).

Engstrom et al examined type A isolates from healthy birds and those with NE or cholangiohepatitis. Subtyping by amplified fragment-length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) revealed that healthy birds carried 2 to 3 subtypes. They examined only one isolate from each diseased bird, but found that subtypes in birds with disease were different from those in healthy birds (64).
Gholamianekhordi et al found a high level of flock-to-to-flock diversity in subtypes, as well as wide genetic variance among multiple isolates from individual healthy chickens. Multiple isolates from birds with NE were of a single PFGE profile or, in rare cases, two profiles (77). Nauerby et al performed the most detailed study of the genetic diversity of *C. perfringens*, testing up to 30 isolates per bird. They found several different PFGE types of *C. perfringens* in individual healthy birds and within nondiseased flocks, whereas flocks (and individual birds in those flocks) affected by NE or cholangiohepatitis carried one or two types only. After treatment, recovered birds again yielded up to four PFGE types of *C. perfringens*. Interestingly, they found identical PFGE types in healthy birds and in birds with NE, suggesting that both the isolate and the appropriate conditions must come together for NE to result (137).

Thus, NE develops when specific strains of *C. perfringens* establish and multiply in the chicken's intestinal tract. Decreased intestinal motility, intestinal mucosal damage, and intestinal obstruction favor growth of *C. perfringens* (5, 33, 44, 46, 49, 52, 58, 68, 75, 118, 169, 174, 180, 185, 196), with early physiological changes including decreased intestinal pH and leakage of serum proteins into the intestinal lumen (53). *Clostridium perfringens* has a generation time of 8 to 10 min, so when growth conditions are optimal, populations can increase very quickly (166). High numbers of *C. perfringens* in the intestinal tract is a characteristic of NE, but alone is not sufficient to produce disease (185).

It is likely that pathogenesis is toxin-mediated, although this has not been definitively demonstrated. CPA is the only major toxin produced by *C. perfringens* type
A (64, 68, 77, 92, 121, 129, 130, 161, 185), and has been detected in feces of poultry
(high levels in NE birds and low levels in healthy birds) (161). Thus, there is some
sentiment that it plays a role in pathogenesis. NE isolates may (92) or may not (77)
produce higher levels of CPA than isolates from healthy birds.

CPA is a 370-amino acid polypeptide (69) with zinc-dependent phospholipase C
and sphingomyelinase activity (22, 77, 99, 134, 135, 185). Its structure and function have
been reviewed elsewhere (179). Its role in pathogenesis of myonecrosis has been
established (12, 34, 35, 69, 164-166, 168). Sakurai et al present a mechanism of action
against cells, including insertion of the C-terminal domain into the cell membrane, attack
by the N-terminal domain on either phosphatidylcholine or sphingomyelin, and
stimulation of signal transduction through phospholipid hydrolysis and activation of
endogenous PLC and/or SMase (149).

Inoculation of ligated intestinal loops in rabbits, rats, and chickens with CPA
results in fluid accumulation, inflammation, and mucosal damage (66). Al-Shiekhly and
Truscott (6) infused broth cultures intraduodenally and recorded the time course of events
in development of lesions. Five hours post inoculation; birds had intestinal necrosis
typical of field cases. After 12 h, necrosis was more severe, with lesions extending from
the site of infusion (posterior duodenal limb) to the distal ileum. Microscopic lesions
appeared much earlier (6). Twenty minutes after inoculation with crude toxin
preparations the intestinal tract showed edema in the lamina propria, dilation of blood
vessels, and some epithelial cell desquamation. After 40 min, the epithelial layer was
detaching from the lamina propria, and there was coagulation necrosis of some villous
tips. Coagulation necrosis was more common after 1 h, with a fibrinous exudate in the intestinal lumen (7). Dilation of blood vessels and slight edema were noted in lamina propria, and in birds challenged with broth cultures, large numbers of Gram-positive rods were found on villi. After 3 h, the epithelial layer detached from lamina propria, and there was a monocytic infiltrate. Blood vessels were blocked with erythrocytes and occasional hyaline thrombi. By 5 h post-challenge, necrotic lesions encompassed the entire length of villi, and this increased from 8 - 12 h. After 20 h, villous regeneration had begun (6, 7).

It should be noted, however, that these and other studies were based upon crude toxin preparations. Given that C. perfringens produces numerous toxic or potentially-toxic molecules (36, 137, 161), it is impossible to separate effects induced by one toxin from those induced by another. Synergistic effects add an order of magnitude to the complexity. Studies in which toxic effects were abrogated by mixing culture supernatant fluids with hyperimmune serum (prepared against toxoids produced from whole culture supernatant fluids) are similarly compromised due to the polyclonality of the antisera (5, 7).

Antibodies arising from immunization with the C-terminal domain of CPA neutralize both PLC and hemolytic activity and protected mice from the lethal effects of toxin or spore challenge (167, 197).

High titers of anti-CPA antibodies have been found in birds with a history of NE (121). Less than 3% of birds from healthy flocks were seropositive, compared to 15.7% of birds in flocks with a history of NE. Eighteen percent of birds from flocks with NE were seropositive at 35 days of age, but the rate of seropositivity was 78% in birds 53
days of age. Low titers of antiCPA IgY(IgG) were found in birds from flocks with low or high incidence of NE-associated hepatitis. Sixty to eighty percent of experimentally-inoculated birds developed antiCPA antibody titers. Thus, the exact role of antiCPA antibodies in immunity to NE remains unknown (120, 121).

Transfer of maternal antibodies [IgY, IgM, and IgA] from hen to chick in the yolk mass are important in protecting newborn chicks from disease (120). Yolk contains > 100 mg of IgY, and IgA and IgM are incorporated into egg albumin during formation (107). However, yolk IgY has a short half-life (120). Newly-hatched chicks have antiCPA antibodies, and chicks produced by older hens have higher levels of antibodies (84, 121). Chicks are immunologically-immature until ~ 3-4 weeks of age (111), and maternal antibodies persist for < 3 weeks (84). This may explain why NE typically occurs seen in chickens 2 - 6 weeks of age (120).

CPA produced by a swan isolate had 58 amino acid and significant structural differences from consensus, and was speculated to have an evolved role in the intestine, especially in avian hosts (99). This may or may not be the case, but examination of *cpa* from field NE strains revealed that most avian *C. perfringens cpa* sequences are homologous to mammal-derived strains. The most common sequence of *cpa* from NE cases had only one amino acid difference from the sequence in strain 13 (158).
1.7 Epidemiology

The major source of *Clostridium perfringens* during outbreaks of NE may be contaminated feed or litter (11, 27, 68, 70, 75, 151, 161, 176, 194, 195). Outbreaks of NE in Australia in the 1960s were associated with one commercial brand of poultry feed (136), and workers in Yugoslavia found that 64% of poultry feed samples were contaminated with *C. perfringens* (90). Contaminated feed components (e.g., fishmeal) may have also been a source of *C. perfringens* in NE outbreaks (57, 194). *Clostridium perfringens* has also been found in the yolk sac of an embryonated egg, suggesting vertical transmission from hens to chicks (157, 195). Dhillon et al (52) found that a possible source of infection for laying hens was flies. Dead flies were found in feed troughs and in intestinal tracts of dead birds, and bacteriologic culture yielded isolates of *C. perfringens*. Thus, flies may be porters of *C. perfringens*, resulting in contamination of feed or direct inoculation of birds (52).

Litter used for bedding can be composed of wood chips, sawdust, wheat straw, peanut hulls, sand, peat moss, or rice hulls; ~ 5.6 million tons of litter dry matter are produced in the US annually (123, 173). Poultry houses are typically cleaned and have litter replaced after raising 8 to 10 broiler flocks; however, rising production costs have led some producers to recycle or "decake" the litter by removing the upper 5 - 10 cm, which consists of manure, bedding material, and spilled feed (159). The practice of recycling litter increases exposure to *Eimeria* oocysts, coliforms, and *C. perfringens* (162).
*Clostridium perfringens* is common in soil around poultry farms, dust in poultry houses, litter, and as poultry intestinal flora (64, 68, 185). Most isolates are type A, although type C is occasionally found (137). In hatcheries, *C. perfringens* has been isolated from shell fragments, chick fluff (47, 48, 185), and paper pads used to transfer chicks from hatchery to grow-out facilities (185). Craven et al found *C. perfringens* on breeder farms, hatchery, previous grow-out flocks, grow-out flocks 3-5 weeks of age, and on processed carcasses (47). Isolates from breeder farm, hatchery, broiler farm, and a processed carcass were of the same ribotype, but they were unable to directly track the movement of any ribotype through the broiler operations (47, 185).

Outbreaks of NE are most common in broiler chickens two to six weeks of age (21, 25, 64, 67, 75, 84, 87, 115, 120, 136, 142), perhaps due to a window of ineffectiveness in immunity. Maternal antibodies disappear at ~ two weeks of age, and the chick immune system reaches maturity only around 3 to 4 weeks of age (111, 120). However outbreaks have also occurred in broilers up to eleven weeks of age (87, 115, 136). NE has also been reported in 3 to 6-month old commercial layers (68, 142), and in 12 to 16-week old replacement pullets (33, 68, 70, 136, 142). An outbreak of NE in 9 month-old chickens was reported in India in 1974, but this is the only reported case of the disease in older birds (109). NE outbreaks have also been reported in species other than chickens, including 7 to 12-week old turkeys (54, 55, 57, 68, 75, 161), 4 to 6-week old ostriches (110), captive wild fowls (17, 161), waterfowl, and wild crows (11).
Patterns of occurrence of NE are affected by management-related stress (46, 58, 156), infection by *Eimeria* spp. (10, 33, 46, 49, 52, 58, 68, 75, 77, 84, 87, 110, 156, 161, 177, 180, 185, 189, 198), changes in diet and other alterations to feeding programs (46, 49, 68, 84, 138, 156, 189), bedding on high fiber litter (10, 27, 68, 75, 84, 110, 180, 189), and dietary restriction or fasting (138). Time of year has a key role in initiation of NE outbreaks, with disease being most common during warmer months (July to October in the Northern Hemisphere and September to December in the Southern Hemisphere) (103). The counterpoint to this is a survey by Kaldhusdal and Skjerive of NE outbreaks in Norway from 1969-1989. They found that incidence of NE was lower during warmer months (103, 119). Identification of potentially-confounding factors, such as differing management styles and genetics of broilers, may resolve these apparently conflicting data.

High energy, protein-rich diets, particularly those with a high content of animal protein (e.g., fishmeal) (21, 33, 53, 75, 77, 109, 110, 115, 177, 185, 198) and dietary form (pelleted versus mash feed) (138) affect the incidence of NE. Increasing the amount of dietary crude protein (as fishmeal, but not as soy protein) increases numbers of *C. perfringens* in cecum (53). It has been suggested that source and amino acid content of the protein affect growth of *C. perfringens* in the lower digestive tract. Glycine and methionine increase the rate and extent of growth of *C. perfringens in vitro*, and CPA production requires addition of glycine-containing peptides to the medium (53). The nutritive value of fishmeal varies considerably from product to product, due to differences in raw materials, processing, and handling. Rations containing > 12%
Fishmeal have been associated with a condition called vomito negro (black vomit) in Mexico and Peru, a disease characterized by black watery fluid in the crop and erosion in the gizzard. Peruvian fishmeal at concentrations $\geq 12\%$ was more likely to cause vomito negro than menhaden fishmeal, which cause no lesions at the same concentration (108). Replacement of 25 - 50\% of soybean meal with fishmeal in poultry feed does not affect body weight, but replacing 75\% does so (187). There was no difference in the intestinal flora when the diet consisted of as little as 9\% or as much as 25\% fishmeal. However, the higher percentage of fishmeal was associated with an increased rate of growth (21).

Fishmeal and wheat both contain zinc (~150 ppm) (13), and this element increases weight gain in broilers (37).

