The development and use of multiplex PCR protocols for the detection of *Clostridium perfringens* toxin encoding genes *cpa*, *cpb*, *etx*, *ia*, *cpe*, *netB*, and *tpeL*

by

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Abstract

Clostridium perfringens (CP) is the etiological agent of necrotic enteritis (NE) and gangrenous dermatitis (GD) in poultry as well as CP enterotoxin (CPE)-mediated food poisoning in humans. All these diseases can cause significant problems for the poultry industry. Both NE and GD cause extensive losses due to mortality, reduced performance, and carcass condemnation at processing and CPE food poisoning is one of the top foodborne illnesses in the US.

Major virulence factors of CP include the production of numerous toxins and isolates are typed A-E depending upon the varied production of four major mouse-lethal toxins, α, β, ε, and ι. All three diseases are associated with type A CP, although poultry isolates are not known to produce CPE. In the past, α-toxin produced prolifically by type A organisms was thought to be the main virulence factor of NE; however, recent studies have shown that is not the case with all strains. A novel toxin, NetB, was shown to be an important virulence factor for some isolates. Evidence also suggests that isolates with both a *netB* gene and a gene encoding a second novel toxin, TpeL, tend to be more virulent than those with only *netB*. The *netB* gene has been found in a majority of NE isolates from countries where studies have been conducted, although there is little data describing the prevalence of *netB* and *tpeL* in the United States.

The objective of this study was to develop a multiplex PCR to detect genes encoding the major lethal toxins as well as *cpe*, *netB*, and *tpeL* and use this to screen 238 isolates of CP from poultry disease outbreaks and poultry litter. Although optimization of a single multiplex was not successful, screening of isolates using a multiplex reaction detecting the major lethal toxins and a

second multiplex detecting *netB* and *cpe* as well as a single loci PCR of *tpeL* completed the toxin genotyping analysis. Results showed that all isolates but 3 from poultry litter (which were determined to be *C. bifermentans*, *Bacteroides caccae*, and a *Wolinella spp.*) were positive for *cpa* and only one was positive for *cpb*. The *netB* gene was present in only two (4%) of the 49 isolates taken from NE outbreaks and were not present in any isolate from GD or poultry litter. The *tpeL* gene was present in one necrotic enteritis isolate and one poultry litter isolate.
These results suggest that *netB* is not a necessary virulence factor for the development of necrotic enteritis for many strains of *C. perfringens*. However, all isolates analyzed in this study originated in Alabama, limiting the conclusions to populations from that region. More research is necessary to determine the prevalence of this gene on a global scale, which would indicate its importance as a virulence factor to the *C. perfringens* population as a whole.

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List of Abbreviations

AAD	Antibiotic associated disease
AE-PCR	Multiplex PCRs which toxin genotype *C. perfringens* into types A, B, C, D, or E
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BHI	Brain-heart infusion
CIAV	Chick infectious anemia virus
CPE	*Clostridium perfringens* enterotoxin
DDGS	Distiller's dried grains with solubles
dNTP	Deoxynucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
ERIC	Electronic RapidID Compendium
GI	Gastrointestinal
IBDV	Infectious bursal disease virus
LCT	Large clostridial toxin
NCBI	National Center for Biotechnology Information
NRRL	USDA Agricultural Research Service Culture Collection
NSP	Non-specific product
PCR	Polymerase chain reaction
PFO	Perfringolysin O
TSA II	Trypticase Soy Agar with 5% Sheep Blood

CHAPTER 1:

Introduction

Clostridium perfringens is one of the main causes of necrotic enteritis and gangrenous dermatitis, which are considered major issues to the poultry industry. Necrotic enteritis outbreaks, in which lesions are characterized mainly by mucosal necrosis in the jejunum and ileum of poultry, occur in all countries where poultry are produced (Ficken and Wages, 1997a). The estimated cost to the worldwide poultry industry is around $2 billion (Martin and Smyth, 2009), both from mortality and reduced bird performance. Gangrenous dermatitis is characterized by necrosis of soft tissue with bloody edema and gas buildup between myonecrotic muscle groups; these lesions occur mainly on the breast, wings, and abdomen of infected birds, and lead to shock and death (Ficken and Wages, 1997b; Li *et al.*, 2010a,b). This condition is responsible for increased costs related to mortality and condemnation of diseased carcasses at processing, resulting in its classification as a major disease issue (Li *et al.*, 2010a,b).

Clostridium perfringens, the pathogen responsible for these diseases, is a Gram-positive, anaerobic bacterium. It is ubiquitous in the environment because of its relative aerotolerance and its ability to produce endospores (Titball *et al.*, 1999; Jean *et al.*, 2004; Myers *et al.*, 2006). In addition to naturally occurring in soil and decaying organic matter, it is also a normal member of the intestinal microflora in animals. *Clostridium perfringens* is a prolific toxin producer with the

capacity to manufacture at least 17 toxins (Bokori-Brown *et al*., 2011). These toxins are believed to be the main virulence factors of this pathogen, providing the ability to degrade numerous substrates from host tissue (Shimizu *et al*., 2002). Four toxins of importance (α, β, ε, and ι-toxin), known as the major lethal toxins because of their ability to kill mice when injected intravenously, are used to type *C. perfringens* isolates into five types: A,B,C,D, and E. This typing method is based on each isolate's differential ability to produce these four toxins (Heikinheimo and Korkeala, 2005; Li and McClane, 2006a; Carman *et al*., 2008).

Each toxinotype of *C. perfringens* is associated with different diseases. For instance, types B, C, D, and E are isolated from cases of enteric disease in various livestock whereas type A isolates are primarily associated with necrotic enteritis and gangrenous dermatitis in poultry (Niilo, 1980). In addition, type A strains are occassionally associated with *C. perfringens* enterotoxin (CPE), which is a known cause of food poisoning and is suspected in the pathogenesis of antibiotic-associated diarrhea, sporadic diarrhea, and sudden infant death syndrome in humans (Lindström *et al*., 2011).

Alpha-toxin produced by type A *C. perfringens* has traditionally been the main toxin implicated in the development of necrotic enteritis. However, recent studies have shown that certain isolates still have the ability to produce lesions in healthy birds even when their α-toxin gene has been deactivated (Keyburn *et al*., 2006). This discovery lead to the isolation of the NetB toxin which was shown to be a necessary factor for the development of necrotic enteritis for certain isolates (Keyburn *et al*., 2008). Another toxin, TpeL, was recently discovered by Amimoto *et al*. (2007) and appears to have some sequence homology to large clostridial toxins (LCTs) in other species of *Clostridium*, although it does not appear to play a major role in *C. perfringens* virulence.

Despite this new information regarding novel *C. perfringens* toxins, there is little data demonstrating how wide-spread the genes encoding the NetB and TpeL toxins (*netB* and *tpeL,* respectively) are distributed in the overall *C. perfringens* population. Existing research identified *netB* in isolates from Australia, Belgium, Denmark, Sweden, and Canada. However, analysis of isolates from the United States has been limited to states in New England, New York, and Pennsylvania (Johansson *et al*., 2010; Keyburn *et al*., 2010).

The purpose of the research presented was to develop a multiplex PCR to detect the genes encoding the four major lethal toxins (α, β, ε, and ι), CPE, NetB, and TpeL and then utilize this protocol to genotype 238 isolates originating from cases of necrotic enteritis and gangrenous dermatitis as well as from poultry litter originating from farms in Alabama. The goal of this genotyping study was to determine the prevalence of *netB* and *tpeL* in Alabama *C. perfringens* populations, which would provide more information regarding the importance this toxin plays in overall *C. perfringens* pathogenicity and lead to a greater understanding of the pathogenesis of poultry diseases caused by *C. perfringens*.

CHAPTER 2:

Literature Review

INTRODUCTION

Clostridium perfringens is an important pathogen in humans as well as animals. The bacterium has been known since at least the late 19th century, when it was shown to cause gas gangrene in humans (Niilo, 1980). Bosworth and Glover (1935) recognized certain strains of *Clostridium welchii* (*C. perfringens*) as the causative agent of some ovine enteric diseases such as lamb dysentery and pulpy kidney disease, a type of acute enterotoxemia of lambs.

By the mid-1900's, the organism had been implicated in certain human gastrointestinal illnesses (GI). Epidemics of *enteritis necroticans* were documented in north-eastern Germany in the years following World War II (WWII). Known as *darmbrand*, this enteric disease in humans was thoroughly studied and *C. perfringens* was identified as the pathogen (Zeissler and Rassfeld-Sternberg, 1949; Murrell *et al.*, 1966; Johnson and Gerding, 1997). A similar disease in Papua New Guinea termed *pigbel* was described soon after and is thought to have been caused by *C. perfringens* beta (β)-toxin. Normally, adequate levels of trypsin produced by well-nourished individuals degrades beta-toxin in the gut, preventing damage to the intestinal mucosa; the diet of those indigenous to the highlands of Papua New Guinea was typically low in protein and high in carbohydrate sources containing trypsin inhibitors. This resulted in an inadequate amount of

enzyme to degrade any β-toxin that may have been ingested with *C. perfringens* contaminated pork (Murrell *et al.*, 1966; Johnson and Gerding, 1997). Similar high carbohydrate, low protein diets following WWII were explained as a causative factor of *darmbrand* in Germany (Murrell *et al.*, 1966).

Enterotoxin (CPE)-mediated food poisoning, one of the most common foodborne illnesses in the world (Li and McClane, 2006a; Lindström *et al.*, 2011), was first described in the 1940's. Those cases involved consumption of previously cooked food contaminated with anaerobic spore-forming bacilli including *C. perfringens*. Symptoms were typical of CPE-mediated GI, i.e. diarrhea and abdominal cramping (Knox and MacDonald, 1943; McClung, 1945; Johnson and Gerding, 1997; Labbe and Juneja, 2006).

In addition to enterotoxemia of lambs, several other diseases of livestock have been attributed to *C. perfringens*. Among them are enteric diseases of sheep, pigs, cattle, goats, and horses as well as necrotic enteritis and myonecrosis (gangrenous dermatitis) in poultry (Niilo, 1980; Quinn *et al.*, 1994; Songer, 1997). Although *C. perfringens* has been identified as the primary organism responsible for these enteric outbreaks, the precise mechanism for its pathogenicity is poorly understood (Shimizu *et al.*, 2002). *Clostridium perfringens* toxins are attributed as the main virulence factors in these diseases and many biochemical properties of the toxins have been elucidated. However, these properties are insufficient in explaining the exact role that each toxin plays in causing specific outbreaks. Adding to the lack of clarity, models of the disease mechanism change frequently, particularly as new toxins and other virulence factors continue to be discovered.

PHYLOGENY AND SPECIES CHARACTERISTICS

A large taxonomic group, the genus *Clostridium* is genetically diverse and includes over 150 species, 35 of which are described as pathogenic. All are anaerobic, endospore-forming, Gram-positive, bacilli. These bacteria produce a multitude of toxins (around 18% of all known bacterial toxins); however, only 15 species of *Clostridium* manufacture toxins that are considered potent (Popoff and Stiles, 2005). Species within this genus are phylogenetically classified into 16 clusters based on similarity between their 16S ribosomal RNA-encoding genes. Most pathogenic species fall into clusters I and II, with the exception of *C. difficile* and *C. sordellii*, which belong to cluster XI (Stackebrandt and Rainey, 1997; Lyras and Rood, 2000; Popoff and Stiles, 2005). This large number of phylogenetic clusters is an example of the heterogeneity within the *Clostridium* genus. It has been suggested that those species outside of core clusters I and II should be reclassified into a separate genus (Lyras and Rood, 2000).

A member of phylogenetic cluster I, *C. perfringens* is a successful organism; it is ubiquitous in soil and decaying organic matter and is normally isolated from the digestive tracts of humans and other animals including chickens (Titball *et al.*, 1999; Keyburn *et al.*, 2008). It is also one of the most common disease-causing microorganisms in the world (Lindström *et al.*, 2011). *Clostridium perfringens* is typical of its genus as it produces endospores when environmental conditions become unfavorable for vegetative growth. The formation of resilient endospores allows the organism to persist under varied conditions and contributes to its widespread presence (Titball *et al.*, 1999). *Clostridium perfringens* is distinct from other *Clostridium spp.* in that it is aerotolerant and can survive stress induced by a relatively high redox potential, further contributing to its survivability in numerous environments (Jean *et al.*, 2004; Myers *et al.*, 2006). It also has the ability to produce numerous lethal toxins and other

proteins of lesser enzymatic activity that contribute to its pathogenicity. In total, *C. perfringens* produces at least 17 different toxins (Bokori-Brown *et al.*, 2011) with the collective ability to degrade a multitude of different substrates. These toxins are important for the organism's survival, as it is missing genes responsible for producing several enzymes necessary for the biosynthesis of amino acids, rendering it dependent upon assimilating organic compounds extracted from damaged host tissues (Shimizu *et al.*, 2002; Popoff, 2004).

