INTRODUCTION

The human population is projected to grow to 9 to 10 billion by the year 2050 (Godfray et al., 2010). As a consequence of the population explosion, food animal and poultry production must confront a new array of challenges. Among these are global food security, climate change, emerging infectious diseases, a regulatory ban on antimicrobials, high-density production conditions, and waste management (Grasty, 1999; Turnpenny et al., 2001; Bohannon, 2004). Approximately 71 million tons of poultry meat were produced worldwide in 2009 (USDA-FAS, 2009). To ensure continuity in the supply of poultry food products, effective control measures against infectious diseases in the framework of environmental change are critical (Dekich, 1998).

In the United States, necrotic enteritis (NE) and Eimeria coccidiosis are among the most important infectious diseases in chickens (Shane, 2004a,b; Smith and Helm, 2008). The etiologic agent of NE is Clostridium perfringens, a gram-positive, anaerobic, spore-forming bacterium. To accomplish these goals, better understanding of host- and environmentally related factors on the development of NE and potential vaccination strategies against C. perfringens infection will be necessary. Furthermore, a reliable and reproducible NE disease model is needed for characterization of C. perfringens pathogenesis and host protective immunity. This review summarizes recent developments in NE disease models, pathogenesis, host immunity, risk factors, and vaccine development for C. perfringens-associated NE in poultry.

ABSTRACT

The increasing trends of legislative restrictions and voluntary removal of antibiotic growth promoters worldwide has already affected, and will continue to affect, poultry production and animal health. Necrotic enteritis (NE) is being considered among the most important infectious diseases in the current poultry production system globally, with an estimated annual economic loss of more than $2 billion, largely attributable to medical treatments and impaired growth performance. Thus, there is an urgent need to develop rational, alternative, and integrated management strategies not only to control NE, but also to prevent it. In both humans and many warm-blooded animals and birds, NE is caused by Clostridium perfringens, a gram-positive, anaerobic, spore-forming bacterium. To accomplish these goals, better understanding of host- and environmentally related factors on the development of NE and potential vaccination strategies against C. perfringens infection will be necessary. Furthermore, a reliable and reproducible NE disease model is needed for characterization of C. perfringens pathogenesis and host protective immunity. This review summarizes recent developments in NE disease models, pathogenesis, host immunity, risk factors, and vaccine development for C. perfringens-associated NE in poultry.

Key words: host immunity, necrotic enteritis, poultry, vaccine

INTRODUCTION

The human population is projected to grow to 9 to 10 billion by the year 2050 (Godfray et al., 2010). As a consequence of the population explosion, food animal and poultry production must confront a new array of challenges. Among these are global food security, climate change, emerging infectious diseases, a regulatory ban on antimicrobials, high-density production conditions, and waste management (Grasty, 1999; Turnpenny et al., 2001; Bohannon, 2004). Approximately 71 million tons of poultry meat were produced worldwide in 2009 (USDA-FAS, 2009). To ensure continuity in the supply of poultry food products, effective control measures against infectious diseases in the framework of environmental change are critical (Dekich, 1998).

In the United States, necrotic enteritis (NE) and Eimeria coccidiosis are among the most important infectious diseases in chickens (Shane, 2004a,b; Smith and Helm, 2008). The etiologic agent of NE is Clostridium perfringens, a gram-positive, anaerobic, spore-forming bacterium that is transmitted by the fecal-oral route as well as through contaminated feed, water, housing structures, and insects (Titball et al., 1999; Wages and Opengart, 2003; Williams et al., 2003; Van Immerseel et al., 2004; Songer and Uzl, 2005; Williams, 2005; Keyburn et al., 2006). Globally, the economic loss attributable to avian NE is estimated to cost the US $2 billion annually, largely because of medical treatments and impaired growth performance (Cooper et al., 2009; Van Immerseel et al., 2009). Thus, there is an urgent need to develop rational, alternative, and integrated management strategies not only to control, but also to prevent, both of these diseases (Williams, 2005). A better understanding of host-pathogen, as well as pathogen-pathogen (Clostridium-Eimeria), interactions in poultry will be required to realize these goals.