Diets high in maize generally protect against NE, while barley- and birds on wheat- or barley-based diets may experience incidence NE at a rate 6 - 10 times higher (189). In fact, the ratio (wheat + barley)/maize can be an important predictor of NE (103). Experimentally-infected birds fed corn-based diets develop lesions which are much less severe than those in birds on a wheat-based diet (27). Mortality in broilers on a corn-based diet was up to 12.5\%, while the rate in those on wheat-, rye-, or barley-based diets was 26 - 35\% (185). Indigestible cell wall components in wheat are believed to be at least partially responsible for this difference. In addition, carbohydrates in corn may be less available for microbial digestion than those in wheat, barley, or rye (148). *Clostridium perfringens* proliferates at a higher rate *in vitro* in digested wheat or barley than in digested corn diets (10, 185). Wheat-, rye-, and barley-based diets also contain soluble-NSP fibers, such as arabinoxylans and $\beta$-glucans, which reduce digestibility,
interfere with absorption, and increase digesta viscosity (2, 28, 44, 77, 93, 113, 125, 160, 185). Higher viscosity leads to decreased weight gain and feed conversion ratios and to longer intestinal transit time (10, 28, 43, 53, 93, 112, 113, 125, 141, 163, 185, 192). Inclusion of NSP-degrading enzymes decreases transit time by nearly 50% and reduces the risk of NE in some cases (103). Soluble NSPs can also increase microbial populations in small intestine (2, 28, 43, 44, 62, 93, 112, 124, 125, 141, 163), since nutrients not digested by the host become available to bacteria in the lower part of the small intestine (2, 44, 93, 112, 113, 125, 141, 150, 163). Soluble NSPs may also interfere with starch digestion, making the starch available for bacterial digestion in the intestinal tract (192). Feeding of whole grains may have a similar effect on transit time. Feed intake may be reduced due to the bird's limited capacity for processing seeds in the gizzard and resulting in slower feed passage through the intestine (62, 72, 88, 172, 175).

The overall form of feed components also influences incidence of NE (185). Birds given pelleted feed gain more weight during the feeding period than birds fed mash (62). However, while the smaller particles in pelleted feeds are more digestible by birds, they are also more available to \textit{C. perfringens} (72). Feeding coarsely ground mash is associated with lower mortality (18.1% in one study) than is the feeding of hammer mill (finely ground) wheat (28.9%) (29, 148, 185).
Seven species of *Eimeria* infect chickens (Table 3). Sporozoites and merozoites invade intestinal epithelium, damaging the mucosa and resulting in severe hemorrhage by the fifth day of infection (97). Poultry coccidiosis occurs worldwide and costs poultry producers $800 million to $1.5 billion annually (18, 78). More than three-quarters of poultry farms have chickens infected with *Eimeria* sp., and subclinical coccidiosis may be present in all flocks (3).

Early outbreaks of NE were associated with coccidiosis (136), specifically *Eimeria brunetti* and *E. maxima* infection (87). Coccidiosis is associated with moisture in the litter, which allows the oocysts to sporulate (51, 196). *Eimeria* infections reduce intestinal pH, cause mucosal damage (4, 14, 53, 97, 196), and increase transit time by depressing peristalsis (53, 87, 156), all of which allow *C. perfringens* to increase in numbers (14, 196), particularly in the ceca (13-16, 26, 86, 97). Numbers of *C. perfringens* in birds in the acute phase of coccidiosis (days 5-6) were several logs higher in small intestine and ceca, but upon recovery (~ day 14), numbers had returned to normal levels. This finding applies to both conventional and specific pathogen-free chickens (86). In addition, numbers of *Lactobacillus* sp., *Bacteroides* sp., and

<table>
<thead>
<tr>
<th>Eimeria spp.</th>
<th>Most common</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>brunetti</em></td>
<td><em>acervulina</em></td>
</tr>
<tr>
<td><em>necatrix</em></td>
<td><em>maxima</em></td>
</tr>
<tr>
<td><em>praecox</em></td>
<td><em>mitis</em></td>
</tr>
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<td><em>tenella</em></td>
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Bifidobacterium sp. decrease (15, 26, 97). In germ-free chicks, C. perfringens adheres at a higher rate to cecal mucosa in Eimeria-infected birds than in noninfected birds (15). Infection with Eimeria spp produced mortality rates more than 25% higher than in Eimeria-free birds, and if Eimeria infection precedes C. perfringens infection, the mortality rate is even higher (53, 156). However, the precise role of coccidiosis in development of NE has not been completely established (196).

Coccidiostatic drugs have been the method of choice for prevention of coccidiosis in broiler chickens for the past fifty years (195). Many drugs are used to treat coccidiosis, and the most popular currently are ionophore antibiotics (63). Anticoccidial drugs, such as the polyether ionophores monensin, lasalocid, salinomycin, narasin, and maduramycin, alter ion transport and disrupt osmotic balance in coccidia, and serendipitously protect against NE (8, 40, 146, 154). However, surveillance has revealed an increase in resistance by Eimeria spp to various ionophores (18, 39, 114, 140, 146, 195), so alternatives are being sought. Anticoccidial vaccines have been available since 1952 (190), and breeders have protected valuable breeder stocks with anti-coccidial vaccines, and the focus of vaccine development has been on these long-lived birds (191). However, vaccines have now been developed for use in broiler chickens. These use unmodified, live, and attenuated strains of Eimeria spp, and four live, multivalent anti-coccidial vaccines are now registered around the world for use in poultry (195). Eimeria spp in these products are resistant to ionophore coccidiostats, thus allowing producers to use ionophores for the 3 - 4 weeks during which immunity develops (114). Immunity is boosted by re-infection
from the oocysts in litter, and this recycling of infection is critical for development of complete immunity (195).

However, the live, attenuated parasites in these vaccines cause intestinal damage, allowing development of NE (185, 195). Vaccination of chickens against coccidiosis protected chickens from challenge with *C. perfringens*, but only if they were challenged with both *Eimeria* and *C. perfringens* (196); birds challenged with *C. perfringens* alone developed NE lesions like nonvaccinated groups. Higher numbers of *C. perfringens* are found in the intestinal tracts of vaccinated birds than in nonvaccinated birds (196). McReynolds et al examined the role of several vaccines, including a commercial coccidial vaccine (CCV) and a commercial bursal disease vaccine (CBDV), on development of NE. CBDV alone and CCV + CBDV groups had higher mortality and more severe intestinal lesions than did nonvaccinated birds challenged with *C. perfringens*. However, the CCV alone group had lower mortality and less severe intestinal lesions than nonvaccinated groups challenged with *C. perfringens* (131). Thus, the evidence strongly suggests that anti-coccidial vaccines increase the risk of developing NE (127, 195).

1.8 Treatment and Prevention

In general, NE can be controlled or prevented by reducing exposure to risk factors such as coccidiosis and unsuitable diets (30). Alterations to feed composition, such as removing fishmeal from the diet, have been effective in reducing incidence of NE (68).
However, poultry producers have for decades prevented and treated NE by adding antimicrobials to feed (120). NE has been treated with lincomycin, bacitracin, oxytetracycline, penicillin, and tylosin in water or bacitracin, lincomycin, virginiamycin, penicillin avoparcin, and nitrovin in feed (49, 68).

Lincomycin, virginiamycin, and bacitracin have all been used in the US to prevent and treat NE (16, 41, 49, 80). Lincomycin at 20 g per ton of feed significantly reduces mortality due to NE (180). Hamdy et al found that lincomycin at 16.9 mg/L in water was effective in treatment of broilers (80, 81).

Virginiamycin at 5 - 40 g per ton of feed is also effective in treating NE (76). Bacitracin is used at 100 mg per gallon in water for prevention of NE and at 200 - 400 mg per gallon for treatment (144). Zinc bacitracin added to feed reduces NE mortality (193), and bacitracin methylene disalicylate in feed, alone or in combination with narasin, reduces mortality as well as severity in survivors (32, 185). Bacitracin alone was a more effective treatment than narasin alone, but the greatest effect was with the two drugs in combination (32).

The ionophore narasin, used primarily as a coccidiostat, at 70 ppm reduces mortality due to NE (30, 58, 185, 189), and also decreases numbers of *C. perfringens* in ceca (30, 58). Narasin also increases feed efficiency and terminal body weight (30). Salinomycin also reduces severity of NE (185).

Penicillin in water at 100,000 IU per L prevented mortality in experimentally-induced NE. Use of this antimicrobial at 110 mg per L in water delayed and reduced NE mortality, but did not completely prevent disease (117). Tylosin phosphate at 100 ppm in
feed was also an effective treatment, preventing mortality and increasing body weight (31, 185).

Heavy use of antimicrobials in feed to prevent NE has led to rising concern about resistance to these drugs. In 1980, *C. perfringens* from pigs, cattle, and poultry in the US were susceptible to avoparcin, furazolidone, monensin, nitrovin, penicillin G, ronidazole, and tiamulin, but were resistant to flavomycin. Poultry isolates were also susceptible to carbadox, chloramphenicol, erythromycin, and virginiamycin, but were resistant at low levels to lincomycin and bacitracin (56). In 1997, most chicken isolates were susceptible to avilamycin, avoparcin, monensin, narasin, and penicillin, but had low level resistance to tilmicosin, tylosin, and virginiamycin and high levels of resistance to bacitracin and lincomycin (30, 189). Turkey isolates had low level resistance to bacitracin, tilmicosin, tylosin, and virginiamycin and high level resistance to lincomycin. Thus, use of virginiamycin, lincomycin, and bacitracin to prevent NE might be leading to resistance (189).

Competitive exclusion (CE) has been used extensively to prevent colonization by pathogenic bacteria. Chicks are given cultures of normal chicken intestinal flora to exclude pathogens (58), and has been widely studied as a method for preventing salmonellosis (58, 185). Several researchers have attempted to use competitive exclusion to prevent NE. Fukata et al and others demonstrated the value of intestinal flora, finding that experimentally-inoculated conventional chickens developed mild disease with a case fatality rate, but the death rate in similarly-inoculated germ-free birds was one-third to one-half (71). A commercial CE product delayed proliferation of *C. perfringens* and
reduced occurrence of NE in 3-4 week-old chicks, but was ineffective after the age of 4 weeks (102). Hofacre et al found that a normal gut flora product reduced severity of NE lesions (compared to non-treated controls and those treated with virginiamycin and bacitracin) and increased feed efficiency (91). A Swedish CE product decreased cecal carriage of *C. perfringens*, decreased the incidence of NE, and reduced mortality due to experimental challenge (49, 58). These effects are apparently restricted to birds on diets containing animal protein (58). *Bacillus subtilis* spores have also been used for exclusion of *C. perfringens* from broiler chicks (111). A single dose of $10^9$ spores, administered 24 h prior to challenge with *C. perfringens* suppressed colonization in specific-pathogen free chicks (20, 111). *Clostridium perfringens* could not be recovered from colon or cecum for up to 36 days after dosing with spores (111). *Bacillus subtilis* colonization of chicks also increased body weight and feed efficiency (176).

Probiotics are live microbial cultures which, when added to feed, beneficially alter intestinal microflora (185). Use of probiotics such as *L. acidophilus* and *S. faecium* reduces severity of NE (68, 161, 185). Several *Lactobacillus* spp have antimicrobial activity against clostridia (176). A *Lactobacillus* probiotic increased resistance of chickens to coccidiosis and enhanced local immune responses against *Eimeria* sp (50).

Prebiotics are non-digestible feed components that affect the host by stimulating growth of beneficial intestinal flora (2, 185). Non-digestible oligosaccharides added to feed pass into large intestine, where they enhance growth of lactobacilli and bifidobacteria (2). Fructo-oligosaccharides and manno-oligosaccharides did not affect weight gain, feed conversion, or NE mortality in broiler chicks (185). $\beta$-mannanase in
feed reduces NE lesion scores and improves performance in broilers challenged with *C. perfringens*. β-mannanase hydrolyzes β-galactomannans (β-mannans), which are recognized as anti-nutritional components of poultry feed. However, inclusion of β-mannanase was not superior to bacitracin or salinomycin in limiting severity of NE lesions (95).

Dietary lactose (174) and blends of essential oils (133) may decrease numbers of *C. perfringens* in intestines. Addition of pectin and guar gum to diets eliminates incidence of NE, but also severely reduces growth rate (27, 148).

Organic acids added to feed or litter inhibit growth of *Salmonella, C. perfringens*, and *E. coli* (2). Spraying of wood-derived litter with organic acids (sodium lignosulfonate, formic acid, and propionic acid) reduced the number of *C. perfringens* present in ceca of birds raised on this litter. It also increased the number of *Lactobacillus* spp in ceca, but reduced the number of ileal *Lactobacillus* spp (74).

Vaccination against *C. perfringens* and its toxins (including CPA) has been studied in mice, lambs, piglets, goats, and calves (185, 188). Active and passive immunization against CPA protect against wild-type challenge (166). Vaccination of broiler breeder hens with type A and type C toxoids resulted in a strong IgY serum response to CPA (120). Those hens vaccinated with type A toxoid had higher serum anti-CPA IgY than hens vaccinated with type C toxoid. These anti-CPA antibodies were transferred to progeny, which developed titers similar to those in the hens. Upon challenge, vaccinated chicks were protected against subclinical NE and hepatitis. The
type C toxoid provided better protection than the type A toxoid, in spite of stimulation of higher anti-CPA titers by the latter (120).