VIRULENCE FACTORS AND RELATED GENES

Clostridium perfringens, having the largest arsenal of toxins in its genus, is the most prolific toxin producer currently known (Popoff and Stiles, 2005). These toxins are considered major virulence factors in the pathogenicity of *C. perfringens*-associated disease. *Clostridium perfringens* strains can be classified into five types (A,B,C,D,E) based on their production of the alpha (α), beta (β), epsilon (ε), and iota (ι)-toxins (Table 2.1). Additionally, type A strains are associated with enterotoxin (CPE), which is the main virulence factor of *C. perfringens* food poisoning and is suspected in the pathogenesis of other GI diseases such as sporadic out-patient diarrhea and nosocomial antibiotic-associated diarrhea (Heikinheimo and Korkeala, 2005; Li and McClane, 2006a; Carman *et al.*, 2008).

Since its initial development, traditional *C. perfringens* toxinotyping has become less useful, as several other toxins have been discovered to be important in the pathogenicity of *C. perfringens* associated diseases. These include: Perfringolysin O, or PFO (also known as θ-toxin), which is associated with myonecrosis in conjunction with α-toxin produced by type A strains (Popoff, 2004; Popoff and Stiles, 2005); β2-toxin, a possible virulence factor of enteric diseases in humans and animals (Fisher *et al.*, 2005); and the recently identified NetB toxin, which appears to be a necrotic enteritis virulence factor (Keyburn *et al.*, 2008). Other toxins

produced by *C. perfringens* that might play a role in its pathogenicity include: the recently identified TpeL toxin, a member of the large clostridial toxins (CTL)(Amimoto *et al.*, 2007); delta (δ)-toxin, a hemolytic toxin only active against artiodactyl erythrocytes and certain other eukaryotic cells; kappa (κ)-toxin, a collagenase also associated with myonecrosis; and several enzymes with different hydrolytic activities such as protease (λ-toxin), lipase, hyaluronidase (μ-toxin), neuraminidase, deoxyribonuclease (ν-toxin), and urease (Popoff, 2004).

The gene encoding α-toxin (*cpa* or *plc*) as well as PFO (*pfoA*) and hydrolytic enzymes also involved in myonecrosis (κ-toxin, hyaluronidase, neuraminadase etc.) are chromosomal and all loci are within variable regions near the origin of replication (Popoff and Stiles, 2005). Studies have shown *cpa* to be highly conserved among strains; however, α-toxin isolated from avian species appears to differ slightly from those of mammals in its phospholipase C activity (Tsutsui *et al.*, 1995; Justin *et al.*, 2002; Popoff, 2004).

Most other toxin encoding genes are located on various plasmids including *cpb* (β-toxin), *etx* (ε-toxin), *ia* and *ib* (ι-toxin), *cpb2* (β2-toxin), *netB*, and *tpeL* (Johnson, 1997; Lepp *et al.*, 2010; Sayeed and McClane, 2010). This characteristic contributes to the instability of these genes, as plasmids are easily lost, and is the most likely reason for the diverse array of *C. perfringens* toxinotypes and the numerous distinct pathogenicities associated with the species. The exception to this is the CPE gene (*cpe*) which is on a large plasmid in certain isolates and on the chromosome in others.

Chromosomal *cpe* sits within a Tn*1565* transposon-like structure situated between the insertion sequences IS*1470* and IS*1469*. Plasmid *cpe* is flanked by insertion sequences as well. The presence of these sequences and the discovery of a circular transposon intermediate (Tn*5565*) indicates that *cpe* can be mobilized between plasmid and chromosome by transposition

(Brynestad and Granum, 1999; Popoff and Stiles, 2005). Interestingly, whether *cpe* is chromosomal or plasmid-borne appears to affect the pathology of a particular strain. Chromosomal *cpe* isolates are associated with food-poisoning in humans, whereas isolates having *cpe* on a plasmid are involved with other diseases in humans and animals (Li and McClane, 2006a; Carman *et al.*, 2008). Although type A strains are isolated from cases of cpe-related disease, the gene has been found in all toxinotypes A-E (Granum and Brynestad, 1999). Type E isolates have a *cpe* gene; however, it is not expressed and is associated with the insertion sequences IS*1151* and IS*1469* located on the same plasmid as *ia* and *ib* (Popoff and Stiles, 2005).

DISEASES ASSOCIATED WITH POULTRY

As previously stated, the wide range of toxins and various toxinotypes of *C. perfringens* contributes to the large number of diseases caused by the organism. Each toxinotype is associated with a unique set of diseases that affect different species of domestic animals as well as humans. In each case, there are certain toxins or combinations of toxins that are known or suspected to be involved in the pathogenicity of each disease (Table 2.2). In poultry, *C. perfringens* is the principle etiological agent of necrotic enteritis and gangrenous dermatitis. Although type C strains have been isolated from some cases of disease such as necrotic enteritis, most cases of disease in chickens have been associated with type A strains.

Necrotic enteritis

Necrotic enteritis, an important disease caused by type A C. perfringens, is a disease in chickens and other domestic poultry reportedly found in most countries around the world where poultry are produced (Ficken and Wages, 1997a). Some estimates have placed the world cost of necrotic enteritis at around $2 billion per year (Martin and Smyth, 2009). This disease presents itself in varying severities with multiple degrees of impact on poultry meat production. An

outbreak may result in minor clinical signs in which slight loss in production is observed or acute outbreaks may result in high mortality and carcass condemnation at slaughter (Johansson *et al.*, 2010).

Lesions in diseased birds are usually found in the small intestine, primarily in the jejunum and ileum. Thickened mucosa and multifocal ulceration are present in the intestine of less severe cases while a yellow or green pseudomembrane representing extensive inflammation and necrosis of the intestinal mucosa are associated with acute cases. Hemorrhage is not a prominent feature, although flecks of blood may be observed (Ficken and Wages, 1997a). Although depression and reduced appetite as well as mortality all have an impact on meat production, losses in minor outbreaks can be primarily attributed to damage of the intestinal mucosa (Van Immerseel *et al*., 2004). As damaged tissue regenerates, scarring takes place manifested as shortened intestinal villi (Long *et al.*, 1974). In birds that survive, damage to intestinal villi leads to protein leakage and ultimately decreased surface area available for diffusion of nutrients from digesta into epithelial cells and blood vessels (Gholamiandekhordi *et al.*, 2008). Reduced absorption of nutrients causes impaired growth and production loss from poor feed conversion (Van Immerseel *et al*., 2004). Other losses in production stem from condemnation at the processing plant. Multifocal or massive necrotic hepatitis frequently accompany necrotic enteritis (Lovland and Kaldhusal, 2001; Van Immerseel *et al.*, 2004). Birds with *C. perfringens* associated hepatic lesions may have their livers condemned at slaughter. If these lesions are severe enough, whole carcasses may also be condemned as unfit for human consumption (Lovland and Kaldhusal, 2001; Johansson *et al.*, 2010).

There are several conditions which appear to be predisposing factors in the development of necrotic enteritis. Sudden disturbances to natural gut microflora caused by the ingredient

composition of the animal's diet can influence the likelihood of developing the disease (Williams *et al.*, 2003; Van Immerseel *et al.*, 2009). High inclusion levels of ingredients containing poorly digested carbohydrates such as wheat and barley can predispose a flock to necrotic enteritis (Ficken and Wages, 1997a; Annett *et al.*, 2002; Williams *et al.*, 2003). Damage to the gut by these fibrous materials in the diet or in litter eaten by birds has been suggested as the reason for this association, as well as increased viscosity and transit time of digesta that gives *C. perfringens* more time to colonize the gut (Williams, 2005). In addition, dietary animal products such as fish meal are excellent substrates for *C. perfringens* growth and high inclusion levels of these ingredients are risk factors of necrotic enteritis (Williams, 2005). In recent years, increases in the amount of corn designated for ethanol biofuel production has raised the price of this crop (Tyner, 2008). Thus, poultry producers have attempted to replace part of the corn diet with cheaper alternative ingredients, many of which have higher inclusion of less-digestible carbohydrates. Distillers dried grains with solubles (DDGS), a byproduct of ethanol production, are considered to be one of these economical alternatives (Loar *et al.*, 2010). Although safe at low inclusion levels, DDGS at high inclusion can lead to an increase in intestinal viscosity due to a greater content of less-digestible carbohydrates that could possibly result in higher *C. perfringens* viability.

Although type A infections are the main cause of necrotic enteritis in poultry, to a lesser extent, type C *C. perfringens* isolates have also been implicated. The pathogenesis of necrotic enteritis linked to type C *C. perfringens* strains has been attributed to the action of β-toxin, which is the main virulence factor of this organism (Songer, 2010). The β-toxin is extremely labile and is readily cleaved by proteases, especially trypsin (Johnson and Gerding, 1997; Popoff and Stiles, 2005). Young animals are particularly vulnerable to infection by type C *C. perfringens* for this

very reason, as they have a lesser ability to produce trypsin and other proteases. This would make broilers susceptible to disease from type C strains, as they are only 1 day of age upon introduction to the farm. Dietary ingredients that could negatively impact trypsin activity in the gut could also be a risk factor for developing type C-associated necrotic enteritis. For example, poorly processed soy bean meal in the diet may contain active trypsin inhibitors that lead to the neutralization of any trypsin produced by the animal. This would allow any β-toxin present to stay active and cause intestinal damage.

Additional factors that predispose a flock to necrotic enteritis outbreaks include reduced immune status caused by prior infections such as infectious bursal disease virus (IBDV), chick infectious anaemia virus (CIAV), and Marek's disease virus, as well as other non-disease related stressors (Williams, 2005). Infection by *Eimeria spp*. is an important risk factor for necrotic enteritis. Although the relationship between coccidiosis and necrotic enteritis is uncertain, it has been proposed that damage caused to intestinal epithelial cells during the various stages of coccidial reproduction can result in the release of plasma proteins which serve as growth factors for *C. perfringens* (Van Immerseel *et al.*, 2004; Williams, 2005). This leads to a shift in gut microflora and increases the likelihood of colonization by *C. perfringens*. Because coccidia are found universally wherever chickens are raised (Ficken and Wages, 1997a) and given the ubiquitous nature of *C. perfringens,* the potential for an outbreak of necrotic enteritis is always possible. Thus, measures to reduce the incidence of coccidiosis are critical for preventing necrotic enteritis.

With the advent of legislation limiting the use of antibiotics in Europe, and the increase in public sentiment against these drugs in other countries, infection by *C. perfringens* will continue to be an important issue in the future (Van Immerseel *et al.*, 2004; Thanissery *et al.*, 2010). This

underscores the need to explore alternative strategies in preventing clostridial infections such as the use of probiotics and prebiotics, preventing development of immunosuppresive diseases, innovative vaccination programs, and optimization of feed ingredients (Li *et al.*, 2010a).

Gangrenous dermatitis

Clostridium perfringens type A strains are responsible for causing myonecrosis, a disease of great medical importance in both human and veterinary medicine. Known as gas gangrene in humans, or gangrenous dermatitis in poultry, this condition is described as necrotizing of healthy soft tissue undamaged by previous trauma (MacLennan, 1962). If untreated, a gangrenous infection will spread from the initial wound and ultimately lead to shock and death (Bunting *et al.*, 1997). Gangrenous dermatitis is responsible for substantial, world-wide production losses in the poultry industry and is regularly considered a disease of great importance by the United States Animal Health Association's Committee on Transmissible Diseases of Poultry and other Avian Species (Li *et al.*, 2010a,b).

In poultry, lesions of gangrenous dermatitis are usually found on the breast, abdomen, or wings and characterized as moist, reddish or dark discolored tissue generally devoid of feathers. Bloody edema and gas buildup between underlying muscle groups are common accompanying characteristics (Ficken and Wages, 1997b; Li *et al.*, 2010a,b). Myonecrosis ordinarily results from deeply penetrating wounds that cut off blood supply from the damaged area. This promotes anoxic conditions and subsequent lowered pH (from anoxic reduction of muscular pyruvate to lactate) which are favorable for the growth of contaminating anaerobic microorganisms, such as *C. perfringens* (MacLennan, 1962; Stevens, 1997). Outbreaks of gangrenous dermatitis in poultry may be preceeded by other viral infections such as IBDV and CIAV, which cause

immunosuppresion and reduced resistance to many infectious agents, including *C. perfringens* (Ficken and Wages, 1997b; Rosenberger and Cloud, 1998).

As the organism proliferates in the lesion it produces several toxins such as α-toxin, PFO, and extracellular enzymes, which serve to further damage host tissue, maintain anaerobic conditions, and neutralize polymorphonuclear leukocytes (Bunting *et al.*, 1997; Awad *et al.*, 2002). In addition, the ability of *C. perfringens* to escape the phagosome of macrophages, even under aerobic conditions, further helps it to spread from localized ischemic tissue into surrounding healthy tissue (O'Brien and Melville, 2000).

Gangrenous dermatitis causes considerable economic losses to the international poultry industry due to the high mortality of the disease and recent pressure (both by government legislation and public opinion) to discontinue the use of antibiotic feed-additives as growth promoters which have kept outbreaks at a minimum (Li *et al.*, 2010a,b). As with necrotic enteritis, preventing gangrenous dermatitis in the future will rely on the exploration of alternative methods to control *C. perfringens* infections.