Necrotic enteritis is difficult to reproduce experimentally with C. perfringens alone, albeit that NE lesion by challenge with C. perfringens alone has been reported (Collier et al., 2008; Park et al., 2008). A variety of factors have been identified that promote the development of experimental NE. These include the C. perfringens strains used to induce the disease, the route, dose, and frequency of bacterial challenge, coinfection with Eime-
rnia or immunosuppressive viruses, dietary factors, and host genetics (Williams et al., 2003; Williams, 2005; Thompson et al., 2006; Park et al., 2008). The present review mainly focuses on NE challenge models and *C. perfringens* vaccines against NE.

**GENERAL OVERVIEW OF C. PERFRINGENS AND NE**

*Clostridium perfringens* grows at temperatures between 15 and 50°C and forms endospores that can tolerate 100°C for 2 h. The bacterial generation time is less than 20 min at 33 to 49°C, and it is difficult to prepare spore-free poultry feed by standard processes (Brynestad and Granum, 2002; Williams, 2005). Necrotic enteritis is not a new disease and has been partially controlled by ionophore anticoccidials and antibiotic growth promoters. In some regions, including in the United States and Asia, both ionophore anticoccidials and antibiotic growth promoters are still used together. Necrotic enteritis has reemerged as a significant problem as a result of restrictions on the use of in-feed antibiotics, modern practices of high-density housing conditions, and the reuse of litter (Van Immerseel et al., 2004; Williams, 2005). Broiler mortality attributable to NE is usually between 2 and 10%, but can be as high as 50%. In layers, mortality has been reported to be approximately 6.5% (Dhillon et al., 2004). Subclinical infection leads to decreased feed intake, which adversely affects growth rate, feed conversion, and flock uniformity (McDevitt et al., 2006). Subclinical NE associated with hepatitis or cholangiohepatitis is often found in broilers at processing, and liver condemnation frequencies may vary between approximately 0.02 to 0.20% (Lovland and Kaldhusdal, 1999).

**C. PERFRINGENS TOXINS**

Seventeen exotoxin- or enterotoxin-encoding genes have been identified (Songer, 1996). Bacterial type classification (A, B, C, D, or E) is based on toxin production, and *C. perfringens* type A is the most common cause of NE in broiler chickens (Engström et al., 2003; Siragusa et al., 2006; Crespo et al., 2007; Cooper and Songer, 2009). α-Toxin, a zinc metalloenzyme with phospholipase, sphingomyelinase, and hemolytic activities, is produced by all 5 types of *C. perfringens* strains without evidence of NE. α-Toxin is thought to be a major virulence factor of the *C. perfringens* strains developed NE and that diseased birds had an accumulation of α-toxin in the gut. This latter study reconfirms the role of α-toxin in the pathogenesis of NE, which needs further investigation.

In addition to α-toxin, it has been proposed that the NE B-like (NetB) toxin is primarily associated with *C. perfringens* type A strains isolated from NE-affected broilers (Keyburn et al., 2008; Van Immerseel et al., 2009). The NetB shares 35% sequence similarity with *C. perfringens* β-toxin and 31% with *Staphylococcus aureus* α-toxin. Martin and Smyth (2009) investigated 12 *C. perfringens* isolates from clinical NE chickens and reported that 7 were NetB positive and 5 were NetB negative. Necrotic enteritis B-like toxin is also present in *C. perfringens* field isolates recovered from chickens without evidence of NE. *Clostridium perfringens* type A also produce β2-toxin, which has been isolated from NE-affected and healthy chickens, as well as from piglets with gastrointestinal disease (Engström et al., 2003; Waters et al., 2003; Gholamiandekhordi et al., 2006; Siragusa et al., 2006; Crespo et al., 2007; Cooper and Songer, 2010), although the role of β2-toxin in NE has been questioned (Crespo et al., 2007). In addition to these toxins, other hydrolytic enzymes (Shimizu et al., 2002; Myers et al., 2006), unknown toxins, or both may contribute to the complex pathogenesis of NE. Indeed, the recent seminal paper by Lepp et al. (2010) provided considerable insight into the disease, suggesting that NE is caused by several novel virulence factors whose genes are clustered on pathogenicity loci, some of which are plasmid born.