Thompson et al vaccinated chickens via inoculation with virulent or avirulent C. *perfringens* daily for 5 days, followed by treatment with bacitracin for 9 days. Upon re-challenge, birds inoculated first with virulent isolates were protected, but those pre-challenged with avirulent isolates were not. Vaccination with a CPA-deficient mutant also protected birds, suggesting that factors other than CPA are involved in immunity (177).

1.9 Reproduction of NE

It has proven difficult to effectively and consistently reproduce NE under experimental conditions (6, 13, 45, 58, 101, 138, 196). Methods which have been successful to one degree or another include oral challenge with *Eimeria* spp and *C. perfringens* (4, 13, 45, 68, 80, 81, 144, 156, 174), intraduodenal challenge with *C. perfringens* liquid cultures (6, 13, 14, 45, 68, 75, 76, 80, 92, 101, 161, 174) or cell-free supernatant fluids (5, 7, 12, 13, 66, 68, 71, 75, 101, 161), offering feed or water mixed with broth cultures of *C. perfringens* (4, 5, 14, 27, 30-32, 45, 68, 71, 75, 76, 80, 81, 92, 117, 143, 148, 156, 161, 174, 180, 196), exposure to litter from infected flocks (13, 14, 80, 81, 128, 161, 196) or rearing in facilities where outbreaks have occurred (14, 45, 68, 76, 80, 81, 193), oral challenge with liquid cultures (24, 46, 49, 68, 76, 128, 131, 138), and intravenous inoculation with liquid cultures (23, 24, 68, 196). Some workers have
used sodium bicarbonate to neutralize acidity in the gut and opium to decrease peristalsis (24). Disease has also been reproduced in turkeys and Japanese quails (68).

Key factors in experimental reproduction of NE are inoculation with a virulent strain of *C. perfringens* and creation of an intestinal environment that encourages anarchic growth of *C. perfringens* (5, 13). Strains vary in virulence, and NE has been reproduced with isolates from field cases of NE and cholangiohepatitis (5, 117). Preparation of the intestinal environment is typically accomplished by creating minor damage to enterocytes (e.g., prechallenge with *Eimeria* spp or feeding a diet high in fishmeal) and employing a diet which results in entry of large amounts of rapidly-fermentable carbohydrate and/or protein into the small intestine (143). Type and percentage of fishmeal are important; in some cases, a diet with as little as 13% fishmeal has been part of a successful scheme to produce lesions, but rations with 50% fishmeal are required for consistent disease production (180).

Coinfection with *Eimeria* is a predisposing factor for field cases of NE. Mortality rates and numbers of *C. perfringens* in the intestinal tract are both higher when birds are coinfected; mortality can be twice as high in coinfected birds (4, 14). Supplemental dietary zinc increases incidence of NE in birds challenged with *Eimeria* and *C. perfringens* (13).

Large amounts of CPA produced in the intestine may facilitate development of lesions, but are apparently not necessary (104). Intestinal damage is caused by soluble products of *C. perfringens* cultivated *in vitro* (6, 7), so these may participate *in vivo*. 
Most challenges are *per os*, but NE has been reproduced by oral *Eimeria* challenge, followed in 6 days by intracloacal inoculation with *C. perfringens* (196). Intracloacal challenge with *C. perfringens* alone did not produce lesions, but 50% of coinfected birds developed NE (196). It is unknown if the infection actually progresses upstream from the cloaca or is the result of environmental fecal contamination.

Several lesion scoring systems have been used (Table 4), but System 1 remains the standard.

Table 4: Lesion scoring systems for necrotic enteritis

<table>
<thead>
<tr>
<th>Score</th>
<th>System 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>System 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>System 3&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No gross lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+</td>
<td>Thin-walled, friable intestine</td>
<td>Small lesions totaling ≤ 2.5 cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>&lt; 10 lesions</td>
</tr>
<tr>
<td>2+</td>
<td>Focal necrosis, ulceration</td>
<td>Lesions covering &gt; 2.5 cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>≥ 10 focal lesions</td>
</tr>
<tr>
<td>3+</td>
<td>Large patches of necrosis</td>
<td>Severe lesions throughout intestine</td>
<td>≥ 1 lesion extending &gt; mucosal circumference</td>
</tr>
<tr>
<td>4+</td>
<td>Severe/extensive necrosis ~ field cases</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>5+</td>
<td>Birds dying with 4+ lesions</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> (30-32, 131, 143, 144, 177, 180, 196)

<sup>b</sup> (76)

<sup>c</sup> (120)

<sup>d</sup> Not applicable
1.10 Research contributions

Effectively and consistently modeling NE in poultry is unquestionably important for future studies on pathogenesis, therapy, and prophylaxis. After development of such a model (Appendix A), we were able to examine strain-to-strain variations in virulence (Appendix B), effect on virulence of in vivo passage (Appendix A), and efficacy of immunization with potential vaccine candidates (Appendix C). In addition, this work includes preliminary investigations into other attributes which may facilitate colonization and lesion production by C. perfringens.
2.0 PRESENT STUDY

2.1 Methods

*Clostridium perfringens* strains used in these studies were listed in Table 5.

Table 5: *Clostridium perfringens* strains used in bacteriocin and *pilA* studies

<table>
<thead>
<tr>
<th>Strain JGS</th>
<th>Source</th>
<th>Bacteriocin</th>
<th><em>pilA</em> PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>4143</td>
<td>type A, field case NE</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>1714</td>
<td>type A, enterotoxigenic, human food poisoning</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>1882</td>
<td>type A, porcine neonatal enteritis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>1936</td>
<td>type A, bovine neonatal enteritis</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>4024</td>
<td>type A, porcine neonatal enteritis, passed <em>in vivo</em> through the porcine intestinal tract</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>4151</td>
<td>type A, sequenced strain, Strain 13</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>4142</td>
<td>type A, bovine jejunal hemorrhage syndrome</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>1473</td>
<td>type A, avian normal flora</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>no numbers (n = 10)</td>
<td>Avian Normal Flora</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>5252</td>
<td>type A, field case of NE</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>1070</td>
<td>type C, porcine hemorrhagic enteritis</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>1120</td>
<td>type A, sequenced strain, ATCC 13124</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>4303</td>
<td>type A, enterotoxigenic, TIGR sequenced strain SM101</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Other strains (n = 71)</td>
<td>See results</td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

Sequencing data (not shown) revealed that JGS4143 has *pilA1* and *pilA2* in its chromosome, and others demonstrated these two chromosomal genes in JGS4151, JGS1120, and SM101.
**Bacteriocin preparation**

To test for bacteriocin production, colonies on brain heart infusion (BHI; Difco) agar with 5% citrated bovine blood were emulsified in BHI broth and the suspension mixed by vortexing. Density was adjusted to that of a MacFarland No. 3 standard by dilution with BHI broth. Aliquots (100 µl) were spread on BHI agar plates to form confluent lawns.

Colonies of strains to be tested for bacteriocin production were then picked with sterile toothpicks and stabbed through the lawn prepared above, through ~ 3/4 of the thickness of the agar. Plates were incubated in an anaerobic environment (5% H₂: 5% CO₂:90% N₂) at 37°C for 24 h and were then examined for zones of inhibition around the stabs.

**PilA PCR**

*Clostridium perfringens* DNA was prepared by suspending 1 - 2 colonies (cultivated on BHI agar) in 200 µl sterile HPLC-grade water and boiling for 20 min. Tubes were then centrifuged (14,000 x g, 5 min) and 9.2 µl aliquots used as template.
Each 20 µl reaction mix contained 2 µl of each primer (50mM, Sigma) (Table 6), 8.8 µl Redmix (Gene Choice) and 9.2 µl DNA template. Cycling was in a Mastercycler (Eppendorf), according to parameters shown in Table 6.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pilA1</td>
<td>TAAAAGGAGGACCATAAATGTTATTA</td>
<td>TATTTATACTATAGACACCGTAACTT</td>
<td>94°C</td>
<td>53°C</td>
<td>72°C</td>
</tr>
<tr>
<td>pilA2</td>
<td>TATTAGCAGCCGTAGCTATACCAAAC</td>
<td>CATTTGTTTTCTCTTTAATAATA</td>
<td>94°C</td>
<td>53°C</td>
<td>72°C</td>
</tr>
</tbody>
</table>
2.2 Results

Bacteriocin studies.

JGS 4143 produces a bacteriocin that inhibits growth of all 10 avian normal flora strains and all pathogenic non-avian strains except JGS 4024 (Table 7). Isolate JGS 4024 inhibited growth of all of pathogenic non-avian strains, as well as all of the avian normal flora strains. Strains JGS 1714 and JGS 4151 did not inhibit growth of any pathogenic non-avian or avian normal flora strains. However, JGS 1882 and JGS 1936 inhibited most of the avian normal flora and other virulent strains, and strains JGS 4142 and JGS 1473 inhibited growth of only a few normal flora strains and two non-avian strains (Table 7).
Table 7. Zone of inhibition results of potential bacteriocin production

<table>
<thead>
<tr>
<th>Lawn Strains</th>
<th>Source of strain</th>
<th>JGS 4143</th>
<th>JGS 1714</th>
<th>JGS 1882</th>
<th>JGS 1936</th>
<th>JGS 4024</th>
<th>JGS 4151</th>
<th>JGS 4142</th>
<th>JGS 1473</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS 4143</td>
<td>Field case of necrotic enteritis</td>
<td>N/A</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JGS 1714</td>
<td>Human food poisoning</td>
<td>+++</td>
<td>N/A</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JGS 1882</td>
<td>Porcine neonatal enteritis</td>
<td>+++</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JGS 1936</td>
<td>Bovine neonatal enteritis</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>N/A</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>JGS 4024</td>
<td>Porcine enteritis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JGS 4151</td>
<td>Strain 13</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>N/A</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>JGS 4142</td>
<td>Bovine jejunal hemorrhage syndrome</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>JGS 1473</td>
<td>Avian normal flora</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>P1 N23</td>
<td>Avian normal flora</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P2 N23</td>
<td>Avian normal flora</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P2 N20</td>
<td>Avian normal flora</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P2 N30</td>
<td>Avian normal flora</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P1 N8</td>
<td>Avian normal flora</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P2 N13</td>
<td>Avian normal flora</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P1 N18</td>
<td>Avian normal flora</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P1 N15</td>
<td>Avian normal flora</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P2 N25</td>
<td>Avian normal flora</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P1 N20</td>
<td>Avian normal flora</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*Note: The avian normal flora strains are labeled for the pen number (P) the birds were placed into for the trial, and the number of the individual birds (N)

*a Each + is equal to 3 mm of a zone of inhibition
**PilA PCR studies.**

Primers for amplification of the two pilA genes were based upon sequence data from JGS 4143, so this isolate was used as the positive control. JGS 5252, a highly virulent NE isolate, was only positive for pilA1. JGS 4120 was PCR positive for both genes, whereas the other five NE strains had only pilA1, pilA2 gene, or neither (Table 8).

Non-avian strains JGS 4024, JGS 1936, and JGS 4142 were positive for pilA1 only. Avian normal flora strain JGS 1473 and type C strain JGS 1070 were positive for both genes and JGS 1882 was positive for pilA2 alone. In addition, enterotoxigenic strain JGS 1714 was PCR negative for both genes (Table 8).

Sequenced strain JGS 1120 (ATCC 13124) had both pilA genes, while JGS 4151 (Strain 13) had only pilA1. JGS 4303 (sequenced enterotoxigenic strain SM 101) contained neither gene, similar to JGS 1714 (another enterotoxigenic strain) (Table 8).