NetB AND ITS ROLE IN NECROTIC ENTERITIS

The molecular pathogenesis of necrotic enteritis is poorly understood, although some evidence implicates certain toxins in the disease development. For years, *C. perfringens* α-toxin was thought to be the main virulence factor in necrotic enteritis (Keyburn *et al.*, 2008). This assumption was based on several important points starting with the fact that α-toxin can be identified in the feces of birds suffering from necrotic enteritis. In addition, strains of *C. perfringens* isolated from cases of necrotic enteritis were shown to produce more α-toxin than strains from healthy birds (Songer, 1997). Some studies have demonstrated that strains from healthy birds produce so little α-toxin on prepared media that they are difficult to type using

traditional toxin neutralization methods (Niilo, 1980). Crude extracts of α-toxin consisting of cell-free filtrates from cultures of *C. perfringens* type A were able to induce necrotic enteritis by intraduodenal infusion in both germ-free and normal chicks (Songer, 1997; Keyburn *et al.*, 2006). Scientists performing these experiments concluded that because α-toxin was the main toxin produced by these *C. perfringens* strains, α-toxin in the filtrate must be responsible for the pathogenicity (Keyburn *et al.*, 2006). Adding credence to this claim, administering anti-α-toxin antibodies to culture filtrates before innoculation of birds was shown to provide protection against necrotic enteritis (Songer, 1997; Lovland *et al.*, 2004).

This array of evidence led researchers to the conclusion that α-toxin is the main virulence factor in necrotic enteritis. However, because many of these experiments were based on crude filtrates there remains an unaddressed possiblility that other toxins were involved in inducing necrotic enteritis. Conflicting evidence has also surfaced; for example, Gholamiandekhordi *et al.* (2006) could not find a correlation between *in vitro* α-toxin production and incidence of necrotic enteritis in isolates taken from diseased and healthy birds. Other studies have shown more inconsistencies such as the absence of leukostasis in infected tissues, which is a normal characteristic of α-toxin activity in gas gangrene, a lack of correlation between intestinal levels of α-toxin and lesion scores, and the construction of a *plc*-null mutant that retained its ability to induce necrotic enteritis *in vivo* (Keyburn *et al.*, 2006).

The lack of evidence implicating α-toxin as the main virulence factor in necrotic enteritis lead to the identification and purification of the NetB toxin described by Keyburn *et al.* (2008). The toxin was first extracted from a *C. perfringens* isolate (EHE-NE18) that expressed the ability to induce necrotic enteritis *in vivo* even with its α-toxin gene deactivated. NetB was shown to have limited sequence similarity to *C. perfringens* β-toxin (38% identity) and to contain a

conserved domain necessary for pore-formation by β-toxin and other toxins such as *Staphylococcus aureus* α-toxin. In a disease model, wild-type EHE-NE18 was able to produce significant levels of necrotic enteritis. However, *netB*-null mutants administered to birds were unable to cause disease. Virulence was restored to the mutants by complementing with plasmids containing a *netB* gene. This disease model thus fulfilled molecular Koch's posulates regarding the importance of NetB in the pathogenesis of necrotic enteritis for EHE-NE18. Additionally, these same researchers detected *netB* in a majority of strains isolated from cases of necrotic enteritis, indicating that NetB might also be important for these strains. In another study, the presence of the *netB* gene paired with *tpeL*, a gene encoding the recently discovered TpeL toxin, seemed to increase the virulence of *C. perfringens* in broiler chicks when compared to isolates with only *netB*. (Coursodon *et al.* 2010).

There is currently limited data indicating the worldwide importance of the NetB toxin. A study by Keyburn *et al.* (2010) reported that 70% of isolates taken from numerous necrotic enteritis cases in Australia, Belgium, Denmark, and Canada were positive for the *netB* gene and expressed NetB *in vitro*. A study in Sweden showed that 91% of isolates taken from disease cases were positive for *netB* (Johansson *et al.*, 2010). However, in another study, only 58.3% of necrotic enteritis-associated isolates from New England, New York, and Pennsylvania were positive for *netB* (Martin and Smyth, 2009). This would indicate that NetB is not an essential virulence factor for many strains, but the limited geographical area sampled necessitates more research to determine the importance of the toxin on a worldwide scale. Saita *et al.* (2009) reported no instances of *netB* in 25 strains of *C. perfringens* type A isolated from diseased turkeys, which would suggest that NetB is not an important virulence factor for necrotic enteritis in that species.

TYPE A GASTROINTESTINAL DISEASES

In addition to necrotic enteritis and gangrenous dermatitis in poultry, Type A *C. perfringens* is suspected to be responsible for several gastrointestinal (GI) diseases in humans including food-poisoning, antibiotic-associated diarrhea (AAD), sporadic diarrhea, and sudden infant death syndrome (SIDS). In AAD and sporadic diarrhea, as well as GI diseases in dogs, horses, and pigs, CPE produced during sporulation of this organism is thought to be the main virulence factor, although accessory toxins are suspected in some cases. The connection between *C. perfringens* and SIDS is even less understood. However, CPE-producing strains have been isolated from a large number of infants afflicted with SIDS, and systemic circulation of CPE has been proposed as a cause of death (Lindström *et al.*, 2011).

While only about 6% of *C. perfringens* isolates carry a *cpe* gene (Popoff and Stiles, 2005), type A food poisoning is of particular importance to the poultry industry from a food safety standpoint, as it is a high-profile foodborne disease in the industrialized world arising primarily from the improper handling of meat contaminated with endospores. As *C. perfringens* is a ubiquitous organism, contamination of any poultry product with the organism's endospores is possible at many different points. Thus, correct handling of the product is essential for disease control.

CPE-mediated food poisoning

With almost 1 million cases a year, food poisoning in humans caused by *C. perfringens* type A is one of the most common foodborne diseases in the US (Li *et al.*, 2011). It is consistently ranked among the second or third most common foodborne diseases in both the US and UK every year, as well as the second leading cause of food-related death in the UK between 1990-2000 (McClane, 2005). Incidences of type A food poisoning usually affect large numbers

of people and are normally associated with mishandling of poultry and other meat dishes. These outbreaks typically occur when food contaminated with *C. perfringens* spores is held at an improper temperature (10-54°C), which is optimal for spore germination and vegetative growth (Lindström *et al.*, 2011). Improper chilling can also lead to propagation of *C. perfringens*. The USDA-FSIS requires that chilling from 54.4-26.7°C should not take longer than 1.5 hours and chilling from 26.7-4.4°C no longer than 5 hours (Labbe and Juneja, 2006). As *C. perfringens* has a relatively fast generation time of only 10-15 minutes, pathogenic levels of vegetative cells in contaminated meat can arise quickly under these improper temperatures (McClane, 2005). When vegetative cells are ingested in high numbers some survive the acidic environment of the stomach and reach the small intestine where they can sporulate and produce CPE. The endospore is released when the cell lyses and it is at this time that the CPE toxin is released (Huang *et al.* 2007). Symptoms of illness arise between 8-12 hours from ingestion, manifested as abdominal cramps and diarrhea, and last around 24 hours with fatalities typically occurring only in the elderly or immunocompromised individuals (Lindström *et al.*, 2011).

Although type A *C. perfringens* may have either a chromosomal or plasmid-borne *cpe* gene, most cases of CPE-mediated food poisoning have been associated with isolates having a chromosomal *cpe* gene. It is not clear why such a bias exists, although it has been shown that strains with this genetic configuration exhibit a greater survivability under conditions used to preserve foods such as high and low temperatures, high salt concentration, and addition of nitrites, which could explain their greater prevalence in food poisoning cases (Li and McClane, 2006a,b). Interestingly, in recent studies, plasmid-borne *cpe* strains have been isolated from outbreaks in Finland, Germany, Japan, and other European countries (Lindström *et al.*, 2011)

indicating that, although less common, CPE-mediated food poisoning is possible from this genotype of *C. perfringens* as well.

METHODS FOR TOXINOTYPING *Clostridium perfringens* ISOLATES

Before the development of modern polymerase chain reaction (PCR) methods, the toxin profiles of various *C. perfringens* isolates were characterized utilizing serotyping methods. The most common of these techniques involves assessing toxin neutralization in guinea pigs or mice by type-specific antisera (Niilo, 1980; Songer and Meer, 1996). There are several problems with this method such as concern for the welfare of animal test subjects, the expense and complex nature of such projects, and insufficient sensitivity and specificity (Songer and Meer, 1996). Other techniques can be utilized for the diagnosis of *C. perfringens* associated disease such as observation of clinical signs and gross lesions in addition to culturing or direct methods to determine an unusual presence of clostridium in diseased tissue. Various immunoassays can be used for the detection of certain toxins such as enzyme-linked imunosorbent assays (ELISA), immunoelectrophoresis, immunodiffusion, and latex agglutination; (Songer and Meer, 1996)

Nucleic acid-based detection methods such as PCR can be used for genotyping of *C. perfringens* and the presence of certain toxin genes used to presumptively toxinotype an isolate (Songer and Meer, 1996; Yoo *et al.*, 1997; Heikinheimo and Korkeala, 2005). This method has the advantages of being much faster than toxin neutralization, relatively inexpensive, and highly specific. A drawback that exists is the possibility of false positives due to non-expression of the detected toxin gene because of deficiencies in regulation or sporulation (Kanakaraj *et al.*, 1998). For example, some strains of type E *C. perfringens* have a *cpe* gene; however, they do not produce CPE because of nonsense and frameshift mutations within the gene as well as the absence of regulatory sequences such as a start codon, promoters, and ribosomal binding sites

(Billington *et al.*, 1998). Because of the possibility of a false positive, other methods which can detect toxin expression are necessary for actual toxinotyping. Nevertheless, PCR genotyping is an efficient diagnostic test that has the potential to reduce the risk of false negative results that have been described as less desirable for certain applications (Kanakaraj *et al.*, 1998).

PCR AND MULTIPLEX TOXINOTYPING

Genotyping by PCR methods is achieved by two steps. The first step involves the amplification of segments within the gene of interest by the action of *Taq* DNA polymerase (Taq). Second, these amplicons are verified for proper size by agarose gel electrophoresis and visualized using a DNA-binding flourescent compound, such as ethidium bromide. To amplify a target segment, two oligonucleotide primers are designed to anneal specifically to the targeted gene: one primer (reverse primer) binds to the 3' end of a reference strand and a second primer binds to the 3' end of the complementary strand (forward primer). First, the temperature is raised to around 95°C to denature the DNA double helix. Then the temperature is lowered to the optimal annealing temperature of the primers (usually around 55°C), which then attach to their complementary sites. The temperature is then raised to around 72°C, the optimal temperature for Taq function. Taq incorporates deoxynucleotide triphosphates (dNTPs) onto the 3' ends of the annealed primers, synthesizing the target gene. This cycle of denaturing, annealing, and extension is then repeated 35-40 times, replicating the targeted gene segment and amplifying it exponentially. Concentrations of reagents, such as dNTPs, $MgCl_2$, reaction buffer, Taq, primers, and template DNA must all be optimized for the amplification to be successful.

In a multiplex PCR, multiple gene segments are amplified in a single reaction. Therefore, separate primer sets for each gene seqment must be incorporated into the reaction mixture. This makes it more difficult to optimize reaction concentrations, as high primer concentrations are

unavoidable when amplifying several different loci at one time. Nevertheless, the use of an optimized multiplex PCR is much more efficient for analyses regarding multiple genes, such as toxin genotyping assays. Because multiple genes are detected in a single reaction, the assay requires less time and fewer reagents than performing a separate amplification for each targeted gene.

THESIS PROPOSAL

Multiplex PCR protocols have been developed which can detect the genes encoding the four major lethal toxins as well as various combinations of major toxin genes and other genes such as *cpb2*, *cpe*, *netB*, and *tpeL*. However, to this date, there has not been a single multiplex which can detect more than six genes at one time. One of the objectives of this project is to develop a multiplex PCR that will detect seven toxin encoding genes: *cpa*, *cpb*, *etx*, *ia*, *cpe*, *netB*, and *tpeL* providing an efficient tool for the characterization of *C. perfringens* isolates. The second objective is to utilize this multiplex protocol to characterize the toxin genotypes of 78 strains isolated from different outbreaks of necrotic enteritis and gangrenous dermatitis in poultry as well as 160 poultry litter isolates, all originating in Alabama. The discovery of novel toxins produced by *C. perfringens*, such as NetB, has brought to question the role of α-toxin in the pathogenesis of necrotic enteritis in poultry. NetB has been shown to play an important role in the development of necrotic enteritis in certain isolates of *C. perfringens*. Despite its apparent importance to the development of the disease, there has been limited research demonstrating the prevalence of NetB producing isolates from necrotic enteritis outbreaks in the United States. TpeL, another novel toxin, has limited data published which characterizes its prevalence in disease outbreaks. Hopefully by determining the toxin genotypes of these isolates more light will

be shed on the importance of these novel genes and also provide more information for the future analysis of *C. perfringens*.