**EXPERIMENTAL MODELS OF NE**

*C. perfringens* Isolates in Experimental NE

A critical factor in the development of experimental NE is use of the appropriate virulent strains of *C. perfringens*. In general, pathogenic *C. perfringens* strains for reproducing experimental NE are selected based on the in vivo studies, using the direct challenge of the strain. In most experimental models, these bacterial strains have been isolated from clinical NE outbreaks. However, not all NE isolates are effective in reproducing disease (Kaldhusdal et al., 1999; Barbara et al., 2008; Cooper and Songer, 2009). Recently, Timbermont et al. (2009) tested a variety of *C. perfringens* strains recovered from healthy or NE-affected chickens, and from cases of calf hemorrhagic enteritis, for their ability to cause experimental disease. α-Toxin-producing bacterial strains from apparently healthy birds or from birds with hemorrhagic enteritis failed to induce NE. By contrast, birds inoculated with 33% of disease isolates developed NE-specific intestinal lesions. Chalmers et al. (2007) compared 5 NE field strains of *C. perfringens* (SNECP43, SNECP44, SNECP47, SNECP49, and SNECP50) and observed that birds inoculated with SNECP50 exhibited reduced BW gain, increased
gut lesion scores, and greater mortality compared with those infected with the other isolates or with uninfected controls. Experimental NE with the presence in the gut of specific lesions has been produced using single isolates (Thompson et al., 2006; Gholamiandehkordi et al., 2007) or combinations of multiple strains (McReynolds et al., 2007; Park et al., 2008; Stringfellow et al., 2009). Overall, Lepp et al. (2010) attributed the difficulty in disease production to the plasmid-born nature of pathogenic loci of C. perfringens. However, the presence of a well-known toxin (i.e., NetB) on C. perfringens strains detected by toxin-specific PCR does not guarantee their pathogenesis in NE.

**Route, Dose, and Frequency of C. perfringens Infection**

The induction of experimental NE has been accomplished by oral gavage, passive inoculation through exposure to C. perfringens-contaminated feed or litter (Hamdy et al., 1983a,b; Keyburn et al., 2006; Chalmers et al., 2007; Si et al., 2007; Barbara et al., 2008; Pedersen et al., 2008), or both. Doses of bacteria and durations of exposure have also differed. Park et al. (2008) used a single dose (1 × 10⁹ cfu/bird) at 26 d posthatch, whereas other investigators have used multiple bacterial doses (10⁶ to 10⁹ cfu/bird) per day for up to 8 d (Dahiya et al., 2005; Olkowski et al., 2006; Barbara et al., 2008; Collier et al., 2008; Mikkelsen et al., 2009; Timbermont et al., 2009). Clostridium perfringens infection initiated as early as 1 d to as late as 26 d posthatch have been reported, with d 17 to 19 and durations of 3 to 4 d being the most frequently used. In any event, NE-specific lesions can be produced several days after the C. perfringens challenge.

**Coinfection with Eimeria**

Eimeria maxima is the most common species used when inducing experimental NE in C. perfringens-infected birds (Williams et al., 2003; Park et al., 2008; Miller et al., 2010). Eimeria acervulina, Eimeria tenella, and Eimeria mitis have also been used in these coinfection model systems (Timbermont et al., 2009). In addition, both field isolates and vaccine strains of Eimeria have been used. For wild-type Eimeria field strains, approximately 10³ to 10⁴ oocysts/bird were given at 4 to 5 d before C. perfringens infection (Collier et al., 2008; Park et al., 2008; Miller et al., 2010). For vaccine strains, 10- to 24-fold higher doses than recommended for vaccination against coccidiosis have been given at 3 d before or 1 d after C. perfringens infection (McReynolds et al., 2004; Pedersen et al., 2008; Timbermont et al., 2009). In general, this step is considered to facilitate subsequent C. perfringens challenge, which induced lesions. This dual-infection system is used in our laboratory, and both E. maxima and C. perfringens act synergistically to reproduce NE. Eimeria maxima alone does not produce NE-specific lesions, whereas C. perfringens alone can produce NE-specific lesions, although the lesion severity is significantly lower than those seen with dual infection (Collier et al., 2008; Park et al., 2008).