Sixty-two of 71 (87.3%) NE strains in our collection contained pilA1, while only 26 (36.6%) contained pilA2. Only 24/71 (33.8%) contained both pilA genes (Table 8).
Table 8. Presence of two \textit{pilA} genes in non-avian strains and necrotic enteritis strains

<table>
<thead>
<tr>
<th>C. \textit{perfringens} isolate</th>
<th>\textit{pilA1}</th>
<th>\textit{pilA2}</th>
<th>Both genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS 4143</td>
<td>Pos.</td>
<td>Pos.</td>
<td>Both</td>
</tr>
<tr>
<td>JGS 5252</td>
<td>Pos.</td>
<td>Neg.</td>
<td>\textit{pilA1} only</td>
</tr>
<tr>
<td>JGS 4024</td>
<td>Pos.</td>
<td>Neg.</td>
<td>\textit{pilA1} only</td>
</tr>
<tr>
<td>JGS 1936</td>
<td>Pos.</td>
<td>Neg.</td>
<td>\textit{pilA1} only</td>
</tr>
<tr>
<td>JGS 1714</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neither</td>
</tr>
<tr>
<td>JGS 4142</td>
<td>Pos.</td>
<td>Neg.</td>
<td>\textit{pilA1} only</td>
</tr>
<tr>
<td>JGS 1070</td>
<td>Pos.</td>
<td>Pos.</td>
<td>Both</td>
</tr>
<tr>
<td>JGS 1473</td>
<td>Pos.</td>
<td>Pos.</td>
<td>Both</td>
</tr>
<tr>
<td>JGS 1882</td>
<td>Neg.</td>
<td>Pos.</td>
<td>\textit{pilA2} only</td>
</tr>
<tr>
<td>JGS 4151</td>
<td>Pos.</td>
<td>Neg.</td>
<td>\textit{pilA1} only</td>
</tr>
<tr>
<td>SM101</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neither</td>
</tr>
<tr>
<td>JGS 1120</td>
<td>Pos.</td>
<td>Pos.</td>
<td>Both</td>
</tr>
<tr>
<td>JGS 4104</td>
<td>Pos.</td>
<td>Neg.</td>
<td>\textit{pilA1} only</td>
</tr>
<tr>
<td>JGS 1521</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neither</td>
</tr>
<tr>
<td>JGS 1235</td>
<td>Neg.</td>
<td>Pos.</td>
<td>\textit{pilA2} only</td>
</tr>
<tr>
<td>JGS 1501</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neither</td>
</tr>
<tr>
<td>JGS 4064</td>
<td>Pos.</td>
<td>Neg.</td>
<td>\textit{pilA1} only</td>
</tr>
<tr>
<td>JGS 4120</td>
<td>Pos.</td>
<td>Pos.</td>
<td>Both</td>
</tr>
<tr>
<td>Necrotic enteritis strains</td>
<td>62/71 (87.3%)</td>
<td>26/71 (36.6%)</td>
<td>24/71 (33.8%)</td>
</tr>
</tbody>
</table>
2.3 Conclusions

The mechanism by which NE strains out-compete avian normal flora has been the subject of speculation, and production of a bacteriocin may be such a method. JGS 4143, an NE strain, produces a substance which suppresses growth of avian normal flora strains, but also inhibits growth strains virulent for other species. It is clear that this phenomenon is not restricted to NE strains, since strains virulent for pigs and calves also produce a bacteriocin; this may be involved in pathogenesis of those infections. These preliminary results suggest that bacteriocins may be virulence factors in NE, but additional work will be needed to confirm a specific role.

Recently a type IV pilus was identified in *C. perfringens* (SM 101, strain 13, and ATCC 13124), and examination of sequence data on NE strain JGS 4143 revealed the same genes. PCR assays for the two *pilA* genes revealed that JGS 1473 has both genes, as did type C strain JGS 1070. However, JGS 1473, which is avirulent, colonized birds, but JGS 1070, which is a pig pathogen, was avirulent for chicks and failed to colonize. Other non-avian strains were either negative for both genes (JGS 1714) or had *pilA1* (JGS 4024, JGS 1936, and JGS 4142) or *pilA2* (JGS 1882) alone.
Examination of NE strains revealed that 87.3% have \( pilA1 \). Interestingly, only about one-third of NE strains have \( pilA2 \), and another one-third have both genes. In addition, JGS 5252, a highly virulent NE strain, has only \( pilA1 \). Among the six virulent NE strains, only JGS 4120 had both genes, while JGS 4104 and JGS 4064 had \( pilA1 \). JGS 1235 had only \( pilA2 \), and JGS 1521 and JGS 1501 had neither.

These results may be affected by sequence variations in \( pilA \) genes that would be expected to cause falsely-negative PCR results. As previously stated, all three sequenced \( C. perfringens \) strains had both \( pilA1 \) and \( pilA2 \). However, \( pilA \) from JGS 4151 (strain 13) and JGS 1120 (ATCC 13124) were \(~98\%\) identical, while the gene in JGS 4303 was only \(~85\%\) identical to the other two; JGS 4151 and JGS 1120 were PCR positive and JGS 4303 was not. \( pilA2 \) in JGS 4151 and JGS 4303 were \(~96\%\) identical, but in JGS 1120 was only \(~76\%\) identical to the others; PCR assays were positive for JGS 1120 only. JGS 1120 also carries \( pilA3 \) and \( pilA4 \), and our sequence data show that JGS 4143 also has these genes.

These results are very preliminary, and PCR negatives must be confirmed by application of other methods of gene detection (e.g., probes). Furthermore, the occurrence of type IV pilus genes in strains which are virulent (or putatively virulent) and in avirulent strains suggests that their role in the biology of \( C. perfringens \) will not be
solely related to virulence. Production and virulence testing of mutants will perhaps
provide a means by which to determine the role of the pil gene family in pathogenesis of
NE and other enteritides.
3.0 REFERENCES


140. **Peeters, J. E., J. Derijcke, M. Verlinden, and R. Wyffels.** 1994. Sensitivity of avian Eimeria spp. to seven chemical and five ionophore anticoccidials in five Belgian integrated broiler operations. Avian Dis **38:**483-93.


APPENDIX A:

NECROTIC ENTERITIS IN BROILER CHICKENS: REPRODUCTION OF DISEASE AND ROLE OF IN VIVO PASSAGE ON VIRULENCE OF CLOSTRIDIUM PERFRINGENS ISOLATES

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________________________________________

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Abstract.

Over the last few decades, necrotic enteritis in poultry has been prevented and treated with the addition of antimicrobials to the poultry’s feed. Yet, due to rising concerns by consumers about the overall negative effect of adding antimicrobials to animal feed, many countries are beginning to ban the use of antimicrobials in livestock feed. These recent bans and other factors have lead to a slow re-emergence of necrotic enteritis in poultry around the world, which is leading researchers to look for alternative prevention and treatment methods for the disease. However, very little is known about the pathogenesis of necrotic enteritis in poultry, due to the fact that there is no established experimental model for the disease which can effectively and consistently produce the disease. We developed an experimental model for necrotic enteritis in broiler chickens that can effectively produce the disease, with an average of greater than 75% of challenged birds developing gross lesions typical of necrotic enteritis. We were also able to show that the model can produce the disease in birds through numerous studies, and consistently produce disease every time birds are challenged. In addition, we were able to show that in vivo passage of a necrotic enteritis strain through the chicken’s intestinal tract can increase the ability of the strain to produce disease in the experimental model. We were also able to produce the disease using several different strains of Clostridium perfringens isolated from field cases of necrotic enteritis
Introduction.

Poultry necrotic enteritis (NE), described by Parish in 1961 in a flock of 6-7 week-old cockerels (3, 15, 23, 41, 48, 59-61), has since been reported worldwide (16, 18, 26, 33, 35, 46, 51, 52, 57, 60, 67, 87). Disease is most common in broiler chickens 2 to 6 weeks of age (11, 14, 33, 35, 40, 46, 48, 59, 63, 69, 72), but has also been reported in 3 to 6 month-old commercial layers (35, 72), 12 to 16 week-old replacement pullets (19, 35, 37, 69, 72), 7 to 12 week-old turkeys (27-29, 35, 40, 78), and other avian species (6, 10, 57, 78). NE symptoms and effects range from subclinical to severe, resulting in reduced weight gain, poor feed conversion, and abnormally-high mortality rates (53, 55, 66, 78, 81, 83, 87). Many in the industry consider NE the most economically-significant poultry disease (25), with annual costs estimated at $2 billion worldwide (49, 67).

The primary etiologic agent of NE is Clostridium perfringens type A (Table 1; (2, 5, 6, 9, 27, 28, 30, 35, 40, 46, 47, 50, 54, 55, 62, 63, 66, 72, 77, 78, 81, 87)), an anaerobic, spore-forming, non-motile, large Gram-positive rod (20, 36, 45, 70, 72, 76, 78, 79). It is commonly a commensal in the intestinal tract of humans and other animals (36, 50, 72), but it produces up to 17 different exotoxins (20, 70, 78) and can be involved in many intestinal and extraintestinal disease processes.
Poultry producers have traditionally treated and prevented necrotic enteritis by use of antimicrobial growth promoters (AGPs) in feed (63), but rising consumer concerns regarding possible human health effects has led to bans on AGP use (39, 63). The European Union has banned use of avoparcin, spiramycin, tylosin, bacitracin, and virginiamycin (21, 31, 32, 83), and this has resulted in increased incidence of NE, particularly in Western Europe (22, 88). Use of ionophore coccidiostats, which are serendipitously anti-clostridial, is also decreasing as more producers implement vaccination with live, attenuated coccidial vaccines (64, 84, 86). This is apparently making further contributions to re-emergence of NE.

Parish reproduced the disease by oral inoculation of chickens with *C. perfringens*, calcium bicarbonate, sodium bicarbonate, and opium (13, 35, 59, 69). However, consistent, effective experimental reproduction of NE has proven difficult (3, 8, 23, 30, 54, 71, 87). Approaches have included co-inoculation with *Eimeria* spp and *C. perfringens* (1, 8, 23, 35, 43, 44, 74, 77, 80), intraduodenal inoculation with *C. perfringens* broth cultures (3, 8, 9, 23, 35, 40, 41, 43, 50, 54, 78, 80), intraduodenal inoculation with toxin-containing culture supernatant fluids (2, 4, 7, 8, 34, 35, 38, 40, 54, 78), mixing *C. perfringens* cultures with feed or water (1, 2, 9, 15-18, 23, 35, 38, 40, 41, 43, 44, 50, 61, 73, 75, 77, 78, 80, 82, 87), rearing birds on litter obtained from
infected flocks (8, 9, 43, 44, 65, 78, 87), rearing broilers in facilities where outbreaks 
previously occurred (9, 23, 35, 41, 43, 44, 85), and oral (13, 24, 25, 35, 41, 65, 67, 
71) or intravenous challenge with *C. perfringens* cultures (12, 13, 35, 87).

We report here the development of an effective experimental model of poultry NE 
and demonstration of a range of virulence among isolates of *C. perfringens* type A from 
diseased birds.
**Methods and Materials.**

**Birds and care**

Commercial jumbo Cornish x Rock broiler chicks were obtained as one-day old hatchlings from Murray McMurray Hatchery (Webster City, IA). Birds were housed in 5 ft diameter brooders constructed of 1/4 inch thick pegboard and divided into three equal parts. Commercial wood shavings provided as bedding were changed at two week intervals.

Birds were fed a commercial chick starter ration (20% protein, Eagle Milling, Casa Grande, AZ) through experimental day 7. Thereafter, birds were fed a high protein feed (28% protein) mixed 50:50 with menhaden fishmeal (SeaLac). On day 14, feed was withheld for 20 h, followed by challenge with *C. perfringens*. Water was available *ab lib* in galvanized steel automatic waterers.

**Challenge inoculum and protocol**

Strains used in these studies are listed in Table 2, with host and disease process (if any) of origin. Strains were stored at -80°C in 50% glycerol. For inoculum preparation, a small amount was transferred to a plate of brain heart
infusion (BHI; Difco, Detroit, MI) agar with 5% citrated bovine blood and streaked for isolation. After incubation under anaerobic conditions (5% H₂: 5% CO₂: 90% N₂ at 37°C for 24 h, 1-2 colonies were transferred into 10 ml of cooked meat medium (CMM) (Difco) in Hungate tubes and incubated in the same atmosphere at 37°C for 18 h. The resulting culture was serially passed, into fluid thioglycollate broth (FTG) medium, then into CMM, and finally into FTG medium, increasing the volume at each step. The final culture, in 1 L FTG medium, was mixed with feed for challenge. A separate serially-passed culture was prepared for each challenge feeding. Numbers of colony-forming units (cfu) were determined by preparing and plating serial 10-fold dilutions on BHI agar.