Table 2.1 *C. perfringens* toxinotypes based on production of major toxins

Toxinotype	Major toxins produced			
	A	B	E	I
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Table 2.2 Human and veterinary diseases attributed to each *C. perfringens* toxinotype

Toxinotype	Diseases in animals	Possible virulence factors[1]	Diseases in humans	Possible virulence factors[1]
A	Myonecrosis/necrotic enteritis in poultry	α, NetB	Myonecrosis	α, PFO
	Enterotoxemia (cows, goats, sheep)	α, β2	Antibiotic-associated diarrhea	CPE, β2
	Porcine necrotic enterocolitis	α, β2, CPE	Sporadic diarrhea	CPE, β2
	Equine colitis	β2, CPE[2]	Food poisoning	CPE
	Canine hemorrhagic gastroenteritis	CPE	Sudden infant death syndrome	CPE
B	Dysentary/chronic enteritis in lambs	β, ε		
	Ovine hemorrhagic enterotoxemia	β, ε		
	Hemorrhagic enteritis (goats, calves, foals)	β, ε		
C	Necrotic enteritis in poultry	α, β	Enteritis necroticans (darmbrand/pigbel)	β
	Neonatal hemorrhagic/necrotic enteritis (cows, sheep, goats, pigs, horses)	β[3]		
	Ovine enterotoxemia (struck)	β[3]		
D	Enterotoxemia (cows, goats, sheep)	ε		
	Caprine eterocolitis	ε		
E	Enterotoxemia in cows and sheep	ι		
	Rabbit enteritis	ι		

[1] Many listed toxins are suspected in the pathogenesis or likely work synergistically with unconfirmed virulence factors

[2] Ochoa and Kern (1980) induced disease in ponies by intravenous injection of CPE; results were inconclusive due to crude extracts of CPE

[3] Songer *et al.* (1996) showed that, although necessary for disease, β-toxin alone cannot cause type C disease in pigs or lambs, indicating the possibility of other necessary factors

CHAPTER 3:

Development of two multiplex PCR protocols for the detection of *Clostridium perfringens* toxin encoding genes *cpa*, *cpb*, *etx*, *ia*, *cpe*, *netB*, and *tpeL*

ABSTRACT Toxin genotyping of *Clostridium perfringens* isolates utilizing PCR has usually required multiple reactions per isolate as the organism produces many different important toxins. The large number of oligonucleotide primers needed to detect these genes in a single multiplex poses an obstacle in developing a protocol that detects more than a few genes in a single reaction. The objective of this project was to develop a single multiplex PCR protocol that can detect seven toxin-encoding genes in reference strains of *C. perfringens,* including genes corresponding to the four major lethal toxins (*cpa*, *cpb*, *etx*, *ia*), the gene encoding enterotoxin (*cpe*), and genes encoding two recently discovered toxins (*netB* and *tpeL*). Fourteen primers (cpaF, cpaR, cpbF, cpbR, etxF, etxR, iaF, iaR, cpeF, cpeR, netBF, netBR, tpeLF, tpeLR) were initially designed using the primer-BLAST function provided by the National Center for Biotechnology website. A commercial master mix, EconoTaq PLUS GREEN, provided Taq-polymerase, $MgCl_2$, and dNTPs for the reaction. Template DNA was extracted from reference strains provided by the American Type Culture, the United States Department of Agriculture,

and Iowa State University. Primers were tested individually for functionality prior to addition to the multiplex and replaced when necessary. Cycling conditions and reagent concentrations were tested systematically using titrations to attempt optimization of the multiplex reaction. These attempts were ultimately unsuccessful, resulting in the need to develop two multiplex reactions: one detecting the four major lethal toxins and a second to detect *cpe*, *netB*, and *tpeL*. These seven genes were successfully detected using the two multiplex PCR reactions, providing a useful tool for the toxin genotyping of *C. perfringens* isolates.

INTRODUCTION

An anaerobic, Gram-positive, endospore-forming bacterium, *C. perfringens* derives its pathogenicity from the production of an array of toxins. This species has the ability to produce at least 17 toxins (Bokori-Brown *et al.*, 2011); although, a given isolate can only produce certain combinations, defined as its toxinotype. Pathology associated with *C. perfringens* is attributed to a particular toxinotype(s), making it diagnostically important to determine which toxins a given isolate can produce. Isolates are typed A-E depending on their ability to produce four major lethal toxins: α, β, ε, and ι.

Toxinotyping has traditionally been performed using toxin neutralization assays in mice and guinea pigs; however, this method has become increasingly obsolete due to animal welfare concerns, cost and complexity of such tests, and their low specificity and sensitivity (Songer and Meer, 1996). Therefore, use of PCR in typing isolates has become more common. This assay genotypes isolates by amplifying short nucleotide sequences within genes associated with the production of a particular toxin. These amplicons can then be separated by DNA gel electrophoresis and visualized on an agarose gel stained with a DNA-binding detection compound such as ethidium bromide. Use of PCR has provided the advantages of being *in vitro*,

relatively cheap, less time consuming, more target-specific, and highly sensitive (Songer and Meer, 1996; Yoo *et al.*, 1997). In addition, multiplex PCR, or the simultaneous amplification of multiple target sequences in a single assay, has made it possible to genotype isolates more efficiently (Songer and Meer, 1997).

Despite their importance to certain diseases, multiplex PCRs that characterize only A-E genotypes (AE-PCR) have become less useful for describing the pathogenicity of some *C. perfringens* isolates as novel toxins and toxin/disease relationships have been discovered. NetB, a relatively novel toxin, was demonstrated to be important in the pathogenesis of necrotic enteritis for some isolates (Keyburn *et al.*, 2008). Despite this apparent importance, the prevalence of *netB* among disease isolates in the US has not been widely investigated. Another toxin, TpeL, was also recently discovered and appears to have some homology to large clostridial toxins (LCT) of other species, although it is less toxic to mice than other LCTs (Amimoto *et al.*, 2007). At this time, the relationship of TpeL to disease does not appear to be significant (Keyburn *et al.*, 2010). Nevertheless, few studies have screened disease isolates for the production of the TpeL toxin, leaving its true importance unclear. A-E genotyping also omits detection of *cpe*, the main virulence factor in *C. perfringens* mediated food poisoning of humans, one of the most common foodborne illnesses in the United States (Li *et al.*, 2011). Because investigations defining the prevalence of *netB* and *tpeL* are yet to be exhaustive in terms of global *C. perfringens* populations, and given the importance of *cpe* to food microbiology, the incorporation of primers sensitive to these genes would expand the pertenance of an AE-PCR assay.

A probable difficulty in the development of this expanded AE-PCR can be forseen in the large number of primers needed to detect all seven genes at once (*cpa, cpb, etx, ia/ib, cpe, netB,*

tpeL). These oligonucleotides must have similar melting temperatures (T_m), and their resulting amplicons must be of sufficient difference in length to allow resolution with gel electrophoresis. These become increasingly limiting factors as more primers are added to the assay. In addition, high primer concentration increases the likelihood of mispriming or the ocurrence of primer-dimers. This leads to the production of non-specific products (NSPs) which compete with desired products for reagents and decrease yield (Innis and Gelfand, 1990).

Successful amplification of a target sequence relies on the optimization of two main sets of variables: cycle parameters and reagent concentrations. A PCR cycle consists of three basic steps: DNA denaturation, primer annealing, and primer extension. The temperature and time of these steps must be adjusted to optimize a reaction. The concentration of reagents such as *Taq* DNA polymerase (Taq), deoxynucleotide triphosphates (dNTP), $MgCl_2$, oligonucleotide primers, as well as the composition of reaction buffer all must be optimized. This experiment attempted to elucidate cycle parameters and reagent concentrations which would optimize the amplification of the seven toxin genes discussed above utilizing primers designed for this experiment.

MATERIALS AND METHODS

Reference strain storage, preparation, and DNA extraction

Reference strains known to be positive for genes of interest (Table 3.1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA), the USDA Agriculture Research Service Culture Collection (NRRL), and Iowa State University. Isolates were stored at -80°C on sterile glass beads within cryotubes containing brain-heart infusion broth (BHI) (BD Diagnostics, Franklin Lakes, NJ) with 20% glycerol. For DNA extraction, isolates were enriched for 24 hours at 37°C in cooked meat medium (HiMedia Laboratories Pvt. Ltd., Mumbai, India) incubated within a Bactron IV Anaerobic Environmental Chamber (Shel Lab, Cornelius, OR)

with an environment of 5% CO_2, 5% H_2, and 90% N_2. Enriched strains were then streaked for isolation on Trypticase Soy Agar with 5% Sheep Blood (TSA II) (BD Diagnostics) and incubated at 37°C for 24-48 hours in the anaerobic chamber. A single colony exhibiting double-zone hemolysis was then transferred to a BHI tube, which was incubated for 24 hours at 37°C in the anaerobic chamber. Bacterial DNA was extracted from the resulting cells using a Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI). Extracted DNA was quantified and evaluated for purity with a Nanodrop 1000 Spectrophotometer (Thermo Scientific Inc., Bremen, Germany) according to the instructions provided by the manufacturer. The standard for purity was a 260nm/280nm ratio between 1.8-2.0.

Primer Design

Primers were designed using the primer-Basic Local Alignment Search Tool (BLAST) function on the National Center for Biotechnology Information's (NCBI) website. All search parameters were left at default except for product size, which was manipulated to yield products for each primer set at least ~100bp difference in length. Primer descriptions are listed in Table 3.2. Primers were synthesized commercially and purified by high-performance liquid chromatography (Eurofins MWG Operon, Huntsville, AL).

Single loci optimization

First, cycle parameters were optimized for each individual loci using the same default reagent concentrations for each reaction. These concentrations were as follows: 1x EconoTaq PLUS GREEN Master Mix (Lucigen Corp., Middleton, WI)(12µL master mix per 25µL reaction), 0.2µM each of target sequence's forward and reverse primer, and 1µg template DNA. Amplification was performed using an iQ5 thermocycler (Bio-Rad, Hercules, CA).

Nonfunctional primers were replaced with either redesigned primers or previously published functional ones (Table 3.3).

Denaturation. The DNA denaturation step must be performed at a high enough temperature to allow maximum separation of DNA strands with minimum loss of enzyme activity. Typical conditions for this step are 95°C for 30 seconds. The half-life of Taq at this temperature is around 40 minutes, more than enough time for 35-40 cycles (Innis and Gelfand, 1990). Thus, these parameters were chosen for the denaturation step.

Annealing. Primer annealing conditions are dependent on the base composition and length of the primers. The higher the G+C content and the longer the primer, the higher its T_m and the longer it takes to anneal to a complementary sequence. In order to maximize yield of specific products and minimize production of non-specifics, annealing temperature should be as close as possible to the T_m without negatively impacting yield (Innis and Gelfand, 1990). Maximum annealing temperature was empiricaly estimated for each primer pair individually by titration from 50-65°C. Annealing temperatures tested in the multiplex were determined based on these temperatures.

Extension. Primer extension parameters are mainly based on the optimal temperature of Taq and the length of the target sequence. Taq has been reported to operate most efficiently between 70-80°C (Lawyer *et al.*, 1993), thus temperatures within this range are desireable. Extension time should consider the speed at which Taq can operate at optimal temperatures. Longer target sequences require more time to replicate. However, it is reported that an extension time of one minute is enough for the synthesis of products up to 2kb long (Innis and Gelfand, 1990). Because the largest amplicon produced by these primer sets is only 868bp long, an extension time of 30 seconds was initially used.

***Gel electrophoresis*.** PCR products were separated by electrophoresis in a 2% agarose gel. Running buffer and gels were made using 1x concentrations of AccuGENE TBE buffer (Lonza Group, Basel, Switzerland). For DNA visualization, 1µL ethidium bromide solution (10mg/mL) was added to each gel prior to solidifying. Electrophoresis was performed at 75v for ~1 hour or until sufficient separation between products was obtained. A 100bp DNA Ladder (Promega) was used as a DNA size standard.

Multiplex optimization

After optimizing cycle parameters for individual loci, reagent concentrations and cycling conditions for the multiplex reaction were adjusted as needed according to a step-by-step protocol for multiplex PCR optimization (Henegariu *et al.*, 1997). However, because of the use of a master mix, concentrations of Taq, $MgCl_2$, and buffer solution had to be adjusted simultaneously. In consequence, fine adjustment options for reagents were limited to primer and template concentrations only.

Verification of PCR products

Bands for each PCR product were excised and purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Isolated products were verified for concentration and purity and sent to the Auburn University Genomics and Sequencing Lab for sequencing. A nucleotide BLAST search of the determined sequences was then utilized to calculate the likelihood that each PCR amplicon originated from its target gene. To determine the significance of each hit from the search, BLAST calculated a statistic known as the Expect value, or E-value. This parameter describes the number of times BLAST would return a particular hit by chance within a given database for a particular query. Thus, the smaller the E-value, the less likely that the hits corresponding to a queried sequence arose out of chance.

RESULTS

Single loci optimization

Single loci optimization was achieved for cpa, cpb, etx, and ia primers. Using the default reagent concentrations described above, these primers produced moderately bright bands until annealing temperature was raised above 64°C, at which point ia, cpb, and etx primers produced only faint bands. Denaturing and extension parameters chosen appeared to be acceptable for high efficiency replication. Primer sets targeting *cpe*, *netB*, and *tpeL* amplicons, however, proved difficult to optimize and did not amplify a product. Attempts to adjust primer/template concentrations and cycling parameters were also not productive. Master mix was adjusted between 0.83x-1.17x for each combination of parameters, to no avail. Thus, the cpe primer set was redesigned (Table 3.3). Previously published netB (AKP78 and AKP79) and tpeL (AKP80 and AKP81) primer sets were already available (Keyburn *et al.*, 2010) and their product sizes were adequate for proper separation by gel electrophoresis (Table 3.3). These primer sets were successfully optimized in single loci PCR using the same cycling conditions and reagent concentrations as for cpa, cpb, etx, and ia primers with the exception that the maximum annealing temperature of the cpe, netB, and tpeL primers were slightly lower, resulting in weak bands above 62°C for cpe and netB and weak bands above 61°C for tpeL.