**Immunosuppression**

Coinfection of chickens with C. perfringens and immunosuppressive viruses, such as infectious bursal disease virus (IBDV), chick anemia virus, and Marek’s disease virus, has been suggested to promote the development of NE in the field, and experimental NE has been produced using IBDV plus C. perfringens (Williams et al., 2003; Gholamiandehkordi et al., 2007; McReynolds et al., 2007; Stringfellow et al., 2009; Timbermont et al., 2009). Infectious bursal disease virus is considered one of the important contributing factors in NE because it affects the lymphoid cells and specifically targets the bursa of Fabricius, which is responsible for the development of humoral immunity. Birds infected with IBDV often have secondary infections such as C. perfringens and Escherichia coli. McReynolds et al. (2004) reported that high doses of an IBDV vaccine increased the severity of NE. Birds immunized with the IBDV vaccine at 14 d posthatch and orally infected with 10⁷ cfu of C. perfringens twice daily for 3 d, beginning on d 17, exhibited increased NE-specific intestinal lesion scores and mortality compared with birds given the vaccine alone or given C. perfringens alone. This study clearly showed that immunosuppression induced by the IBDV vaccine increased the severity of NE, further implying that dietary or environmental factors affecting the immune system of the birds would increase the likelihood of NE development in commercial poultry operations.

Indeed, environmental stresses (e.g., heat or cold stress) have been known to exacerbate any disease challenge, including NE. Tsouriis et al. (2009a,b) conducted a series of experiments using artificially controlled air temperatures to assess the effect of heat or cold stress on experimental NE in broiler chickens. Using a conventional NE model system of C. perfringens challenge, coccidiosis vaccination, and a high-cereal, high-protein diet, they observed that heat stress (35°C, 12 h/d, 5 d) increased the severity of NE clinical symptoms. The authors postulated that the effect of heat stress on experimental NE was due to suppressed host immune competence, altered intestinal microbiota, elevated levels of heat shock proteins, the induction of hemodynamic changes, or both. Tsouriis et al. (2009a) also reported that cold stress predisposed chickens to the development of NE through the immunosuppressive mechanism. Similarly, Burkholder et al. (2008) observed that heat stress for 24 h disrupted the normal protective microbiota community and altered intestinal morphology, which rendered birds susceptible to Salmonella Enteritis infection.
Dietary Factors

Diets enriched with cereals (wheat, rye, barley, or oats), or diets abruptly altered from low to high protein content, have been used to produce experimental NE (Williams, 2005; Dahiya et al., 2006; McDevitt et al., 2006; Cooper and Songer, 2009). Both practices favor *C. perfringens* colonization of the intestine and the development of NE-specific lesions (McReynolds et al., 2009). Increasing dietary protein levels to 24 to 38% has been reported to increase NE (Park et al., 2008; Mikkelsen et al., 2009). Paliyeguru et al. (2010) compared diets containing potato, fishmeal, and soybean meal for the induction of experimental NE. Although protein concentration and amino acid composition were comparable between the different diets, birds fed the potato protein-based diet exhibited a higher incidence of intestinal hemorrhagic lesions compared with birds fed the other diets. The authors attributed the increased incidence of subclinical NE with the potato protein-based diet to its antinutrient factor (high trypsin inhibitor activity) and low lipid content. The timing of dietary change (before, during, or after *C. perfringens* challenge infection) may also influence disease development.

Host Genetics

Until now, very limited information has existed on the effect of chicken breeds on NE susceptibility, although there is an indication (van der Most et al., 2011) that the selection for growth compromises the host immune functions. Siegel et al. (1993) first reported a natural outbreak of NE and its association with the major histocompatibility complex (MHC) in laying hens. Their retrospective study revealed large differences between MHC genotypes (genotype B21B21 being more resistant to NE compared with genotype B13B13) in relation to mortality and hen-day egg production in NE-affected laying hen flocks. In an extensive epidemiological survey on NE conducted in the United Kingdom, a certain type of broiler breed was shown to be at an increased risk for the development of NE (Hermans and Morgan, 2007). Unfortunately, no further information on the susceptible breed is available. Olkowski et al. (2006) speculated that current broiler breeds may have been selected to be more resistant to the development of NE compared with those used 30 or more years ago. However, this speculation has not been verified experimentally. In our unpublished work with the commercial meat-type fast-growing breeds, we observed breed differences in response to NE with respect to clinical and immunological parameters such as BW, mortality, NE lesions, and humoral immunity (S. I. Jang, H. S. Lillehoj, S. H. Lee, and K. W. Lee, all of Animal Parasitic Diseases Laboratory, ARS-USDA, Beltsville, MD; unpublished data). Further studies on breeds susceptible or resistant to NE are now ongoing in this laboratory, which will provide insights into the disease.