On days 15 - 18, the birds were offered inoculated feed twice per day. Feed and FTG medium culture were mixed in a ratio of 3 parts culture to 4 parts high protein feed with fishmeal (v/v). The mixture, which had a paste-like consistency, was then placed in galvanized steel feed trays and offered to birds. Trays were cleaned and remaining feed disposed of prior to each feeding. Water was available ad lib throughout the challenge period. Negative control birds were challenged with uninoculated FTG mixed with high protein feed.
On day 19, birds were euthanized by CO₂ asphyxiation. Necrotic intestinal lesions were scored (0: no gross lesions; 1+: thin-walled or friable small intestine; 2+: focal necrosis or ulceration; 3+: large patches of necrosis; 4+: severe or extensive necrosis typical of field cases) and segments fixed in 10% buffered formalin or retained fresh for bacteriological culture. Intestinal contents (1 g) were mixed with 9 ml phosphate buffered saline [PBS; pH 7.2, 0.1369 M NaCl (Sigma), 0.00695 M K₂HPO₄ (Sigma), 0.0025 M KH₂PO₄ (Sigma)] and mixed by use of a vortex mixer. Serial dilutions were plated on \textit{Clostridium perfringens} selective agar (CPSA) [brain-heart infusion (BHI) agar supplemented with 0.5% yeast extract (Becton Dickinson), 0.1% sodium metabisulfite, 5% bovine blood, and D-cycloserine (400 µg/ml)] and incubated under anaerobic conditions (as above) for 24 h. Colonies with double zones of hemolysis were counted and calculations made to determine numbers of colony-forming units (cfu) in sections of intestinal tract.

**PCR-\textit{Clostridium perfringens} genotyping**

One or two colonies from each intestinal segment were streaked for isolation on BHI agar with 5% bovine blood, plates were incubated under anaerobic conditions for 18-24 h, and isolates genotyped by a multiplex PCR assay (Meer and
Songer (68)). Isolated colonies were added to 200 µl of HPLC-grade water (Mallinckrodt) and boiled for 20 min to prepare DNA template. Five µl of template were added to 20 µl of a master mix solution containing primers, Taq polymerase (Promega), MgCl₂ (25mM, Sigma), dNTPs (5mM, Promega), and 10x Assay Buffer (Promega) to give a total reaction volume of 25µl.

**Pulsed-field gel electrophoresis**

Genotyped strains were embedded in 2% CleanCut (Bio-Rad) agarose plugs according to the method of Lin and Labbe (58). After cells lysis and DNA digestion with SmaI (New England Biolabs), electrophoresis was performed on a Chef Mapper (Bio-Rad) at 6.0 V/cm for 20 h with an angle of 120°, an initial pulse time of 0.50 sec, and a final pulse time of 40 sec. After staining with ethidium bromide, gels were examined and photographed via UV transillumination.
**Results.**

Birds were inoculated with NE strain JGS 4143, and genotype- and PFGE-matched isolates (Figure 1) obtained from those developing intestinal lesions were used to inoculate further groups of birds. In this manner, we were able to assess the impact of up to three *in vivo* passages on virulence of JGS 4143 for chicks (Table 3). Virulence, as measured by proportion of inoculated birds which developed gross lesions (Figures 2 & 3) and average lesion score, increased with each passage. Overall, percent of birds developing gross lesions increased by nearly 50% and average lesion score by one-third. In all cases, lesions were most common in jejunum (data not shown); although a few birds challenged with JGS 4143 after 3 *in vivo* passages developed gross lesions in duodenum. Negative controls did not develop gross lesions in any case (Table 3). We have continued to use JGS 4143 for experimental inoculation of birds, without further increases in virulence (data not shown).

Other NE strains were of variable virulence for birds, with most producing gross lesions in inoculated birds. However, the proportion of inoculated birds developing lesions was quite low for most, averaging 13.9%, as compared to 79%
(across several trials) for JGS 4143 (Table 4). Strains JGS 1066 and JGS 1498 failed to produce gross lesions in any challenged birds. There was no statistically significant difference among strains, measured either as percent of birds developing lesions or average lesion scores ($p = 0.5929$). Percentages of birds with lesions were similar to those seen with JGS 4143 prior to *in vivo* passage. It may be that virulence of these strains would increase in the same manner as in JGS 4143.

Recently-isolated NE strain JGS 5252, on the other hand, produced gross lesions in nearly 90% of challenged birds (Table 4). This isolate produced average lesion scores of 2.82, which was not significantly different from the average lesion score produced by inoculation with JGS 4143 ($p = 0.3388$).

Across all strains, gross lesions were most common in jejunum, although JGS 5252 produced some lesions in duodenum (data not shown). No gross lesions were observed in any negative controls (Table 4).
Discussion.

Effective and consistent experimental reproduction of NE has proven complicated at best, but use of the model described here results in dependable lesion rates across multiple trials. Various strains have different degrees of virulence (from avirulent to highly virulent), perhaps due (at least in part) to an unknown and almost certainly variable number of *in vitro* passages. Based upon experience with strain JGS 4143, it seems likely that *in vivo* passage enhances the ability of strains to produce disease. This suggests upregulation of important virulence attributes *in vivo*, a phenomenon which should be investigated in other strains and also as to mechanism.

Beta2 toxin (CPB2) may have a role in the pathogenesis of NE, but surveillance of healthy chickens and chickens with NE has not revealed a direct correlation between occurrence of disease and presence of *cph2* in isolates (42). Our data neither support nor refute this assumption; we examined too few CPB2-negative strains, and no apparent pattern of virulence was associated with presence or absence of *cph2*. It may be possible in future to test a hypothesis regarding the role of CPB2 by examining virulence of specific *cph2* mutants, or by vaccination with CPB2
toxoids and subsequent challenge. It is also important to note that CPB2 is not produced by all strains which are PCR positive for \textit{cpb2}; JGS 4143 is PCR positive, but a frameshift mutation eliminates CPB2 production.

Overall, we showed that our conditions allow consistent and reliable experimental reproduction of NE. In field cases, lesions are most common in jejunum, followed by ileum, duodenum, and ceca (1, 56, 57, 60, 69, 71, 72, 83). The same occurred in experimentally-infected birds, although we never observed gross lesions in ileum or cecum.
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new method for the experimental production of necrotic enteritis and its use for
studies on the relationships between necrotic enteritis, coccidiosis and
perfringens in the broiler fowl gastrointestinal tract by real-time PCR. Appl
Table 1. Major toxin production by *Clostridium perfringens*

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Table 2. Strains used in these studies

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<tr>
<td>JGS 5252</td>
<td>A</td>
<td>Avian necrotic enteritis</td>
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Table 3. Effect of *in vivo* passage on virulence of *Clostridium perfringens* strain JGS 4143

<table>
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<tr>
<th>Treatment</th>
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<td>20/29 (69.0%)</td>
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<td>3</td>
<td>22/27 (81.4%)</td>
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<td>Negative control</td>
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Table 4. Response of birds to challenge with various necrotic enteritis strains

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<th>NE Strain</th>
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<th>Average lesion score</th>
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<tr>
<td>JGS 4104</td>
<td>7/16 (43.8%)</td>
<td>2.14</td>
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<td>JGS 1235</td>
<td>1/15 (6.7%)</td>
<td>2.00</td>
</tr>
<tr>
<td>JGS 1521</td>
<td>5/16 (31.3%)</td>
<td>2.00</td>
</tr>
<tr>
<td>JGS 1066</td>
<td>0/16 (0.0%)</td>
<td>0.00</td>
</tr>
<tr>
<td>JGS 1498</td>
<td>0/15 (0.0%)</td>
<td>0.00</td>
</tr>
<tr>
<td>JGS 1501</td>
<td>1/15 (6.7%)</td>
<td>2.00</td>
</tr>
<tr>
<td>JGS 4064</td>
<td>2/12 (16.7%)</td>
<td>2.00</td>
</tr>
<tr>
<td>JGS 4120</td>
<td>1/17 (5.9%)</td>
<td>2.00</td>
</tr>
<tr>
<td>JGS 5252</td>
<td>64/72 (88.9%)*</td>
<td>2.50</td>
</tr>
<tr>
<td>JGS 4143</td>
<td>79/100 (79.0%)*</td>
<td>2.70</td>
</tr>
<tr>
<td>Negative controls</td>
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*Multiple studies
Figure 1. Pulsed field gel electrophoresis of recovered JGS 4143 isolates from chicken intestinal samples

Lane 1: JGS 4151 (Strain 13), Lane 2: JGS 4143, Lane 3: Trial 1 isolate #1, Lane 4: Trial 1 isolate #2, Lane 5: Trial 1 isolate #3, Lane 6: Trial 1 isolate #4, Lane 7: Trial 1 isolate #5, Lane 8: Trial 1 isolate #6*, Lane 9: Trial 1 isolate #7, Lane 10: Trial 1 isolate #8, Lane 11: JGS 4143, Lane 12: JGS 4151

*Denotes the isolate that was selected for the challenge inoculums in the next trial
Figure 2. Gross lesions typical of necrotic enteritis from reproduction studies

A. (Mucosa) Typical +2 lesion seen during reproduction studies; B. (Mucosa) Typical +3 lesion seen during reproduction studies, beginning formation of pseudomembrane; C. (Mucosa) Typical +4 lesion seen during reproduction studies, pseudomembrane throughout upper 10 inches of jejunum; D. (Serosa) Typical +3 lesions visible from the serosal surface of the intestinal tract.
Figure 3. Histopathology of the jejunum of challenged and unchallenged chickens

A. Histology from typical +2 lesion, extensive superficial fibrinonecrotic exudates including numerous bacteria; B. Histology from typical +3 lesion, rather extensive area of erosion with superficial necrosis with heavy population of large, rod-shaped bacteria, lesion extends into submucosa; C. Histology from typical +4 lesion, diffuse fibrinonecrotic enteritis, containing numerous bacteria, with extensive erosions; D. Histology of normal intestinal tract from negative control bird. (Pictures 40x magnification)
Acknowledgements.

The authors thank Dr. Kevin Keel (current address: Southeast Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia, Athens, GA) for assistance in model development. Hien Trinh provided technical assistance and guidance in the laboratory, and Drs. Lynn Joens and Michael Riggs contributed helpful commentary.
APPENDIX B:

VIRULENCE FOR CHICKENS OF CLOSTRIDIUM PERFRINGENS ISOLATED FROM POULTRY AND OTHER SOURCES

Cooper, K.K., Songer, J.G.*
Abstract.

*Clostridium perfringens* type A is a common cause of poultry necrotic enteritis (NE). We investigated the virulence for poultry of isolates from a variety of enteric sources. Newly-hatched Cornish x Rock chicks were fed a low protein diet for one week, a high protein diet for a second week, and then challenged with log-phase cultures mixed 3:4 (v/v) with high protein feed. Strain JGS 4143 (genotype A, beta2 (CPB2) positive, from a field case of NE) produced gross lesions compatible with NE in > 80% of challenged birds, but strains JGS 1714 (enterotoxigenic genotype A, CPB2+, human food poisoning), JGS 1936 (genotype A, bovine neonatal enteritis), JGS 4142 (genotype A, CPB2+, bovine jejunal hemorrhage syndrome), JGS 1473 (genotype A, CPB2+, chicken normal flora), JGS 1070 (genotype C, CPB2+, porcine hemorrhagic enteritis), JGS 1882 (genotype A, CPB2+, porcine neonatal enteritis), JGS 1120 (ATCC 13124), JGS 4151 (strain 13), and JGS 4303 (SM101) failed to produce disease. *In vivo* passage failed to increase virulence of the non-NE strains. NE strains apparently have specific poultry-associated virulence attributes, and comparison of sequences of NE isolates to those of JGS 1120, JGS 4151, and JGS 4303 (sequenced and published) may yield information about NE-specific virulence attributes.
**Introduction.**

Poultry necrotic enteritis (NE) is most commonly caused by *Clostridium perfringens* type A (1-4, 9, 10, 12, 17, 20, 29, 30, 37, 40, 43, 47, 53), although occasional cases are due to type C infection (17, 19, 24, 25, 27, 33, 38). This species is anaerobic, spore-forming, Gram-positive, and rod-shaped, and is divided into five toxinogenic types based on the pattern of production of four major toxins (Table 1) (6, 18, 22, 36, 37, 39, 43, 44, 55).

Control of NE has been most commonly achieved by use of antimicrobials for treatment or growth promotion (30). However, concerns over downstream human health effects, such as those arising from resistance transfer from animal pathogens to human pathogens, have led to European Union bans on use of antimicrobial growth promoters (7, 13-15, 48). In consequence, NE has re-emerged as a major problem for the poultry industry (7, 8, 11, 21, 31, 53, 54).