Multiplex optimization

Although optimization of single loci PCR was achieved for each amplicon, attempts at optimization of a multiplex PCR with all seven primer sets were unsuccessful. Consistent replication of the *cpa* and *ia* amplicons was achieved. However, other primers were inconsistently functional and the production of short NSPs (<100bp) was common. Doubling extension time and/or individually increasing primer concentration incrementally from 0.2µM to

1.0µM did not benefit production of long amplicons nor did increasing annealing temperature from 55-60°C. Touchdown PCR with annealing temperatures from 65-50°C followed by 25 cycles of 50°C annealing temperatures succeeded in producing faint bands corresponding in size to the *cpb* and *etx* amplicons in addition to a strong band representing *cpa*; however, the production of an *ia* band did not occur.

Separation into two multiplex PCRs

Because optimization of a multiplex reaction with all seven primer sets was not achieved by adjusting available parameters, an attempt was made to optimize two separate multiplex reactions utilizing the same primer sets. After substituting AKP78 and AKP79 for a newly designed primer set, netB5F and netB5R (Table 3.3), this goal was successfully achieved. Figures 3.1 and 3.2 show the electrophoresis results of multiplex 1 and 2, respectively. These figures show that bands of the proper size were produced for each loci. Results of a nucleotide BLAST search using the sequence data for each PCR product (Table 3.4) showed at least a 90% max identity of each product to its expected sequence as well as low E-values for all results. The final protocols for these reactions are listed as follows:

Multiplex 1 (gel photograph shown in Figure 3.1)

- Reagent concentrations
  - Master mix 1.17x concentration (14µL per 25µL reaction)
    - Taq: 1.4 units
    - dNTP: 224µM each
    - $MgCl_2$: 1.68mM
  - Template: 1µg per 25µL
  - Primers
    - cpaF: 0.2µM
    - cpaR: 0.2µM
    - cpbF: 0.5µM
    - cpbR: 0.5µM
    - etxF: 1.0µM
    - etxR: 1.0µM
    - iaF: 0.2µM
    - iaR: 0.2µM
- Cycle parameters
  - Initial denaturing: 95°C for 5 minutes
  - 15 Cycles Touchdown PCR
    - Denaturing: 95°C for 30 seconds
    - Annealing: 65-50°C for 30 seconds
    - Elongation: 72°C for 30 seconds
  - 25 Cycles
    - Denaturing: 95°C for 30 seconds
    - Annealing: 50°C for 30 seconds
    - Elongation: 72°C for 30 seconds
  - Final elongation: 72°C for 6 minutes

Multiplex 2 (gel photograph shown in Figure 3.2)

- Reagent concentrations
  - Master mix 1x concentration (12µL per 25µL reaction)
    - Taq: 1.2 units
    - dNTP: 192µM each
    - $MgCl_2$: 1.44mM
  - Template: 1µg per 25µL
  - Primers
    - cpe4F: 0.2µM
    - cpe4R: 0.2µM
    - netB5F: 0.2µM
    - netB5R: 0.2µM
    - AKP80: 0.8µM
    - AKP81: 0.8µM
- Cycle parameters
  - Initial denaturing: 95°C for 5 minutes
  - 40 Cycles
    - Denaturing: 95°C for 30 seconds
    - Annealing: 55°C for 30 seconds
    - Elongation: 72°C for 30 seconds
  - Final elongation: 72°C for 6 minutes

DISCUSSION

Although single loci optimization was achieved for primer sets targeting *cpa*, *cpb*, *etx*, and *ia*, it was not the case with initial primer sets targeting *cpe*, *netB*, and *tpeL*. There are several variables which could influence a primer set's inablility to amplify its intended target. Some of these characteristics are low T_m, self complementarity, primer-primer complementarity, and tendency to misprime. Primers that are too short or have too low of a G+C content tend to have a low T_m which makes it necessary to use lower annealing temperatures, leading to greater production of NSPs. All primers were designed with sufficient length and G+C content to have a T_m of at least 60°C, so this is unlikely to be a factor in the failure of these primer sets. The possibility of self complementarity was addressed by the primer-BLAST secondary structure alignment function, although self complementarity scores for cpeR, netBR, and tpeLF were 8, 6, and 6, respectively, close to the "max self complementarity" parameter for the default primer-BLAST search. As this score was so high for these primer sets (an ideal score is as low as possible), it may be possible that self complementarity played a role in the failure of the procedure by causing the formation of primer self-dimers. Primers cpeF and netBR received a 3' complementarity score of 3, which was the "max 3' complementarity" parameter, indicating the possible formation of hairpin structures which could interfere with proper annealing. No analysis of primer-primer complementarity was performed between forward and reverse primers, thus it is indeterminate if the formation of dimers within a primer set interfered with amplification. Tendency to misprime is a factor that influences the production of NSPs; however, that was not an issue with the nonfunctional primer sets.

Regarding functional primers, annealing temperature titrations showed that the maximum annealing temperature for each primer set varied between 60-64°C. This played a large role in

deciding what range of annealing temperatures to try for optimization of the multiplex reaction. As all primers produced bright bands with an annealing temperature of 55°C, and an annealing temperature 5°C lower than the actual T_m of each primer is recommended for optimization (Innis and Gelfand, 1990), the gradient of 55-60°C was chosen to determine the optimal annealing temperature of the multiplex reaction. Likewise, parameter ranges for other cycling steps and reagent concentrations tested in the multiplex were determined by considering optimal parameters in single loci amplification. Because of this, there is a possibility that the failure to optimize the reactions in multiplex stemmed from the avoidance of parameter settings outside these ranges. Another possibility for the failure could be the use of a master mix. The amplification of PCR products longer than the *ia* fragment was not possible with the full multiplex; thus, it could have been beneficial to decrease the buffer concentration while keeping $MgCl_2$ and Taq constant. This is because high salt concentrations tend to inhibit the amplification of long amplicons and benefit the production of short ones (Henegariu *et al.*, 1997). As the buffer used in EconoTaqPLUS GREEN was a proprietary mix, the absolute salt concentration was unknown. Additionally, the ability to independently increase the concentration of $MgCl_2$ and/or decreasing enzyme concentration could have reduced the production of the small NSPs; Henegariu *et al*. (1997) demonstrated that increasing $MgCl_2$ while keeping dNTP concentration constant at 200µM resulted in more specific and efficient amplification. The same study established that independently decreasing Taq helped prevent unbalanced replication of loci and reduced non-specific amplification. This could have been the case in this study, as a lower enzyme concentration would decrease overall enzymatic activity and make it more difficult for free enzyme to replicate non-specifically.

Despite the failure of the full multiplex optimization, two separate multiplex PCR protocols for the amplification of all seven genes of interest were successfully developed. Gel electrophoresis of the reaction products displayed bands that corresponded to the sizes of the target sequences (Figures 3.1 and 3.2). A nucleotide BLAST search using sequences obtained from the gel purified products provided a list of sequences in the database with which significant alignments could be made. Top hits for each product sequence corresponded to the gene containing the target sequence, with at least 90% max identity between the product and target (Table 3.4). The E-value for these hits were all significantly small, indicating the high likelihood that the hits were relevant. Because the size of the products matched the size of the target and the product sequences matched that of the target with high significance, it can be safely concluded that the two multiplex reactions were successful in detecting the presence of their respective target genes, providing another tool for the analysis of *C. perfringens* isolates.

Table 3.1 Reference strains utilized in multiplex PCR optimization

Isolate ID	Source	Type	Genes of interest
27324	ATCC	E	*cpa, ia, cpe*[1]
3626	ATCC	B	*cpa, cpb, etx*
B-23850	NRRL	A	*cpa, cpe*
JGS-1870	Iowa State	A	*cpa, netB, tpeL*

[1] Isolate 27324 contains a mutated, nonfunctional *cpe* gene

Table 3.2 Specifications of oligonucleotide primers designed for multiplex PCR

Primer ID	Sequence	Target Gene	Reported Tm (°C)	Product Length (bp)
cpaF[1]	GCAGCAAAGGTAACTCTAGCTAACT	*cpa*	62.9	230
cpaR[1]	CCTGGGTTGTCCATTTCCCATT		62.7	
cpbF[1]	ACGGATGCCTATTATCACCAACTTT	*cpb*	61.3	868
cpbR[1]	TGTCCTACCCAGTTAGCACCAT		62.7	
etxF[1]	CGCATCAGCGGTGATATCCAT	*etx*	62.6	680
etxR[1]	TCTCTCCCCATTCACTTCCACTT		62.8	
iaF[1]	CGATGAAAAGCCTACACCACTACTT	*ia*	62.9	319
iaR[1]	TGCGGTATATCCTCCACGCA		62.4	
cpeF[2]	AGGGGAACCCTCAGTAGTTTCAA	*cpe*	62.8	464
cpeR[2]	CTGTAGCAGCAGCTAAATCAAGGA		62.9	
netBF[2]	ATGGCTTTAGCATTAACAGCACCT	*netB*	61.2	111
netBR[2]	TCGCCATTGAGTAGTTTCCCAATTT		61.3	
tpeLF[2]	GGCTACTCAAGGAGCTTTTGCT	*tpeL*	62.7	723
tpeLR[2]	CATTCCTCCAACCCAAACAAAATGA		61.3	

[1] Actual primers used in Multiplex 1

[2] Single loci amplification was unsuccessful indicating possible nonfunctionality of these primers

Table 3.3 Specifications of redesigned and previously published primers

Primer ID	Sequence	Target Gene	Reported Tm (°C)	Product Length (bp)
cpe4F[1]	GGCGTTCTTCTAACTCATACCCT	*cpe*	62.8	169
cpe4R[1]	ACTCCATCACCTAAGGACTGTT		60.8	
netB5F[1]	CGCTTCACATAAAGGTTGGAAGGC	*netB*	64.6	316
netB5R[1]	TCCAGCACCAGCAGTTTTTCCT		62.7	
AKP78[2]	GCTGGTGCTGGAATAAATGC	*netB*	60.4	384
AKP79[2]	TCGCCATTGAGTAGTTTCCC		60.4	
AKP80[12]	ATATAGAGTCAAGCAGTGGAG	*tpeL*	58.7	466
AKP81[12]	GGAATACCACTTGATATACCTG		58.9	

[1] Actual primers used in Multiplex 2

[2] netB and tpeL primers previously published by Keyburn et. al (2010)

Table 3.4 BLAST results for sequenced PCR products

Target gene	Primers Used	Highest significant sequence to PCR product	E-value	Max Identity (%)
cpa	cpaF, cpaR	*C. perfringens* strain Z3 alpha toxin gene, partial cds	1×10^{-92}	99
cpb	cpbF, cpbR	*C. perfringens* type B beta-toxin gene, complete cds	0	90
etx	etxF, etxR	*C. perfringens* D strain IVRI Vac1 epsilon toxin (etx) gene, partial cds	0	91
ia	iaF, iaR	*C. perfringens* DNA for iota toxin polypeptides Ia and Ib	1×10^{-116}	95
cpe	cpe4F, cpe4R	*C. perfringens* strain CN1183 enterotoxin (cpe) gene, complete cds	9×10^{-57}	98
netB	netB5F, netB5R	*C. perfringens*strain 200302-1-1-Ba necrotic enteritis toxin B (netB) gene, complete cds	9×10^{-135}	99
tpeL	AKP80, AKP81	*C. perfringens* A strain CP4 TpeL (tpeL) gene, complete cds	5×10^{-169}	93

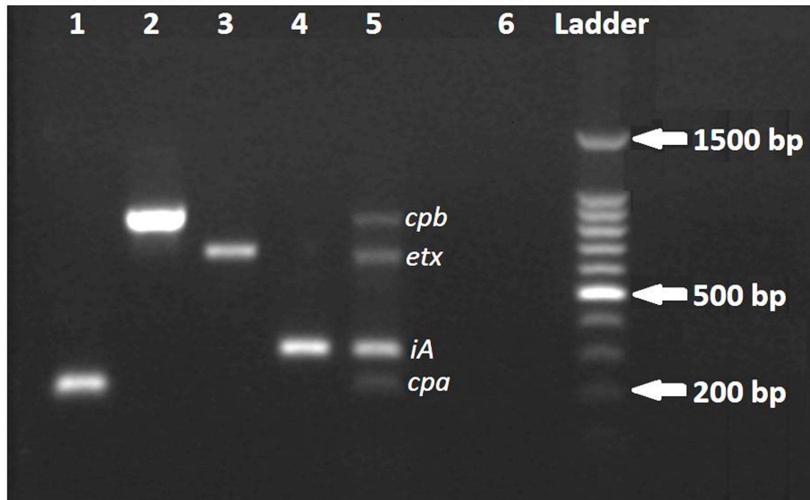

Figure 3.1 Gel photograph of PCR products from Multiplex 1

(Lanes 1-3): *cpa* (230bp), *cpb* (868bp), and *etx* (680bp) were successfully detected in ATCC 3626. (Lane 4): *ia* (319bp) was successfully detected in ATCC 27324. (Lane 5): Multiplex containing template from ATCC 3626 and ATCC 27324 successfully detected all toxin genes used for typing *C. perfringens*. (Lane 6): Negative control (no template).