Clinical Parameters of Experimental NE

Intestinal Lesions

Gut lesions are commonly used to assess the severity of experimental NE (Cooper and Songer, 2009). Lesions scoring systems have varied from 0 to 3 (Gholamiandehkordi et al., 2007; Miller et al., 2010), 0 to 4 (McReynolds et al., 2004; Chalmers et al., 2007), 0 to 5 (Collier et al., 2008; Paliyeguru et al., 2010), and 0 to 6 (Keyburn et al., 2006). Thus, direct comparison of NE lesions between different experimental NE model systems may not be meaningful. Nonetheless, it is generally understood that the severity of NE lesions is closely related to BW gain and mortality.

BW Gain and Mortality

Experimental NE was reported to cause a 17% reduction in BW gain between 1 and 21 d after *C. perfringens* infection and a 7% decreased BW gain between d 22 and 35 compared with uninfected controls (Chalmers et al., 2007; Mikkelsen et al., 2009; Liu et al., 2010). Of note, however, other investigators have reported no significant alteration of BW gain during experimental NE (Pedersen et al., 2008). Mortality during experimental NE ranged from 0% (Olkowski et al., 2006; Pedersen et al., 2008; Liu et al., 2010) to 3 to 64% (Kaldhusdal et al., 1999; Chalmers et al., 2007; Collier et al., 2008; Park et al., 2008; Miller et al., 2010).

Microbiological Parameters of Experimental NE

*C. perfringens* Colonization

Intestinal *C. perfringens* bacteria have been enumerated to characterize experimental NE models (Kaldhusdal et al., 1999; Dahiya et al., 2005) because cecal *C. perfringens* can translocate to the upper intestine. This parameter, however, needs to be differentiated from simple colonization of *C. perfringens*, which is not a disease in itself and is far easier than inducing NE, which requires the presence in the gut of specific lesions. Thus, it is advisable to enumerate the bacteria in different intestinal sections in addition to scoring lesions in the affected area. In experimental NE, *C. perfringens* counts are positively associated with the severity of NE-specific lesions. Kaldhusdal et al. (1999) reported that all examined intestinal samples collected during the NE outbreak showed counts above 6 log10/g of cecal contents, indicating a high-level colonization.
by *C. perfringens*. Only birds that died spontaneously were examined postmortem; thus, the presence of mild (sublethal, subclinical) NE in all experimental groups cannot be excluded. Similarly, Pedersen et al. (2008) observed a high level of colonization of *C. perfringens* in the jejunum and ileum in their NE model induced by infection with *C. perfringens* in conjunction with a coccidial vaccine compared with uninfected controls or compared with birds infected with *C. perfringens* alone or given the coccidial vaccine alone. No differences in *C. perfringens* colonization of the cecum were noted between the NE-induced and clinically healthy controls. In the NE model system reported by Dahiy et al. (2005), *C. perfringens* colonization of the cecum was directly correlated with an increased dietary level of glycine, a predisposing factor for NE. On the other hand, another study reported no differences in *C. perfringens* colonization of the jejunum or cecum between NE-free and NE-induced birds (Mikkelsen et al., 2009). Overall, *C. perfringens* counts can be a useful indicator of the NE model (Kaldhusdal et al., 1999), under the premise of quantitative identification of the challenge strains as well as the incorporation of strict measures to prevent cross-contamination.

**Gut Microbiome**

Several research groups have examined changes in the gut microbiome during experimental NE, but the results have been variable. In a study by Collier et al. (2008), a dramatic shift in the microbiota between birds with experimental NE and healthy controls was reported. They amplified the V3 region of the 16S ribosomal RNA gene on microbial DNA in ileal digest by PCR and analyzed this region by denaturing gradient gel electrophoresis. Although this study provided altered gut bacterial community profiles in NE vs. healthy chickens, further family or species levels were not characterized. Mikkelsen et al. (2009) and Jia et al. (2009) found that the number of *Lactobacillus* spp. in NE-induced birds was equal to the number in healthy controls. By contrast, Liu et al. (2010) observed that the *Lactobacillus* and *E. coli* populations in the ileum were increased in chickens with experimental NE compared with healthy birds. McReynolds et al. (2004) observed a significant and consistent increase in the population of *E. coli* in the jejunum of NE birds. A recent study by Feng et al. (2010), however, showed that the number of *C. perfringens* in the ileum of chickens with experimental NE was negatively correlated with the total population of lactobacilli and specifically with the number of *Lactobacillus aviaris*. In the future, more studies in this field are required to reveal the close interaction between pathogenic *C. perfringens* and non-pathogenic commensal bacteria, which can provide new insights into potential dietary approaches (e.g., probiotics, prebiotics, exogenous enzymes, and essential oils) to prevent NE.