A further increase in incidence of NE has resulted from introduction of products for immunoprophylaxis of coccidiosis (50, 51). Attenuated coccidia in vaccines may produce transient damage to intestinal epithelia, facilitating establishment of *C. perfringens* and production of NE (32, 48, 52, 53). A more important factor may be cessation of use of ionophore coccidiostats. These compounds have been extraordinarily effective in preventing losses due to infection by *Eimeria* spp, but have also provided serendipitous anti-clostridial effects (5, 12, 16, 21, 23, 26, 48, 49). Withdrawal of coccidiostats has fueled re-emergence of NE.
A vital element in development of prevention and control measures for NE is better understanding of pathogenesis. We developed an experimental model of *C. perfringens* type A infection; strains isolated from field cases of NE are virulent, although variably so. We report here the results of studies in which other type A strains, from normal chickens or from enteric disease processes in other species, were examined for virulence in our experimental model. These results reveal that normal flora strains do not produce NE, nor do those with apparent virulence for other species.
Methods and Materials.

Birds and care

Commercial jumbo Cornish x Rock broiler chicks were obtained as one-day old hatchlings from Murray McMurray Hatchery (Webster City, IA). Birds were housed in 5 ft diameter brooders constructed of 1/4 inch thick pegboard and divided into three equal parts. Commercial wood shavings provided as bedding were changed at two week intervals.

Birds were fed a commercial chick starter ration (20% protein, Eagle Milling, Casa Grande, AZ) through experimental day 7. Thereafter, birds were fed a high protein feed (28% protein) mixed 50:50 with menhaden fishmeal (SeaLac). On day 14, feed was withheld for 20 h, followed by challenge with *C. perfringens*. Water was available *ab lib* in galvanized steel automatic waterers.

Challenge inoculum and protocol

Strains used in these studies are listed in Table 2, with host and disease process (if any) of origin. Strains were stored at -80°C in 50% glycerol. For inoculum preparation, a small amount was transferred to a plate of brain heart infusion (BHI; Difco, Detroit, MI) agar with 5% citrated bovine blood and streaked for isolation. After incubation under anaerobic conditions (5% H₂: 5% CO₂: 90% N₂) at 37°C for 24 h, 1-2 colonies were transferred into 10 ml of cooked meat medium (CMM) (Difco) in Hungate tubes and incubated in the same atmosphere at 37°C for 18 h. The resulting culture was serially
passed, into fluid thioglycollate broth (FTG) medium, then into CMM, and finally into FTG medium, increasing the volume at each step. The final culture, in 1 L FTG medium, was mixed with feed for challenge. A separate serially-passed culture was prepared for each challenge feeding. Numbers of colony-forming units (cfu) were determined by preparing and plating serial 10-fold dilutions on BHI agar.

On days 15 - 18, the birds were offered inoculated feed twice per day. Feed and FTG medium culture were mixed in a ratio of 3 parts culture to 4 parts high protein feed with fishmeal (v/v). The mixture, which had a paste-like consistency, was then placed in galvanized steel feed trays and offered to birds. Trays were cleaned and remaining feed disposed of prior to each feeding. Water was available ab lib throughout the challenge period. Negative control birds were challenged with un-inoculated FTG mixed with high protein feed.

On day 19, birds were euthanized by CO₂ asphyxiation. Necrotic intestinal lesions were scored (0: no gross lesions; 1+: thin-walled or friable small intestine; 2+: focal necrosis or ulceration; 3+: large patches of necrosis; 4+: severe or extensive necrosis typical of field cases) and segments fixed in 10% buffered formalin or retained fresh for bacteriological culture. Intestinal contents (1 g) were mixed with 9 ml phosphate buffered saline [PBS; pH 7.2, 0.1369 M NaCl (Sigma), 0.00695 M K₂HPO₄ (Sigma), 0.0025 M KH₂PO₄ (Sigma)] and mixed by use of a vortex mixer. Serial dilutions were plated on Clostridium perfringens selective agar (CPSA) [brain-heart infusion (BHI) agar supplemented with 0.5% yeast extract (Becton Dickinson), 0.1% sodium metabisulfite, 5% bovine blood, and D-cycloserine (400 µg/ml)] and incubated under anaerobic
conditions (as above) for 24 h. Colonies with double zones of hemolysis were counted and calculations made to determine numbers of colony-forming units (cfu) in sections of intestinal tract.

**PCR-Clostridium perfringens genotyping**

One or two colonies from each intestinal segment were streaked for isolation on BHI agar with 5% bovine blood, plates were incubated under anaerobic conditions for 18-24 h, and isolates genotyped by a multiplex PCR assay (Meer and Songer (34)). Isolated colonies were added to 200 µl of HPLC-grade water (Mallinckrodt) and boiled for 20 min to prepare DNA template. Five µl of template were added to 20 µl of a master mix solution containing primers, Taq polymerase (Promega), MgCl₂ (25mM, Sigma), dNTPs (5mM, Promega), and 10x Assay Buffer (Promega) to give a total reaction volume of 25µl.

**Pulsed-field gel electrophoresis**

Genotyped strains were embedded in 2% CleanCut (Bio-Rad) agarose plugs according to the method of Lin and Labbe (28). After cells lysis and DNA digestion with Smal (New England Biolabs), electrophoresis was performed on a Chef Mapper (Bio-Rad) at 6.0 V/cm for 20 h with an angle of 120°, an initial pulse time of 0.50 sec, and a final pulse time of 40 sec. After staining with ethidium bromide, gels were examined and photographed via UV transillumination.
Assays of CPA (Lethicinase) Activity

Egg yolk emulsion was prepared by adding one aseptically-removed egg yolk to 20 ml DMG buffer [0.04 M 3,3 dimethylglutaric acid (Sigma)], in 50 ml HPLC-grade water [pH 7.0] with 0.008 M CaCl₂ (Sigma), 0.0002 M ZnSO₄ (Sigma), and 0.1% bovine serum albumin (Sigma). Serial 2-fold dilutions (final volume 100μl) of purified recombinant CPA or test samples were made in a 96-well plate. An equal volume of egg yolk emulsion was added to each well and the plate sealed to prevent evaporation. After incubation at 37°C for 1 h, results were read spectrophotometrically at 540 nm. Amount of CPA in test samples was derived by comparison of results to a plot generated from results of testing of standards.
Results and Discussion.

We examined the ability of various isolates of *C. perfringens* (Table 2) to produce NE in our model. Disease did not result from challenge with these isolates, although 54/66 (81.8%) positive controls (inoculated with NE isolate JGS 4143) developed gross lesions (Figures 1 and 2) with average lesion scores of 2.46 (Table 3). Negative controls did not develop lesions. There were no statistically-significant differences in CPA production across strains and, in fact, positive control strain JGS 4143 produced the least CPA *in vitro* (Figure 3). These results, combined with the finding that inoculation of birds with CPA-containing culture supernatant fluid did not produce gross lesions (data not shown), lead us to conclude that the quantity of CPA in inocula does not significantly affect the ability of an isolate to produce disease. There were no statistically significant differences among inoculum titers (Table 3).

In initial trials, attempts to recover challenge strains were successful only from birds inoculated with strains JGS 4143 and JGS 1473 (avian normal flora) (Figure 4). Further groups of birds were challenged with JGS 1070, JGS 1714, JGS 1882, JGS 1936, and JGS 4142, as well as strain JGS 4151 [strain 13 (42)]. No challenged birds developed lesions, but genotyping of at least half the colonies (up to 100 per plate), with subsequent PFGE analysis, resulted in recovery of all challenge isolates except JGS 1070 (a type C porcine isolate).

Birds were then inoculated with these isolates (JGS 1882, JGS 1714, JGS 1936, JGS 4142, and JGS 4151) and JGS 1473, all after passage through chicks, to determine if
*in vivo* passage led to increased virulence. However, no challenged birds developed lesions (Table 4). Positive control birds developed gross lesions [15/16 (93.7%), average lesion score 3.07, when challenged with JGS 4143; 13/15 (86.6%), average lesion score 2.69, when challenged with JGS 5252] and negative controls did not. Inoculum titers for the various isolates were very similar (Table 4). This should be studied further, perhaps by inoculating birds with various numbers of cfus of virulent strains to determine the minimum infectious dose.

Only JGS 4143 and JGS 1473 were recovered from jejunum, the most common site of lesions development. JGS 1882, JGS 1936, JGS 4142, and JGS 4151 were isolated from ileum post-challenge, which is in keeping with findings of others that *C. perfringens* most commonly colonizes ileum and cecum of chickens as part of the normal flora (26, 41, 45, 46). JGS 1714 was isolated from duodenum, but not jejunum or ileum, suggesting possible lack of colonization factors that might allow avian strains to colonize jejunum.

Failure of sequenced strains {JGS 4151 [Strain 13 (42)], JGS 1120 [ATCC 13124 (35)], and JGS 4303 [SM101 (35)]} to produce disease (Table 3) suggests that these strains will provide an excellent background for comparisons with genome sequences of NE strains, possibly allowing identification of specific virulence factors.
References.


genome sequence of Clostridium perfringens, an anaerobic flesh-eater. Proc Natl Acad Sci U S A 99:996-1001.
Table 1. Major toxin production by types of *Clostridium perfringens*

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Table 2. *Clostridium perfringens* strains used in studies

<table>
<thead>
<tr>
<th>JGS #</th>
<th>Genotype</th>
<th>Source of Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS 1070</td>
<td>C, cpb2+</td>
<td>Porcine hemorrhagic enteritis</td>
</tr>
<tr>
<td>JGS 1120</td>
<td>A</td>
<td>ATCC 13124&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>JGS 1473</td>
<td>A, cpb2+</td>
<td>Avian normal flora</td>
</tr>
<tr>
<td>JGS 1714</td>
<td>A, cpe+</td>
<td>Human foodborne disease</td>
</tr>
<tr>
<td>JGS 1882</td>
<td>A, cpb2+</td>
<td>Porcine neonatal enteritis</td>
</tr>
<tr>
<td>JGS 1936</td>
<td>A</td>
<td>Bovine neonatal enteritis</td>
</tr>
<tr>
<td>JGS 4303</td>
<td>A, cpe+</td>
<td>SM101&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>JGS 4142</td>
<td>A, cpb2+</td>
<td>Bovine jejunal hemorrhagic syndrome</td>
</tr>
<tr>
<td>JGS 4143</td>
<td>A, cpb2+</td>
<td>Field case NE</td>
</tr>
<tr>
<td>JGS 4151</td>
<td>A, cpb2+</td>
<td>Strain 13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>JGS 5252</td>
<td>A, cpb2+</td>
<td>Field case NE</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sequenced (ref. 35)
<sup>b</sup>Sequenced (ref. 41)
Table 3. Response of birds to challenge with non-avian strains of *Clostridium perfringens*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Birds with gross lesion/Total (%)</th>
<th>Average lesion score</th>
<th>Average challenge inoculum titer (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS 1473</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>6.98 + 1.6</td>
</tr>
<tr>
<td>JGS 1070</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>5.70 + 0.41</td>
</tr>
<tr>
<td>JGS 1882</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>5.86 + 0.99</td>
</tr>
<tr>
<td>JGS 4142</td>
<td>0/15 (0.0%)</td>
<td>0.0</td>
<td>5.88 + 0.88</td>
</tr>
<tr>
<td>JGS 1714</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>5.88 + 0.87</td>
</tr>
<tr>
<td>JGS 1936</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>8.60 + 0.47</td>
</tr>
<tr>
<td>JGS 4303 (SM101)</td>
<td>0/18 (0.0%)</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>JGS 4151 (Strain 13)</td>
<td>0/20 (0.0%)</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>JGS 4120 (ATCC 13124)</td>
<td>0/18 (0.0%)</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>JGS 4143</td>
<td>54/66 (81.8%)</td>
<td>2.46 + 0.58</td>
<td>8.48 + 0.65</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0/69 (0.0%)</td>
<td>0.0</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 4. Response of birds to challenge with re-isolated non-avian strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Birds with gross lesions/Total (%)</th>
<th>Average lesion score</th>
<th>Average challenge inoculums titer (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS 1473</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>9.86 ±0.66</td>
</tr>
<tr>
<td>JGS 1714</td>
<td>0/15 (0.0%)</td>
<td>0.0</td>
<td>11.01 ±0.66</td>
</tr>
<tr>
<td>JGS 1882</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>11.00 ±0.33</td>
</tr>
<tr>
<td>JGS 1936</td>
<td>0/15 (0.0%)</td>
<td>0.0</td>
<td>11.16 ±0.59</td>
</tr>
<tr>
<td>JGS 4142</td>
<td>0/14 (0.0%)</td>
<td>0.0</td>
<td>10.60 ±0.36</td>
</tr>
<tr>
<td>JGS 4151</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>10.69 ±0.35</td>
</tr>
<tr>
<td>JGS 4143</td>
<td>15/16 (93.7%)</td>
<td>3.07±0.59</td>
<td>10.92 ±0.39</td>
</tr>
<tr>
<td>JGS 5252</td>
<td>13/15 (86.6%)</td>
<td>2.69±0.63</td>
<td>10.37 ±0.84</td>
</tr>
<tr>
<td>Negative control</td>
<td>0/16 (0.0%)</td>
<td>0.0</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 1. Gross examination of intestinal tract of birds challenged with various *C. perfringens* strains