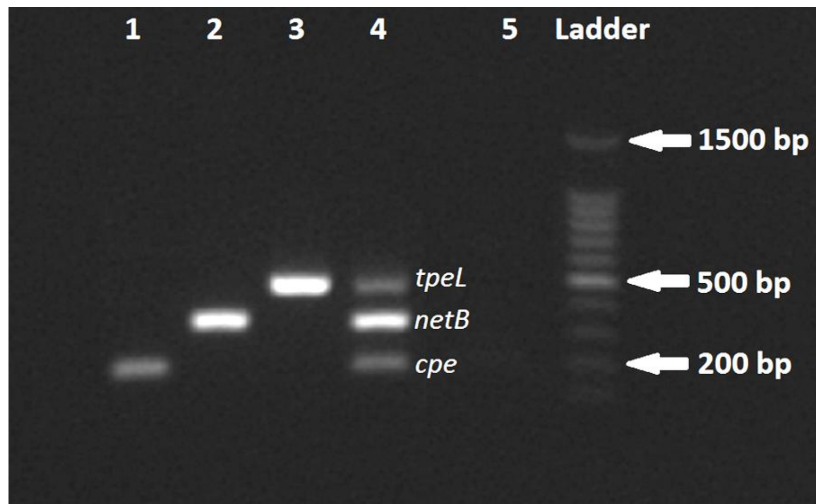

Figure 3.2 Gel photograph of PCR products from Multiplex 2

(Lane 1) *cpe* (169bp) was successfully detected in B-23850. (Lanes 2-3) *netB* (316bp) and *tpeL* (466bp) were successfully detected in isolate JGS-1870. (Lane 4) Multiplex containing template from B-23850 and JGS-1870 successfully detected all three toxin genes of interest. (Lane 5) Negative control (no template).

CHAPTER 4:

Toxin genotyping of *Clostridium perfringens* strains isolated from poultry disease outbreaks and poultry litter using multiplex PCR

This chapter is written as a paper to be submitted to The Journal of Poultry Science

ABSTRACT The discovery of novel toxins produced by *Clostridium perfringens*, such as NetB and TpeL, has created some questions as to their importance in the pathogenesis of disease. It has been shown in some isolates that the presence of the *netB* gene is necessary for the pathogenesis of necrotic enteritis and that α-toxin is not an essential virulence factor for these strains. However, despite its apparent importance, there is little data demonstrating the prevalence of this gene in outbreaks of necrotic enteritis in the United States and there is no data showing how often *netB* appears in *C. perfringens* isolated from poultry litter. Although current evidence supports that TpeL is a lesser toxin and does not play a crucial role in disease, the prevalence of the gene encoding this toxin is also largely unknown. In this study, 49 isolates from outbreaks of necrotic enteritis, 29 from outbreaks of gangrenous dermatitis, and 160 from poultry litter samples were toxin genotyped by the polymerase chain reaction (PCR). Prior to

genotyping, isolates were analyzed by Gram-stain and biochemical tests. In addition to *netB* and *tpeL*, isolates were also screened for the four major lethal toxin genes *cpa*, *cpb*, *etx*, and *ia* and the enterotoxin gene, *cpe*. Results showed that *netB* was detected in only two necrotic enteritis isolates and were not detected in any gangrenous dermatitis or poultry litter isolates. The *tpeL* gene was detected in one necrotic enteritis isolate and one poultry litter isolate. The PCR assay failed to detect α-toxin in three of the isolates, suggesting that these were not *C. perfringens*. Almost all other isolates were identified as *C. perfringens* type A (one was type C) and they were all *cpe* negative. The low indicidence of *netB* (4%) and *tpeL* (2%) within the necrotic enteritis isolates indicated that these two genes are not important virulence factors for the development of necrotic enteritis in a many isolates in Alabama.

INTRODUCTION

Clostridium perfringens¸ the etiological agent of necrotic enteritis and gangrenous dermatitis in poultry, is an important pathogen to the poultry industry. Necrotic enteritis alone has been estimated to cause nearly $2 billion in increased costs to the poultry industry worldwide (Martin and Smyth, 2009). Gangrenous dermatitis, another important disease in poultry, is an economic and animal welfare concern; the United States Animal Health Association's Committee on Transmissible Diseases of Poultry and other Avian Species annually recognizes clostridial dermatitis as a major, global disease issue (Li *et al.*, 2010a,b). Main *C. perfringens* virulence factors include genes for the production of at least 17 different toxins (Bokori-Brown *et al.*, 2011), of which four are known as major lethal toxins. These major lethal toxins include the alpha (α), beta (β), epsilon (ε), and iota (ι)-toxins and isolates of *C. perfringens* have traditionally been grouped into five toxinotypes (A-E) based on which combination of these toxins is produced by a given isolate (Table 2.1). Organisms from each of these toxinotypes have

been asserted to be etiologies of separate pathological effects. For example, type A and, less frequently, type C organisms are associated with necrotic enteritis in poultry (Keyburn *et al.*, 2006; Van Immerseel *et al*., 2009) whereas types B-E are responsible for various enteric diseases of other livestock (Songer, 1997; Songer, 2010).

Pathogenesis of necrotic enteritis by type A *C. perfringens* was originally deduced to be dependent mainly on the production of α-toxin based on two experimental observations: **1)** isolates taken from outbreaks of the disease generally produce large amounts of the toxin and **2)** that crude culture filtrates of these isolates applied by intraduodenal infusion as well as dosing with live broth cultures successfully produced lesions in young chickens (Niilo, 1980; Keyburn *et al.*, 2006). Pathogenesis of gangrenous dermatitis, on the other hand, is attributed to invasion of soft tissues by the organism facilitated by the action of α-toxin in combination with several other toxins of various enzymatic activity. Among these are perfringolysin O (PFO), a hemolysin active against erythrocytes and leukocytes that also exhibits leukostatic activity; κ-toxin, a collagenase; and μ-toxin, a mucin with hyaluronidase activity (Niilo, 1980).

Despite the apparent importance of α-toxin production for most strains of *C. perfringens* that cause necrotic enteritis, some studies have revealed the presence of isolates that produced experimental lesions even after the deletion of almost half of the α-toxin gene (*cpa*), which effectively deactivated the production of functional α-toxin (Keyburn *et al*., 2006). The discovery of a novel toxin, NetB, produced by this same isolate was shown to be a crucial factor in that strain's pathogenicity (Keyburn *et al*., 2008).

The discovery of the TpeL toxin appears to have less of an impact on previous understanding of *C. perfringens* pathology. Although the toxin has some amino acid sequence homology to large clostridial toxins (LCT) produced by *C. difficile*, *C. sordellii*, and *C. novyi*, it

exhibited much lower toxicity in mice (Amimoto *et al.*, 2007). A study by Keyburn *et al.* (2010) showed that only 9% of 44 necrotic enteritis isolates contained the *tpeL* gene, and these isolates were also positive for the *netB* gene. Despite *tpeL*'s apparent infrequency, a recent study has shown that isolates containing both the *netB* and *tpeL* genes may be more virulent to broiler chicks than those that are *tpeL* negative (Coursodon *et al.*, 2012).

Although the implications of these two toxins have been discussed over the past few years, there has been little research determining the prevalence of these genes in *C. perfringens* populations in the United States. Various studies have illustrated a prevalence of *netB*-positive isolates that varies between 58-91% in sampled organisms (Martin and Smyth, 2009; Johansson *et al*., 2010; Keyburn *et al*., 2010), although existing studies in the United States have been limited to isolates sampled in northeastern states (Martin and Smyth, 2009).

In response to this lack of data, a collection of 238 isolates gathered from outbreaks of necrotic enteritis and gangrenous dermatitis as well as from poultry litter samples was analyzed. These isolates were Gram-stained and biochemically tested and were then genotyped by PCR using primers designed for this experiment and *tpeL* specific primers designed by Keyburn *et al*. (2010). In addition to screening for *netB* and *tpeL*, isolates were also screened for genes encoding the major lethal toxins, *cpa*, *cpb*, *etx*, and *ia* and for the enterotoxin gene, *cpe*. Although detection of *etx*, *ia*, and *cpe* was not likely, as these genes are typically associated with non-poultry sources, they were also included. Screening for *netB* and *tpeL* may provide more data describing the prevalence of the *netB* and *tpeL* genes in disease populations as well as environmental populations of *C. perfringens* and hopefully provide more insight into the pathogenesis of *C. perfringens*-related disease in poultry.

MATERIALS AND METHODS

Isolate collection and identification

Isolates originating from cases of necrotic enteritis (49 isolates) and gangrenous dermatitis (29 isolates) were provided by the Mitchem-Sparks Regional Diagnostic Laboratory located in Boaz, AL. These isolates were identified by this diagnostic lab as *C. perfringens* by production of double-zone hemolysis on Difco Blood Agar Base (BD Diagnostics, Franklin Lakes, NJ) with 5-10% sheep blood in Alsever's solution (Colorado Serum Co., Denver, CO) and verification by Gram-staining. One hundred sixty isolates were taken from litter grab samples obtained from commercial poultry houses in Alabama using *C. perfringens* selective media. Isolates utilized as positive controls were obtained from the American Type Culture Collection (ATCC) (3626 and 27324), the USDA-ARS Culture Collection (NRRL) (B-23850), and Iowa State University (JGS-1870). All isolates were Gram-stained and biochemically analyzed via the RapID ANA II System (Innovative Diagnostic Systems, Atlanta, GA). Isolates were placed on sterile glass beads in cryotubes containing brain-heart infusion broth (BHI) (BD Diagnostics) with 20% glycerol and stored at -80°C.

DNA extraction

For isolate retrieval, a glass bead was removed from the storage cryotube and placed into cooked meat medium (HiMedia Laboratories Pvt. Ltd., Mumbai, India) which was incubated in a Bactron IV Anaerobic Environmental Chamber (Shel Lab, Cornelius, OR) containing an atmosphere of 5% CO_2, 5% H_2, and 90% N_2 for 24 hours at 37°C. After enrichment, isolates were streaked onto Trypticase Soy Agar with 5% Sheep Blood (TSA II) (BD Diagnostics) and incubated in the anaerobic chamber for 24 hours at 37°C. A single colony exhibiting double-zone hemolysis was then transffered into BHI and incubated anaerobically at 37°C for 24 hours. DNA

was then extracted from the resulting bacterial cells using a Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI). Extracted DNA was tested for concentration and purity with a Nanodrop 1000 Spectrophotometer (Thermo Scientific Inc., Bremen, Germany) according to the manufacturer's instructions. Standards for purity were a 260nm/280nm ratio between 1.8-2.0.

Detection of toxin genes

Detection of the genes *cpa*, *cpb*, *etx*, *ia*, *cpe*, and *netB* were carried out using sets of primers designed for this experiment (Table 4.1). Primers were designed using the primer-Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology (NCBI) website. The primer set detecting *tpeL* was from a previously published study (Keyburn *et al.*, 2010). Detection of the seven genes was performed using two multiplex PCRs and one single loci amplification. Parameters for multiplex 2 and the single loci amplification were identical except that the single PCR only contained the *tpeL* primers. The two multiplex protocols were as follows:

Multiplex 1

- Reagent concentrations
  - Master mix 1.17x concentration (14µL per 25µL reaction)
    - Taq: 1.4 units
    - dNTP: 224µM each
    - $MgCl_2$: 1.68mM
  - Template: 1µg per 25µL
  - Primers
    - cpaF: 0.2µM
    - cpaR: 0.2µM
    - cpbF: 0.5µM
    - cpbR: 0.5µM
    - etxF: 1.0µM
    - etxR: 1.0µM
    - iaF: 0.2µM
    - iaR: 0.2µM
- Cycle parameters
  - Initial denaturing: 95°C for 5 minutes
  - 15 Cycles Touchdown PCR
    - Denaturing: 95°C for 30 seconds
    - Annealing: 65-50°C for 30 seconds
    - Elongation: 72°C for 30 seconds
  - 25 Cycles
    - Denaturing: 95°C for 30 seconds
    - Annealing: 50°C for 30 seconds
    - Elongation: 72°C for 30 seconds
  - Final elongation: 72°C for 6 minutes

Multiplex 2

- Reagent concentrations
  - Master mix 1x concentration (12µL per 25µL reaction)
    - Taq: 1.2 units
    - dNTP: 192µM each
    - $MgCl_2$: 1.44mM
  - Template: 1µg per 25µL
  - Primers
    - cpe4F: 0.2µM
    - cpe4R: 0.2µM
    - netB5F: 0.2µM
    - netB5R: 0.2µM
- Cycle parameters
  - Initial denaturing: 95°C for 5 minutes
  - 40 Cycles
    - Denaturing: 95°C for 30 seconds
    - Annealing: 55°C for 30 seconds
    - Elongation: 72°C for 30 seconds
  - Final elongation: 72°C for 6 minutes

Gel electrophoresis and verification of amplified products

PCR products were separated by gel electrophoresis at 100V in a 2% agarose gel for one hour or until sufficient migration of bands was reached. A commercial running buffer, AccuGENE TBE (Lonza), was used at 1x concentration. 1µL ethidium bromide solution (10mg/mL) was added to the molten agarose for DNA visualization. Four bands representing the *cpa* amplicon were excised and purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). All bands representing any other amplicons were also excised and purified. The purified DNA was then analyzed with a nanodrop spectrophotometer before sending to the Auburn University Genomics and Sequencing Laboratory for sequencing. Sequences were then subjected to a nucleotide BLAST search to determine the likelihood that each amplicon was replicated from the target gene.