**Cytokine and Toll-Like Receptor Expression**

In humans, *C. perfringens* has stimulated monocyte production of interleukin (IL)-12, a cytokine involved in the differentiation of naïve T cells into T helper (Th)0 cells, which further develop into Th1 or Th2 cells. (Hessele, 2000). In human neonatal umbilical cord cells, however, *C. perfringens* induced higher levels of IL-10 and IL-6 compared with IL-12 and tumor necrosis factor-α (Karlsson et al., 2002). Cultured human endothelial cells exhibited increased neutrophil adhesion in response to treatment with *C. perfringens* α-toxin, which was mediated through platelet-activating factor (Bunting et al., 1997). Our own studies of intestinal intraepithelial lymphocytes from an NE model system using chickens coinfected with *C. perfringens* and *E. maxima* revealed that gene transcripts encoding interferon (IFN)-α, IFN-γ, IL-1β, and IL-10 were significantly increased in NE-infected birds compared with uninfected controls (Park et al., 2008). Similarly, Collier et al. (2008) observed that chickens with induced NE exhibited significantly increased levels of IFN-γ, IL-4, and IL-10 in the intestine compared with healthy controls.

Two previous studies (Collier et al., 2008; Park et al., 2008) observed that the expression patterns of 2 genes, IFN-γ and IL-10, were increased in NE-infected birds compared with healthy controls. Generally, IFN-γ regulates adaptive immunity by activating lymphocytes and enhancing the expression of MHC class II antigens. In addition, IFN-γ is a common marker of cellular immunity, and high levels have been correlated with protective immune responses to enteric coccidial infections (Lee et al., 2010). In addition, IL-10 is a pleiotropic Th2-type cytokine involved in innate and adaptive immune responses, and it functions to inhibit the production of IL-12 by activated macrophages, cluster of differentiation (CD)4+ lymphocytes, and CD8+ T lymphocytes (Park et al., 2008). Whether the enhanced production of either IFN-γ or IL-10 can be considered a common marker that is associated with protective immunity against NE needs to be investigated further.

Given that Toll-like receptors (TLR) participate in pathogen detection and activate innate immune responses, Lu et al. (2009) investigated the expression levels of chicken TLR after infection with *C. perfringens*. In summary, TLR1 type 1 levels in the spleen were upregulated at 1 d postinfection, whereas in the intestine, the levels were increased at d 2. Transcripts for TLR1 type 2, TLR2 type 1, TLR4, and TLR7 also were increased in the spleen at 1 to 2 d postinfection. Transcripts for TLR15 were significantly upregulated in the spleen at 1 to 2 d, but were downregulated in the intestine at d 4 postinfection. Expression of TLR2 type 2 in the spleen or intestine, and the levels of TLR1...
type 2, TLR4, and TLR7 in the intestine were unaffected after *C. perfringens* infection. Taken together, it is clear that various immunological parameters, at least cytokines and TLR, are altered during the course of experimental NE.