A. Gross lesions typical of necrotic enteritis from bird challenged with JGS 4143; B. Gross picture of intestinal tract of bird challenged with JGS 4142, no gross lesions (Picture of JGS 4142 is typical of lack of gross lesions observed from other non-necrotic enteritis strains used to challenge birds in these studies).
Figure 2. Histopathology of intestinal tract of birds challenged with various *C. perfringens* strains

A. Histology from intestinal tract of bird challenged with JGS 4143, extensive fibrinonecrotic inflammation with bacteria and distended crypts and inflammatory infiltrate in the lamina propria;

B. Histology from intestinal tract of bird challenged with JGS 1473, no significant lesions (Picture of JGS 1473 is typical of lack of microscopic lesions observed from other non-necrotic enteritis strains used to challenge birds in these studies).
Figure 3. Alpha toxin assays of challenge inocula
Figure 4. Pulsed field gel electrophoresis of recovered JGS 1473 isolates from chicken intestinal samples

Lane: JGS 4151 (Strain 13), Lane 2: JGS 1473, Lane 3: Sample isolate #1, Lane 4: Sample isolate #2, Lane 5: Sample isolate #3, Lane 6: Sample isolate #4, Lane 7: Sample isolate #5, Lane 8: Sample isolate #6, Lane 9: Sample isolate #7*, Lane 10: Sample isolate #8

*Denotes the sample isolate that was selected to challenge birds in the second study with the non-avian isolates.
Acknowledgements.

The author thanks Bernie Stewart and James Theoret for technical assistance with the animal model, Drs. Robert Glock and Sharon Dial for assistance with the histology from the studies, Hien Trinh for technical assistance and guidance in the laboratory, and Drs. Lynn Joens and Michael Riggs for helpful commentary.
APPENDIX C:

IMMUNIZATION WITH RECOMBINANT ALPHA TOXIN PROTECTS BROILER CHICKS AGAINST EXPERIMENTAL CHALLENGE WITH CLOSTRIDIUM PERFRINGENS

Cooper, K.K., Trinh, H., Songer, J.G.*
**Abstract.**

Alpha toxin (CPA) is considered a critical virulence factor for the pathogenesis of necrotic enteritis in poultry. Challenging germ-free birds with *Clostridium perfringens* type A culture supernatant produced lesions typical of necrotic enteritis, but not if CPA in the supernatant was neutralized with anti-CPA antibodies. In this paper, we show that vaccinating birds with HIS-tagged CPA can provide birds with partial protection against necrotic enteritis. Non-vaccinated birds challenged with *C. perfringens* developed necrotic enteritis at the rate of 72.0%, while only 30.0% of vaccinated birds developed lesions. In addition, non-vaccinated birds had average lesion scores of 1.61, while scores in vaccinated birds averaged 0.74. Vaccination produced a strong immune response, with IgG titers in vaccinated birds more than 2-fold greater than in non-vaccinated birds. After challenge, vaccinated birds had average IgG titers >20-fold higher than those in non-vaccinated birds. These results suggest that CPA has a role in pathogenesis of necrotic enteritis in poultry.
**Introduction.**

Prevention of many diseases of food animals has commonly been accomplished by administration of antimicrobials in feed or water. However, there are rising concerns about effects on human health of such routine use of antimicrobials, particularly as this pertains to transfer by animal pathogens of antimicrobial resistance to human pathogens (43). The European Union has banned many antimicrobial growth promoters (AGPs), and the bans have been accompanied by increased incidence of poultry necrotic enteritis (6, 7, 10, 12-14, 22, 35, 45-47).

Necrotic enteritis is most commonly caused by *Clostridium perfringens* type A (1-3, 9, 11, 18, 24, 25, 30, 34, 39, 41, 46), an anaerobic, spore-forming, large Gram-positive rod (5, 19, 23, 38-42, 48). Two forms of necrotic enteritis are observed in the field; subclinical disease results in decreased feed conversion ratios and accompanying retarded growth rates (15, 29, 32, 33, 35, 36, 45), while acute disease is marked by clinical signs including diarrhea, huddling, depression, and death (4, 17, 26, 36, 37, 45). The annual cost of necrotic enteritis to the poultry industry worldwide is estimated at $2 billion (27, 36).

The only so-called major toxin produced by toxinotype A is alpha toxin (CPA) (25, 28, 38, 41, 42, 48). There has been considerable sentiment, although relatively little evidence, that CPA plays an important role in pathogenesis of necrotic enteritis. CPA induces mucosal damage in chicken intestinal loops (16); crude toxin preparations reportedly produce lesions compatible with necrotic enteritis in germ-free chickens, an effect which is neutralized with antiCPA serum (8, 18, 20, 21, 34, 41). A few
investigators have examined the value of antiCPA immunity in protection against necrotic enteritis. Vaccination of broiler breeder hens with type A toxoids resulted in passage of antiCPA antibodies to progeny and partial protection against subclinical necrotic enteritis (34).

The goal of the work described here was to investigate the value of recombinant CPA as a parenteral immunogen against necrotic enteritis. We present results which suggest that a significant degree of protection can be achieved, but that this varies substantially with the adjuvant.
Methods and Materials.

Birds and care

Commercial jumbo Cornish x Rock broiler chicks were obtained as one-day old hatchlings from Murray McMurray Hatchery (Webster City, IA). Birds were housed in 5 ft diameter brooders constructed of 1/4 inch thick pegboard and divided into three equal parts. Commercial wood shavings provided as bedding were changed at two week intervals.

Birds were fed a commercial chick starter ration (20% protein, Eagle Milling, Casa Grande, AZ) through experimental day 7. Thereafter, birds were fed a high protein feed (28% protein) mixed 50:50 with menhaden fishmeal (SeaLac). On day 24, feed was withheld for 20 h, followed by challenge with *C. perfringens*. Water was available *ab lib* in galvanized steel automatic waterers.

Production of histidine-tagged CPA and vaccination

The *cpa* gene from *C. perfringens* had previously been cloned into pTrcHis (creating plasmid pJGS 211) and this construct used to transform *E. coli* DH5α (creating strain JGS 2445). This recombinant expresses *cpa*, producing CPA with a hexahistidine N-terminal tag (HIS-CPA). JGS 2445 was cultivated in LB broth with 100 µg ampicillin per ml, incubated with shaking at 37°C to OD₆₀₀ 0.6. It was then induced with isopropyl-beta-D-thiogalactopyranoside (IPTG, Gold Biotechnology) and incubated for a further 3
h, after which cells were harvested by centrifugation (5000 rpm, 10 min) and resuspended in binding buffer [20 mM Tris, 100 mM NaCl (pH 8.0)]. Cells were then disrupted in a French pressure cell and the resulting solution centrifuged (15,000 x g, 15 min). DNase (160 U, Promega) was added to the supernatant fluid (~ 40 ml) and incubated at 37°C for 15 min. Recombinant CPA was then purified on TALON resin (Clontech), according to the manufacturer’s instructions. Bound proteins were eluted with imidazole (50 mM, pH 8.0) and 1.5 ml fractions were collected. An aliquot of each fraction was electrophoresed in a 10% SDS-PAGE gel to assess purity of the HIS-CPA, which appears as a band of ~48 kDa. Protein concentrations were determined by Bradford assay (Pierce, Rockford, IL).

Birds were vaccinated subcutaneously (SQ) with 20 µg of HIS-CPA, emulsified 1:1 (w/v) with a proprietary adjuvant with a Quil A base (Benchmark BioLabs, Lincoln, NE). Vaccinations were administered at 5 and 15 days of age. Serum was collected from birds on arrival, prior to challenge (at 25 days of age), and at necropsy (on day 29).

**Challenge inoculum and protocol**

*Clostridium perfringens* type A challenge strain JGS 4143, obtained from a chicken with necrotic enteritis, was stored at - 80°C in 50% glycerol. For inoculum preparation, a small amount was transferred to a plate of brain heart infusion (BHI; Difco, Detroit, MI) agar with 5% citrated bovine blood and streaked for isolation. After incubation under anaerobic conditions (5% H₂: 5% CO₂: 90% N₂) at 37°C for 24 h, 1-2 colonies were transferred into 10 ml of cooked meat medium (CMM) (Difco) in Hungate
tubes and incubated in the same atmosphere at 37°C for 18 h. The resulting culture was serially passed, into fluid thioglycollate broth (FTG) medium, then into CMM, and finally into FTG medium, increasing the volume at each step. The final culture, in 1 L FTG medium, was mixed with feed for challenge. A separate serially-passed culture was prepared for each challenge feeding. Numbers of colony-forming units (cfu) were determined by preparing and plating serial 10-fold dilutions on BHI agar.

On days 25 - 28, the birds were offered inoculated feed twice per day. Feed and FTG medium culture were mixed in a ratio of 3 parts culture to 4 parts high protein feed with fishmeal (v/v). The mixture, which had a paste-like consistency, was then placed in galvanized steel feed trays and offered to birds. Trays were cleaned and remaining feed disposed of prior to each feeding. Water was available *ab lib* throughout the challenge period. Negative control birds were challenged with uninoculated FTG mixed with high protein feed.

On day 29, birds were euthanized by CO₂ asphyxiation. Necrotic intestinal lesions were scored (0: no gross lesions; 1+: thin-walled or friable small intestine; 2+: focal necrosis or ulceration; 3+: large patches of necrosis; 4+: severe or extensive necrosis typical of field cases) and segments fixed in 10% buffered formalin or retained fresh for bacteriological culture.

**Detection of antiCPA antibodies**

Wells of microtitration plates were coated with HIS-CPA by addition of 100 µl of 0.06 M carbonate buffer (0.018 M Na₂CO₃; 0.045 M NaHCO₃, pH 9.6) containing 1 ng
antigen. Plates were incubated overnight at 4°C and washed twice with phosphate buffered saline (pH 7.2, 0.1369 M NaCl (Sigma), 0.00695 M K₂HPO₄ (Sigma), 0.0025 M KH₂PO₄ (Sigma)) with 0.3% Tween 20 (ICN Biomedicals)(PBS-T). One percent bovine serum albumin (BSA) in PBS-T (200 µl) was then added to each well and the plate incubated for 1 h at 4°C. Plates were again washed with PBS-T, and then 100 µl of 10-fold serial dilutions (1:10 to 1:10,240) of test sera added. After incubation for 1 h at 37°C, plates were washed nine times with PBS-T, 100 µl of goat anti-chicken IgG peroxidase conjugate (KPL; diluted 1:8000) were added, and plates were incubated 1 h at 37°C. After washing six times with PBS-T, 100 µl of o-phenylenediamine (OPD) substrate (200 µg/ml) (Sigma) was added and the plate incubated for 30 min at room temperature in the dark. The reaction was stopped with 50 µl of 3M HCl and color development allowed to continue for an additional 15 min. Results were read spectrophotometrically at 490 nm. End-point ELISA titers were reported as the reciprocal of the last dilution which had an OD₄₉₀ ≥ 0.4.

Statistical analysis

A two-tailed Fisher exact test was used for statistical comparison of rates of lesion development and mortality across treatment groups. A paired t test was used to examine differences in average lesion scores across groups.
Results.

Parenteral immunization with HIS-CPA protected birds against challenge (Table 1). Differences in percent of birds with gross lesions were statistically significant across treatment groups (p < 0.0001), and uninoculated controls were significantly different from the remaining two groups in average lesion score (p < 0.0001). The difference between average lesion scores for vaccinated and nonvaccinated/challenged groups nearly achieved statistical significance. The mortality rate in nonvaccinates was higher than in vaccinates, but the difference was not statistically significant (data not shown).

Birds had high average titers of antiCPA antibodies at hatching (average titer: 150.2 + 3.6) (Table 2), but these hen-derived antibodies decreased in titer during the studies, as demonstrated by titers in negative (nonvaccinated, nonchallenged: 18.1 + 2.1) and positive controls (nonvaccinated, challenged: 13.5 + 1.9) at necropsy at 29 days of age. In contrast, prechallenge titers in vaccinated birds were 2-fold higher (69.6 + 1.8).
Discussion.