RESULTS

Isolate identification

All isolates were morphologically consistent with *C. perfringens* (Gram-positive rods) except for two isolates (250-4 and 259-14) which were Gram-negative rods. All but 18 of the disease-originating isolates were biochemically verified to be *C. perfringens*. One of these 18 isolates was inadequately identified as *C. limosum* (72.88%) while the other 17 were identified as *C. novyi* (99%). Three poultry litter isolates, including the Gram-negative isolates, were identified as *C. bifermentans* (232-3), *Bacteroides caccae* (259-14), and a *Wolinella spp*. (250-4). In addition, 40 (25%) of the *C. perfringens* isolates obtained from poultry litter samples did not produce any hemolytic activity on TSA II after incubation up to 72 hours at 37°C under anaerobic conditions (Figure 4.1A).

Isolate genotyping results

Results of PCR genotyping for 15 isolates are summarized in Table 4.2. Overall results revealed that all of the disease-originating isolates were positive for *cpa*, including those that were biochemically identified as *C. novyi* and *C. limosum*. Most isolates were negative for other major toxin genes, making them type A *C. perfringens.* The 3 poultry litter isolates identified as separate species were negative for all 7 genes. One isolate taken from a litter sample (C12), was positive for *cpb* as well as *cpa*, making it type C (Figure 4.2). This isolate was also positive for *tpeL* (Figure 4.4). Only 2 (4%) of the isolates taken from cases of necrotic enteritis were positive for *netB* (Figure 4.3); one of which was also positive for *tpeL* (Figure 4.4).

Sequencing results

Gel electrophoresis bands representing the *cpa* amplicon from four isolates (384-11, 384-12, 384-17, 384-20) were excised, purified, and sequenced. Other bands excised and purified for sequencing included those representing the *cpb* and *tpeL* amplicons from isolate C12, those representing the *netB* amplicon from isolate C103-99, and the *netB* and *tpeL* amplicons from isolate C103-100. The BLAST searches for all sequenced PCR products had hits showing at least 90% max identity to the target sequence with significantly low E-values except for the *tpeL* product from isolate C12, which had a top significant hit of 83% max identity (Table 4.3).

DISCUSSION

Although all disease-related isolates were obtained from one of the Alabama State Diagnostic Labs, which identified them as *C. perfringens*, 17 of these isolates matched a biochemical profile consistent with *C. novyi* and one was inadequately described as *C. limosum* when tested with the RapID ANA II system. It is possible that the isolates were misidentified by the diagnostic lab, however it is important to note that the probable species identity determined

by the commercial biochemical test is calculated using a database compiled from analyzing the metabolic profiles of bacteria of human clinical significance. Research has shown that when used to identify clinical isolates, this commercial test is relatively accurate, correctly identifying 78% of 566 isolates when paired with recommended additional tests (Marler *et al*., 1991). No data could be found evaluating the RapID ANA II system's ability to identify isolates of veterinary origin. However, the manufacturer did provide a warning in the instruction booklet underlining the test's limitations with veterinary isolates. The first-generation version of this test was evaluated by Adney and Jones (1985), resulting in a much lower rate of success with isolates of veterinary origin. In that study, only 59.6% of 183 isolates were identified, including 14.2% that were misidentified at the genus level and 4.4% that could not be identified. Because of these possible limitations regarding veterinary isolates, it is conceivable that these isolates were indeed *C. perfringens*, but were misidentified by the test. Genotyping evidence supports this conclusion, as the *cpa* gene was detected in these 18 isolates and no other bacterial species is known to have the *cpa* gene. Although *C. novyi* does produce a toxin with phospholipase C activity (*C. novyi* γ-toxin), it shares only 58% amino acid sequence homology and 69% nucleotide sequence homology with α-toxin (Tsutsui *et al*., 1995). Additionally, when *cpa* primers were designed, BLAST output failed to predict any products on potentially unintended templates. Indeed, a primer search was performed using the γ-toxin gene as a template with cpaF and cpaR as the forward and reverse primers. This search showed that the specified forward primer (cpaF) could not be found in the template, further validating the specificity of these primers. Therefore, it is reasonable to conclude that the diagnostic lab correctly classified these isolates as *C. perfringens* by the production of double-zone hemolysis and Gram-staining.

Out of the 160 poultry litter isolates, 40 did not show any hemolytic activity on TSA II. This does not necessarily preclude the classification of these isolates as *C. perfringens*, however, since naturally non-hemolytic strains of the organism have been isolated in the past (Schoepe *et al.*, 2001). In any case, these 40 isolates all tested biochemically as *C. perfringens* and PCR genotyping also detected *cpa* in all 40, indicating that they were of this species. Three poultry litter isolates tested biochemically as distinctive species and did not produce hemolysis on blood agar. These three also tested negative for *cpa*, providing more evidence that they undoubtedly were not *C. perfringens*.

Out of the necrotic enteritis derived isolates, all were genotyped as type A *cpe*-negative organisms. This is not surprising, as most documented cases of this particular disease have been associated with type A *C. perfringens* (Keyburn *et al.*, 2006; Van Immerseel *et al.*, 2009). All gangrenous dermatitis isolates were also genotyped as type A, which is the dominant type associated with this disease (Li *et al.*, 2010a,b). For the poultry litter isolates, all but one was type A. Once again, this was not unexpected, because type A organisms are the most common type associated with chickens. Assuming chickens living on the litter would be a major source of the *C. perfringens* population in this environment, it would also be the largest environmental representative of *C. perfringens* in the litter. One isolate from poultry litter was type C, which is also associated with poultry, although less frequently. Thus, it is consistent with the results of past studies that it would be isolated only rarely from a poultry environment.

The *netB* gene was detected in only two of the 238 isolates. Both of them, C103-99 and C103-100, were from separate cases of necrotic enteritis in chickens. As a percentage of the 49 total necrotic enteritis isolates in this collection, the *netB*-positive genotype had a much lower representation (4% *netB*-positive) than what was previously reported (58-91% *netB*- positive)

(Martin and Smyth, 2009; Johansson *et al*., 2010; Keyburn *et al*., 2010). Likewise, *tpeL* was only detected in two of the isolates: one from necrotic enteritis (C103-99) and one from poultry litter (C12). This representation of *tpeL* in necrotic enteritis isolates (2%) appears to be consistent with previous studies, which detected *tpeL* with low frequency as well (9%) (Keyburn *et al*., 2010). These genotyping results imply that *netB* is not an important virulence factor for some *C. perfringens* populations. Results also support existing evidence that *tpeL* is not an essential virulence factor.

The traditional model describing the pathology of necrotic enteritis involves the excessive propagation of *C. perfringens* naturally occuring as gut microflora. This imbalance is thought to be triggered by several possible dietary factors. Inclusion of feed ingredients such as wheat, barley, or DDGS, which contain high amounts of poorly-digestible carbohydrates increases the viscosity of digesta, slowing down its passage and providing more time for newly introduced *C. perfringens* to colonize the gut. High inclusion levels of animal protein sources, such as fish meal, is thought to provide ideal substrates for *C. perfringens*, leading to massive propagation. Additionally, damage to the intestinal mucosa by coccidiosis may release plasma proteins into the gut, providing excellent substrates for *C. perfringens* propagation. However, with the discovery of the *netB* gene, this model as the exclusive pathology has been challenged. In previous studies, a majority of isolates from diseased birds contained the *netB* gene (Martin and Smyth, 2009; Johansson *et al*., 2010; Keyburn *et al*., 2010), which was shown to be an essential virulence factor for certain isolates (Keyburn *et al*., 2008). Additionally, this same gene has been detected in very few strains isolated from healthy birds (Keyburn *et al*., 2010). From these observations, it has been suggested that lesions are induced when clonal populations containing the *netB* gene overwhelm the commensal population. Essentially, this asserts that

disease is not related to the increase in the total *C. perfringens* population, but only to the propagation of certain clones within the population.

The results of this present study support the traditional model of necrotic enteritis pathology, as *netB*-positive isolates were infrequent within this collection, even among those isolated from disease cases. The *netB* gene may be an important virulence factor for some isolates. However, the fact that 96% of necrotic enteritis-originating isolates were negative for *netB* indicates that the gene's importance is limited to certain strains. As the particular isolates tested in this study were all from Alabama, it is clear that the implications of the data are limited to that particular area. Although this study provided data on a previously unrepresented region, more research on isolates from diverse locations is needed to determine the importance of both *netB* and *tpeL* on a worldwide scale.

Table 4.1 Specifications of primers used in multiplex and single loci PCR

Primer ID	Sequence	Target Gene	Reported Tm	Product Length
cpaF[1]	GCAGCAAAGGTAACTCTAGCTAACT	*cpa*	62.9	230
cpaR[1]	CCTGGGTTGTCCATTTCCCATT		62.7	
cpbF[1]	ACGGATGCCTATTATCACCAACTTT	*cpb*	61.3	868
cpbR[1]	TGTCCTACCCAGTTAGCACCAT		62.7	
etxF[1]	CGCATCAGCGGTGATATCCAT	*etx*	62.6	680
etxR[1]	TCTCTCCCCATTCACTTCCACTT		62.8	
iaF[1]	CGATGAAAAGCCTACACCACTACTT	*ia*	62.9	319
iaR[1]	TGCGGTATATCCTCCACGCA		62.4	
cpe4F[2]	GGCGTTCTTCTAACTCATACCCT	*cpe*	62.8	169
cpe4R[2]	ACTCCATCACCTAAGGACTGTT		60.8	
netB5F[2]	CGCTTCACATAAAGGTTGGAAGGC	*netB*	64.6	316
netB5R[2]	TCCAGCACCAGCAGTTTTTCCT		62.7	
AKP80[3]	ATATAGAGTCAAGCAGTGGAG	*tpeL*	58.7	466
AKP81[3]	GGAATACCACTTGATATACCTG		58.9	

[1] Primers used in Multiplex 1

[2] Primers used in Multiplex 2

[3] Primers used in single loci PCR

Table 4.2 Genotyping results from the analysis of 15 out of 238 isolates of *C. perfringens*

Isolate	Toxin genes present							Hemolysis	Source
	cpa	*cpb*	*etx*	*ia*	*cpe*	*netB*	*tpeL*		
C103-7	+	-	-	-	-	-	-	+	Necrotic enteritis
C103-8	+	-	-	-	-	-	-	+	Necrotic enteritis
C103-9	+	-	-	-	-	-	-	+	Necrotic enteritis
C103-99	+	-	-	-	-	+	+	+	Necrotic enteritis
C103-100	+	-	-	-	-	+	-	+	Necrotic enteritis
C103-46	+	-	-	-	-	-	-	+	Gangrenous dermatitis
C103-47	+	-	-	-	-	-	-	+	Gangrenous dermatitis
C103-48	+	-	-	-	-	-	-	+	Gangrenous dermatitis
C103-49	+	-	-	-	-	-	-	+	Gangrenous dermatitis
C103-50	+	-	-	-	-	-	-	+	Gangrenous dermatitis
C12	+	+	-	-	-	-	+	+	Poultry litter
228-2	+	-	-	-	-	-	-	-	Poultry litter
236-4	+	-	-	-	-	-	-	-	Poultry litter
250-1	+	-	-	-	-	-	-	-	Poultry litter
301-18	+	-	-	-	-	-	-	+	Poultry litter

Table 4.3 BLAST results for sequenced products from genotyping PCRs

Target gene	Isolate	Highest significant sequence to PCR product	E-value	Max Identity (%)
cpa	384-11	*C. perfringens* strain Z3 alpha toxin gene, partial cds	$4x10^{-92}$	99
cpa	384-12	*C. perfringens* strain RT40 alpha toxin gene, partial cds	$1x10^{-87}$	97
cpa	384-17	*C. perfringens* strain PLCO80612 alpha-toxin (plc) gene, partial cds	$2x10^{-75}$	96
cpa	384-20	*C. perfringens* strain PLCO80612 alpha-toxin (plc) gene, partial cds	$8x10^{-94}$	99
cpb	C12	*C. perfringens* type B beta-toxin gene, complete cds	0	99
netB	C103-99	*C. perfringens* strain 200302-1-1-Ba necrotic enteritis toxin B (netB) gene, complete cds	$6x10^{-142}$	99
netB	C103-100	*C. perfringens* strain 200302-1-1-Ba necrotic enteritis toxin B (netB) gene, complete cds	$5x10^{-138}$	98
tpeL	C103-99	*C. perfringens* A strain CP4 TpeL (tpeL) gene, complete cds	$4x10^{-175}$	93
tpeL	C12	*C. perfringens* B strain CN1795 plasmid TpeL (tpeL) gene, partial cds; conserved hypothetical protein and transposase genes, complete cds; and beta toxin (cpb) gene, partial cds	$6x10^{-74}$	83