**Microarray Analysis**

Zhou et al. (2009) used a low-density cDNA chicken immune-specific microarray to analyze gene expression during experimental NE. *Clostridium perfringens* infection downregulated the expression of genes encoding cytokines, chemokines, and their receptors [IL-1b, IL-2, IL-16, IL-18, IFN-γ, tumor necrosis factor receptor-associated factor 6 (TRAF6), c-avian musculoaponeurotic fibrosarcoma (C-maf), osteoprotegerin, IL-8, macrophage inflammatory protein 1 β (MIP1b), CXCR receptor 1 (CXCR1), chemokine (C-C motif) receptor 8 (CCR8), AH294, T cell activation gene (TCA)-homolog, other cell surface molecules (CD11b, CD28, CD62L), and apoptosis-related molecules [casparase 8, casparase 9, granzyme 9, Fas-associated death domain (FADD)-like IL-1β-converting enzyme-inhibitory protein (FLIP), apoptosis-associated protein, Bcl-2-related ovarian killer protein (Bcl-2ow)]. It is notable that IFN-γ mRNA expression was downregulated (Zhou et al., 2009) or upregulated (Park et al., 2008) after *C. perfringens* infection. Although a clear explanation for the inconsistent observations in IFN-γ mRNA expression between the 2 studies is not readily available, differences in experimental designs (e.g., single- or dual-infection models and target organs used for gene expression) may account for the discordant results. For example, broilers were challenged with *C. perfringens* alone (Zhou et al., 2009) or with a combination of *E. maxima* and *C. perfringens* (Park et al., 2008). In addition, intestine intraepithelial lymphocytes (Park et al., 2008) or splenocytes (Zhou et al., 2009) were sampled for gene expression. Indeed, we observed different patterns of cytokine mRNA expression in the intestine and spleen of broiler chickens naturally exposed to reused litters contaminated with *Eimeria* spp. and *C. perfringens* [K. W. Lee, H. S. Lillehoj, S. H. Lee (Animal Parasitic Diseases Laboratory, ARS-USDA, Beltsville, MD), S. I. Jang (Animal Parasitic Diseases Laboratory, ARS-USDA), D. A. Bautista (University of Delaware, Georgetown), D. Ritter (Mountaire Farm, Millsboro, DE), A. P. Neumann (Danisco, Waukesha, WI), and G. R. Siragusa (Danisco); unpublished data]. In the future, it is considered important to characterize the local or systemic immune responses to the different NE models.

In addition, genes encoding MHC class I [B-F, β2-microglobulin (B2m), calreticulin, calnexin, transport-associated protein 2 (TAP 2), CD83, T cell receptor β (TCRb)] and class II [B-Lb, IgG, IgA, invariant chain, CD45, restriction fragment pattern γ (RFp-γ), IL-2aR, Bu-1] molecules, as well as proteins [growth factor receptor-binding protein 2 (GRB2), B cell adaptor containing src homology 2 domain (BASH), signal transducer and activator of transcription-2 and -4 (STAT2 and -4)] involved in transcription and signal transduction, were upregulated (Zhou et al., 2009). Based on these results, the authors concluded that both cell-mediated and antibody-mediated immune responses were responsible for the host defense against *C. perfringens* infection. In a subsequent microarray study, Sarson et al. (2009) confirmed that the expression of genes involved in inflammation, humoral immunity, antigen recognition, apoptosis, and immune-related metabolic processes were significantly altered after experimental infection of chickens with *C. perfringens*.

**C. PERFRINGENS VACCINES**

Because maternally derived antibodies against *C. perfringens* can reduce the incidence of NE in broiler chickens (Heier et al. 2001), vaccination of laying hens against the bacterium may afford hatched chicks protection against NE. Broiler chickens hatched from breeder hens that had been immunized with vaccines for *C. perfringens* type A or C were more protected against experimental NE compared with birds hatched from unvaccinated hens (Lovland et al., 2004). Vaccinated hens had high serum IgG antibody titers against *C. perfringens* α-toxin, and the *C. perfringens* type C vaccine was superior to the type A vaccine in protecting against experimental NE. Thompson et al. (2006) found that broiler chicks immunized with virulent *C. perfringens* strains, followed by antibiotic treatment for 9 d, were protected against challenge infection with *C. perfringens*, as assessed by decreased intestinal lesion scores compared with unvaccinated and challenged controls. This result indicates a close link between virulence factors and immunizing ability. To support this view, Thompson et al. (2006) found that immunization with a nonpathogenic *C. perfringens* did not reduce NE lesions after infection with virulent *C. perfringens*. However, immunization with an avirulent α-toxin mutant of a virulent strain also protected against challenge by virulent *C. perfringens*, suggesting that protective immunity may involve bacterial antigens other than α-toxin.