Protection against poultry NE by vaccination with CPA toxoids has been a controversial topic. On the one hand, Lovland and coworkers (34) vaccinated hens with crude “type A” or “type C” toxoids (containing CPA) and progeny were protected against subclinical NE. However, this provides little information about the role of an antiCPA response in protection; these toxoids contained many other antigens against which the birds may have produced a protective immune response. On the other hand, birds inoculated with a CPA mutant were protected against subsequent challenge with a virulent isolate (Thompson et al (44)). Furthermore, birds challenged with a cpa-deleted NE strain developed disease at a rate and severity comparable to that in birds inoculated with the parent strain (Keyburn et al (31)). Thus, the role of CPA in pathogenesis of and immunity to NE has been called into question.

Results reported here are not conclusive, but suggest that antiCPA immunity provides at least partial protection against challenge with C. perfringens. These findings should be confirmed by more extensive trials. The relatively low degree of protection in these experimental trials may translate into better protection against natural challenge in the field, but it seems unlikely to be sufficient to deal effectively with the problem. It may be that better responses would result from use of improved adjuvants, higher doses of immunogen, or alternate routes of delivery. Yolk antibodies may interfere with immune responses to the vaccine. However, it is likely that immunization against effects of other, yet-undiscovered, virulence attributes will be required to provide complete protection.
It remains to rationalize the results of this work with those of Keyburn and coworkers (31), in which CPA was shown to be an unnecessary attribute in pathogenesis of NE. It may be that other attributes are required for establishment of infection and initiation of lesion development, and that CPA adds to the severity of disease.
References.


Table 1. Response of vaccinated birds and controls to challenge with *Clostridium perfringens*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of birds with gross lesions(^a) (%)</th>
<th>Average lesion score(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCPA vaccination</td>
<td>15/50 (30.0%)(^b)</td>
<td>0.74(^e)</td>
</tr>
<tr>
<td>Nonvaccinated, challenged</td>
<td>36/50 (72.0%)(^c)</td>
<td>1.61(^e)</td>
</tr>
<tr>
<td>Nonvaccinated, nonchallenged</td>
<td>0/48 (0.0%)(^d)</td>
<td>0.00(^f)</td>
</tr>
</tbody>
</table>

\(^a\) Composite of three trials; values with different superscripts are statistically different (\(p > 0.0001\))
Table 2. AntiCPA antibody response of birds following vaccination with recombinant CPA and challenge with *C. perfringens*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Titors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hatching</td>
</tr>
<tr>
<td><strong>rCPA vaccination</strong></td>
<td>150.2 ± 3.6</td>
</tr>
<tr>
<td><strong>Nonvaccinated, challenged</strong></td>
<td>11.5 ± 1.4</td>
</tr>
<tr>
<td><strong>Nonvaccinated, nonchallenged</strong></td>
<td>34.8 ± 3.8</td>
</tr>
</tbody>
</table>

End-point ELISA titers are reported as the reciprocal of the last dilution which had an $OD_{490} \geq .400$. 
Acknowledgements.

The author would like to thank James Theoret for technical assistance with the experimental model, and Dr. Robert Glock, Dr. Sharon Dial, Dr. Lynn Joens and Dr. Michael Riggs for helpful commentary.
APPENDIX D:

SUPPLEMENTAL MATERIAL: THE ABILITY OF CRUDE TOXINS FROM CLOSTRIDIUM PERFRINGENS TYPE A TO PRODUCE NECROTIC ENTERITIS IN BROILER CHICKENS
**Methods & Materials.**

**Birds and care**

Commercial jumbo Cornish x Rock broiler chicks were obtained as one-day old hatchlings from Murray McMurray Hatchery (Webster City, IA). Birds were housed in 5 ft diameter brooders constructed of 1/4 inch thick pegboard and divided into three equal parts. Commercial wood shavings provided as bedding were changed at two week intervals.

Birds were fed a commercial chick starter ration (20% protein, Eagle Milling, Casa Grande, AZ) through experimental day 7. Thereafter, birds were fed a high protein feed (28% protein) mixed 50:50 with menhaden fishmeal (SeaLac). On day 14, feed was withheld for 20 h, followed by challenge with *C. perfringens* or culture supernatant fluids. Water was available *ab lib* in galvanized steel automatic waterers.

**Challenge inoculum and protocol**

Strains were stored at - 80°C in 50% glycerol. For inoculum preparation, a small amount was transferred to a plate of brain heart infusion (BHI; Difco, Detroit, MI) agar with 5% citrated bovine blood and streaked for isolation. After incubation under anaerobic conditions (5% H₂: 5% CO₂: 90% N₂ at 37°C for 24 h, 1-2 colonies were transferred into 10 ml of cooked meat medium (CMM) (Difco) in Hungate tubes and incubated in the same atmosphere at 37°C for 18 h. The resulting culture was serially passed, into fluid thioglycollate broth (FTG) medium, then into CMM, and finally into
FTG medium, increasing the volume at each step. The final culture, in 1 L FTG medium, was mixed with feed for challenge. A separate serially-passed culture was prepared for each challenge feeding. Numbers of colony-forming units (cfu) were determined by preparing and plating serial 10-fold dilutions on BHI agar.

Culture in FTG medium was centrifuged (5000 x g, 10 mins), and the supernatant was decanted. The pellet was washed twice with PBS and used as washed cells. Culture supernatant fluid was filtered [220 nm pore diameter (Millpore)] and used to challenge birds. These preparations were mixed to produce washed cells + filtered culture supernatant fluid.

On days 15 - 18, the birds were offered inoculated feed twice per day. Feed and FTG medium culture were mixed in a ratio of 3 parts culture to 4 parts high protein feed with fishmeal (v/v). The mixture, which had a paste-like consistency, was then placed in galvanized steel feed trays and offered to birds. Trays were cleaned and remaining feed disposed of prior to each feeding. Water was available ab lib throughout the challenge period. Negative control birds were challenged with uninoculated FTG mixed with high protein feed. Washed cells were administered by mixing with feed at a 3:4 ratio (v/v). Filtered culture supernatant fluids were administered in feed by mixing at same ratio as previous treatment groups.

On day 19, birds were euthanized by CO₂ asphyxiation. Necrotic intestinal lesions were scored (0: no gross lesions; 1+: thin-walled or friable small intestine; 2+: focal necrosis or ulceration; 3+: large patches of necrosis; 4+: severe or extensive necrosis typical of field cases) and segments fixed in 10% buffered formalin or retained fresh for
bacteriological culture. Intestinal contents (1 g) were mixed with 9 ml phosphate buffered saline [PBS; pH 7.2, 0.1369 M NaCl (Sigma), 0.00695 M K₂HPO₄ (Sigma), 0.0025 M KH₂PO₄ (Sigma)] and mixed by use of a vortex mixer. Serial dilutions were plated on *Clostridium perfringens* selective agar (CPSA) [brain-heart infusion (BHI) agar supplemented with 0.5% yeast extract (Becton Dickinson), 0.1% sodium metabisulfite, 5% bovine blood, and D-cycloserine (400 µg/ml)] and incubated under anaerobic conditions (as above) for 24 h. Colonies with double zones of hemolysis were counted and calculations made to determine numbers of colony-forming units (cfu) in sections of intestinal tract.

**Assays of CPA (Leticinase) Activity**

Egg yolk emulsion was prepared by adding one aseptically-removed egg yolk to 20 ml DMG buffer [0.04 M 3,3 dimethylglutaric acid (Sigma), in 50 ml HPLC-grade water [pH 7.0] with 0.008 M CaCl₂ (Sigma), 0.0002 M ZnSO₄ (Sigma), and 0.1% bovine serum albumin (Sigma)]. Serial 2-fold dilutions (final volume 100µl) of purified recombinant CPA or test samples were made in a 96-well plate. An equal volume of egg yolk emulsion was added to each well and the plate sealed to prevent evaporation. After incubation at 37°C for 1 h, results were read spectrophotometrically at 540 nm. Amount of CPA in test samples was derived by comparison of results to a plot generated from results of testing of standards.
Gavage Challenge

Birds were gavaged with either 5 ml of 1 M NaHCO₃ (Sigma) only or 5 ml of 1 M NaHCO₃ + 10 ml of filtered culture supernatant. Birds were gavaged by placing a 3mm-cuffed endotracheal tube (Hudson RCI) into the crop of the birds, and the crop was filled with the selected solution.

Statistical analysis

A two-tailed Fisher exact test was used for statistical comparison of rates of lesion development and mortality across treatment groups. A paired t test was used to examine differences in CPA titers between whole cell culture and supernatant inocula.
Results and Discussion.

Negative controls (including controls gavaged with bicarbonate alone) did not develop signs or lesions. However, inoculation with JGS 4143 produced lesions in 75% of birds (Table 1). Mortality and average lesion scores were in keeping with those found in other trials. Results were similar in birds inoculated with washed cells or washed cells reconstituted with filtered culture supernatant were much the same. Rate of development of gross lesions was ~ 20% lower in groups challenged with only washed cells, but results of a two-tailed Fisher exact test revealed that the difference was not statistically relevant (p=0.1262). Mortality was somewhat higher in the washed cells group. There was no significant difference among lesion scores or inoculum titers for these three groups. Small amounts of CPA were found in washed cell preparations, but at concentrations ~ 40 times lower than in any of the other challenge inocula. In fact, it seems justified to conclude that presence of CPA in challenge inocula has little or no effect on incidence or severity of disease. This is not surprising, even if CPA plays an important role in pathogenesis of NE. Conditions in the upper gastrointestinal tract of chicks are such that CPA introduced by gavage might have little chance of remaining intact to enter duodenum and jejunum. This would be in keeping with the presumed mode of natural infection in which birds would be expected to ingest *C. perfringens* but not preformed toxin.

Administration of filtered supernatant fluid of *C. perfringens* cultures by mixing it with feed failed to produce gross lesions. Thus, we gavaged birds with filtered culture supernatant fluid buffered with bicarbonate, but this also failed to produce lesions (Table
1). There was no mortality in these groups. We compared CPA titers in filtered culture supernatant fluid to those in whole culture and found no statistically-significant differences \((p = 0.4736)\).

These findings provide further information about the experimental model of NE. Of particular importance is the fact that preformed CPA in inocula has little or no effect on establishment or progression of the infection. However, accurate evaluation of the roles of toxins (alpha and others) will require other approaches.
Table 1: Response of birds to challenge with filtered culture supernatant fluid from *Clostridium perfringens*<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Birds with gross lesions/total birds (%)</th>
<th>Mortality (%)</th>
<th>Average lesion score</th>
<th>Inoculum titer (log&lt;sub&gt;10&lt;/sub&gt;)</th>
<th>Average CPA titer (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS 4143</td>
<td>24/32 (75.0%)</td>
<td>4/32 (12.5%)</td>
<td>2.58 ± .72</td>
<td>8.85 ± .93</td>
<td>1300.60 ± 247.76</td>
</tr>
<tr>
<td>Washed cells</td>
<td>19/34 (55.9%)</td>
<td>12/34 (35.3%)</td>
<td>2.63 ± .50</td>
<td>10.52 ± .82</td>
<td>29.17 ± 38.44</td>
</tr>
<tr>
<td>Supernatant + washed cells</td>
<td>21/32 (65.6%)</td>
<td>4/32 (12.5%)</td>
<td>2.43 ± .60</td>
<td>10.85 ± 1.51</td>
<td>1288.51 ± 239.59</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0/33 (0.0%)</td>
<td>0/33 (0.0%)</td>
<td>0.00</td>
<td>0.00</td>
<td>1296.47 ± 217.60</td>
</tr>
<tr>
<td>Bicarbonate + supernatant gavage</td>
<td>0/16 (0.0%)</td>
<td>0/16 (0.0%)</td>
<td>0.00</td>
<td>0.00</td>
<td>1189.73 ± 191.56</td>
</tr>
<tr>
<td>Bicarbonate gavage</td>
<td>0/15 (0.0%)</td>
<td>0/15 (0.0%)</td>
<td>0.00</td>
<td>0.00</td>
<td>N/A</td>
</tr>
<tr>
<td>Negative control</td>
<td>0/33 (0.0%)</td>
<td>0/33 (0.0%)</td>
<td>0.00</td>
<td>0.00</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<sup>a</sup> Composite of two trials