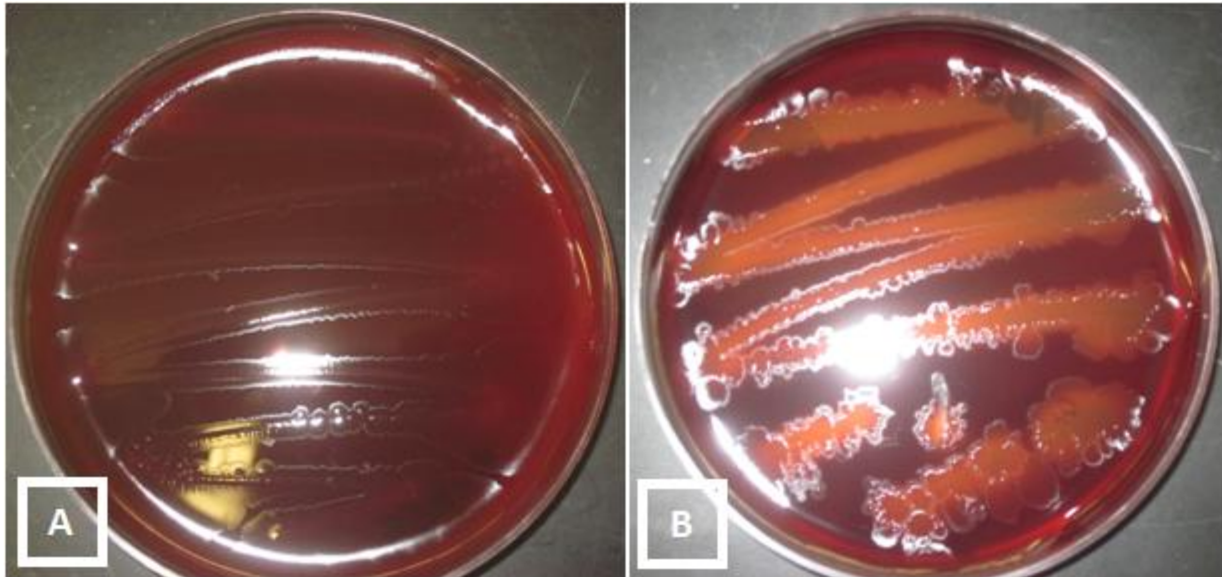

Figure 4.1 Comparative hemolytic properties of environmental and disease-originating *C. perfringens* isolates

Both A and B show two different isolates grown under the following identical conditions: enriched in cooked meat medium (HiMedia) at 37°C for 24 hours anaerobically (5% CO_2, 5% H_2, 90% N_2) and then streaked onto TSA II and incubated at 37°C for 72 hours anaerobically. (A): Isolate 332-2, taken from a poultry litter sample, shows no hemolytic activity after 72 hours. (B): Isolate C103-100, taken from a case of necrotic enteritis in poultry, shows complete hemolysis after 72 hours.

Figure 4.2 Gel electrophoresis photograph of Multiplex 1 genotyping results

(Lanes 1-5): Correspond respectively to isolates C103-7, C103-8, C103-9, C103-99, and C103-100 which were taken from separate cases of necrotic enteritis in chickens. Only *cpa* was detected in these isolates, making them type A *C. perfringens*. (Lanes 6-10): Correspond respectively to isolates C103-46, C103-47, C103-48, C103-49, and C103-50 which were taken from separate cases of gangrenous dermatitis in chickens. These were also type A. (Lanes 11-15): Correspond respectively to isolates C12, 228-2, 236-4, 250-1, and 301-18 which were taken from poultry litter samples. C12 was positive for *cpa* and *cpb*, making it a type C organism. All others were type A. (Lane 16): Negative control containing no template. (Lane 17): Positive control containing a mixture of DNA from ATCC 3626 and ATCC 27324.

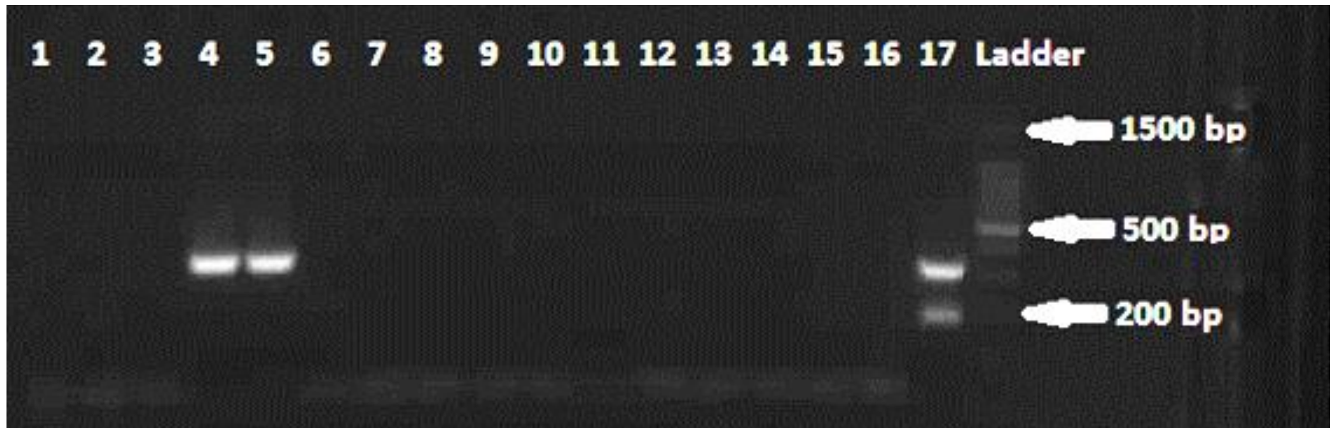

Figure 4.3 Gel electrophoresis photograph of Multiplex 2 genotyping results

All isolates were negative for *cpe*. (Lanes 1-5): Correspond respectively to isolates C103-7, C103-8, C103-9, C103-99, and C103-100 taken from separate cases of necrotic enteritis in chickens. C103-99 and C103-100 were found positive for the *netB* gene. (Lanes 6-10): Correspond respectively to isolates C103-46, C103-47, C103-48, C103-49, and C103-50 taken from separate cases of gangrenous dermatitis; none were positive for *netB*. (Lanes 11-15): Correspond respectively to isolates C12 , 228-2, 236-4, 250-1, and 301-18 taken from poultry litter samples. None of these were positive for either gene. (Lane 16): Negative control lacking template. (Lane 17): Positive control containing DNA from isolates B-23850 and JGS-1870.

Figure 4.4 Gel electrophoresis photograph of single loci detection of *tpeL*

(Lanes 1-5): Correspond respectively to isolates C103-7, C103-8, C103-9, C103-99, and C103-100 which were taken from separate cases of necrotic enteritis in chickens. Only C103-99 from this group was positive for *tpeL*. (Lanes 6-10): Correspond respectively to isolates C103-46, C103-47, C103-48, C103-49, and C103-50 which were taken from separate cases of gangrenous dermatitis in chickens. None were *tpeL* positive. (Lanes 11-15): Correspond respectively to isolates C12, 228-2, 236-4, 250-1, and 301-18 which were taken from poultry litter samples. C12 was positive for *tpeL*. All others were negative. (Lane 16): Negative control containing no template. (Lane 17): Positive control containing DNA from JGS-1870.

CHAPTER 5:

General Discussion and Conclusions

The work presented in this thesis has provided additional information regarding the uniformity of *C. perfringens* toxinotypes within a poultry environment. Specifically, the observation of type A, *cpe* negative organisms as the major representative in cases of necrotic enteritis and gangrenous dermatits has been further substantiated. From a more novel perspective, the prevalence of toxin genes *netB* and *tpeL* within populations of *C. perfringens* has been determined within a collection of isolates from Alabama poultry farms, increasing the geographical area within which the incidence of these genes can be described. Additionally, novel PCR primers specific for six *C. perfringens* toxin encoding genes (*netB*, *cpe*, *cpa*, *cpb*, *etx*, and *ia*) were reported (the primer set used to detect the *tpeL* gene was described previously by Keyburn *et al*., (2010)) and the protocols for two multiplex PCR reactions detecting these genes in reference strains ATCC 3626, ATCC 27324, B-23850, and JGS-1870 were described. Unfortunately, due to time constraints, multiplex 2 (which detected *netB*, *tpeL*, and *cpe*) was unable to be optimized for isolates other than reference strains. Various non-specific products (NSPs) were produced inconsistently when this multiplex was utilized with other isolates, resulting in the need to use two reactions: a multiplex detecting two genes (*netB* and *cpe*) and a single loci amplification of the *tpeL* amplicon.

One explanation for the difficulty in the optimization of this multiplex with other *C. perfringens* isolates is similar to attempts at creating the single multiplex described in Chapter 2, namely the use of a commercial master mix to provide buffer, Taq, dNTPs, and $MgCl_2$. This resulted in the inability to adjust the concentrations of these reagents independently. For instance, changing the buffer concentration can allow for the adjustment of salt levels without impacting $MgCl_2$, which must be at a concentration that allows for the optimal functionality of Taq. In the event of NSP formation, as was the case in this study, it can be beneficial to adjust salt concentration up or down considering the size of the NSPs. Decreasing the Taq concentration can reduce the incidence of NSPs by lowering the probability of stray Taq molecules replicating short-lived, non-specific primer-DNA associations. Finally, increasing the $MgCl_2$ concentration has been shown to increase the specificity of a reaction (Henegariu *et al.*, 1997), although this might require a simultaneous reduction in buffer to keep salt concentrations from becoming too high.

An additional complication that arose regarded 17 of the necrotic enteritis isolates, identified previously by an Alabama State Veterinary Diagnostic Lab as *C. perfringens*, which were biochemically identified by previous lab workers as *C. novyi*. This could merely be an artifact introduced by the limitations of the Electronic RapidID Compendium (ERIC), a database compiled from the analysis of human clinical isolates. As the isolates analyzed in this study were of veterinary origin, it is possible that a misinterpretation arose due to a lack of data regarding isolates of this nature. Furthermore, these isolates were all genotyped as type A, meaning they tested positive for the *cpa* gene encoding α-toxin, a *C. perfringens* toxin with phospholipase C activity. This makes it even less likely that the biochemical tests were correct. Although *C. novyi* γ-toxin shares limited amino acid and nucleotide sequence homology with α-toxin (58% and

69%, respectively) (Tsutsui *et al.*, 1995), Basic Local Alignment Search Tool (BLAST) output failed to predict any products on potentially unintended templates. For further verification of the PCR's specificity, a primer search was performed using the γ-toxin gene as a template with cpaF and cpaR as the forward and reverse primers. As a result, BLAST could not detect cpaF on the template, indicating the specificity of the cpa primer set for *cpa*. Despite these results, no *C. novyi* reference strains were present in the available collection, so empirical tests of this assumption have yet to be performed.

An important result of this research involved the prevalence of *netB* within the isolate collection. Since the discoveries of *netB* and *tpeL*, previous research has described a low prevalence (around 9%) of *tpeL* in *C. perfringens* isolated from cases of necrotic enteritis (Keyburn *et al.*, 2010). Results from this research supported those findings, as only one necrotic enteritis-associated isolate in the collection tested positive for *tpeL*. Previous studies regarding *netB*, however, described a much higher frequency of the gene. Between 58-91% of strains isolated from cases of necrotic enteritis have been reported to be positive for *netB* (Martin and Smyth, 2009; Johansson *et al.*, 2010; Keyburn *et al.*, 2010), whereas only 4% of disease-isolates genotyped in this study had the gene.

Reasons for this discrepancy are unclear; however, there are a few factors that may be important. Most isolates analyzed in previous studies were from European countries, Australia, and Canada. Differences in management practices between those countries and the United States could possibly introduce different selective pressures, resulting in the greater survival of different genotypes of *C. perfringens*. For example, these regions differ in the major ingredients used in poultry feed; namely, in the United States, corn serves as the primary ingredient, whereas these other countries primarily use wheat (Blair, 2008). It is known that poultry diets high in

non-starch polysaccharides, such as wheat-based diets, are correlated with higher incidences of necrotic enteritis (Ficken and Wages, 1997a; Annett *et al.*, 2002; Williams *et al.*, 2003). Since the *netB*-positive genotype has been associated with most cases of disease in countries which provide wheat-based diets, and this study has found that genotype far less frequently in birds fed a corn-based diet, it could be possible that a wheat diet provides some mechanism of selection for *netB*-positive organisms, thereby changing the balance of the *C. perfringens* population in favor of this genotype.

Another possible factor in the discrepancy between *netB* prevalence observed in this study and the prevalence reported by past research could be related to the length of storage time the isolate collection was subjected to. Isolates were obtained over 15 years ago and have been stored at -80°C since this time. One possibility is that the *netB* positive isolates described in previous studies belong to a relatively new population that has arisen due to selective pressure, perhaps recent changes in management practices or feed formulations that may affect gut microflora.

These explanations for the comparatively low incidence of *netB* genotypes in this study are speculative. Currently, this data appears to support the accepted model of necrotic enteritis pathogenicity: that an increase in total *C. perfringens* population in the intestine leads to disease. Furthermore, the data presented in this thesis supports the non-necessity of *netB* as a virulence factor for most necrotic enteritis-originating isolates; however, additional research must be done to increase the analysis of *netB* prevalence in the United States and other countries. Another unaddressed question regards the possibility of increased virulence in isolates that are positive for both *netB* and *tpeL* (Coursodon *et al.*, 2012), although the low incidence of *tpeL* reported thus

far, including results in this study, indicates that this may not be a significant factor in overall poultry disease pathogenicity.

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