In spite of the results using the α-toxin mutant *C. perfringens* vaccine, Cooper et al. (2009) reported that broiler chicks subcutaneously immunized with a recombinant α-toxin protein in a Quil A adjuvant and challenged with virulent *C. perfringens* had significantly reduced intestinal lesion scores compared with birds given the adjuvant alone. α-Toxin-specific antibody titers were greater in the immunized birds compared with the unvaccinated controls, indicating that α-toxin may play a role in pathogenesis and function as a potential immunogen against NE. Recently, Coursodon et al. (2010) provided evidence that challenge with an α-toxin-deficient *C. perfringens* paradoxically induced NE le-
sions in birds that were positively associated with the amount of α-toxin in the gut contents. Thus, the role of α-toxin in pathogenesis and in the vaccine candidate needs further investigation.

Apart from α-toxin, other \textit{C. perfringens} proteins have been evaluated as vaccine candidates, including glyceraldehyde-3-phosphate dehydrogenase, pyruvate:ferredoxin oxidoreductase, fructose 1,6-biphosphate aldolase, endo-β-N-acetylglucosaminidase, and phosphoglyceromutase (Kulkarni et al., 2006, 2007; Jiang et al., 2009). In general, vaccinated birds were more resistant to challenge infection and the development of experimental NE compared with the unvaccinated controls. All vaccinated birds produced antigen-specific IgY in the serum and IgY and IgA in the intestine, regardless of their disease protection status. Orally delivered attenuated \textit{Salmonella} expressing recombinant forms of some of these \textit{C. perfringens} proteins also have been evaluated as NE vaccines. Promising results were reported in reducing gut lesions and improving BW gain compared with controls (Kulkarni et al., 2008, 2010; Zekarias et al., 2008). However, commercial vaccines using recombinant proteins against \textit{NE} for broiler chickens are not available at this stage, and no clinical studies on NetB as a candidate for the NE vaccine in broiler chickens have been addressed. Recently, Lanckriet et al. (2010) reported that immunoprotective properties of the components secreted by \textit{C. perfringens} were dependent on the strains, suggesting that an effective combination of immunogens is needed for full protection against \textit{NE}.

In our laboratory, the genes encoding \textit{C. perfringens} elongation factor Tu (EF-Tu), pyruvate:ferredoxin oxidoreductase, α-toxin, and NetB toxin were cloned and their corresponding recombinant proteins were purified (Lee et al., 2011). Ongoing studies are directed at evaluating whether these purified clostridial proteins, either alone or in combination with novel adjuvants, can serve as potential subunit vaccines for \textit{NE}. In addition, as routinely performed in the commercial broiler industry for selected pathogens, in ovo administration of protein-based subunit vaccines may be an effective method of immunization. Thus, the vaccination of eggs can be a safe, effective, and convenient method of administering a single immunogen or a combination of immunogens against \textit{C. perfringens}.

**CONCLUSIONS**

Poultry eggs and meat are a major source of protein for the global human population. Efficient and safe production of nutritious poultry food products will become increasingly important to meet growing consumer demands. A better understanding of the potential effects of changing environmental factors, emerging infectious diseases, and modern poultry-rearing practices on the efficiency and profitability of future poultry production will be essential for these processes to be realized. In this regard, the negative effect of \textit{NE} on the poultry industry will likely increase in the near term because of the absence of effective \textit{NE} vaccines and the current trend of a legislative or voluntary ban on subtherapeutic antibiotic growth promoters.

Considerable progress has been made in understanding the pathogenesis of \textit{NE} with the use of the reproducible experimental \textit{NE} models. As stated in this review, many laboratories have reported reliable and reproducible \textit{NE} models. However, it may not be relevant to judge the \textit{NE} models described in this review because many factors are likely to affect the clinical, microbiological, and immunological parameters of \textit{NE}. Thus, it would be absurd to conclude which model is better or more reliable than others. Rather, it needs to be established what the similarities and differences are in various experimental \textit{NE} models in relation to the \textit{NE} parameters described in this review. Currently, we are conducting studies to compare the host immune responses in different \textit{NE} models (single- vs. dual-infection systems). This experiment will shed light on the possible homogeneous or heterogeneous nature between \textit{NE} models.

With the recent development of a reliable \textit{NE} experimental model system, future studies that are focused on the immunobiology of host-pathogen interactions will contribute to novel control strategies against this disease, including second-generation recombinant vaccines, new delivery vectors, and novel adjuvants, as well as dietary immunomodulating agents such as pre- or probiotics. In addition, the role of the microbiome in the pathogenesis and prevention of \textit{NE} needs to be characterized and may provide insights into the complex interaction of host-pathogen-microbiota in broiler chickens.

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