CELLULITIS IN TURKEYS: CHARACTERIZATION OF CAUSATIVE AGENTS AND PREVENTIVE MEASURES

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Dedicated

to

my parents

D.r T. K. Johny and Prof.Pushpi Johny

ABSTRACT

Cellulitis continues to cause extensive losses in turkey production in USA due to severe mortality, carcass condemnation and treatment costs. *Clostridium perfringens* and *Clostridium septicum* have been recognized as the primary causative agents of cellulitis in turkeys. In this study, cellulitis lesions and mortality in turkeys were successfully reproduced with *Clostridium perfringens* and *Clostridium septicum* isolated from cellulitis cases. *Clostridium perfringens* and *Clostridium septicum* isolates varied in their ability to produce spores as well as toxins. We observed differences in the toxicity and biological effects of different strains of *C. perfringens* and *C. septicum in vitro*, and *in vivo*. .Though the spore count and hemolytic effects of *C. perfringens* were found to be higher than *C. septicum in vitro,* mortality studies in mice and turkeys showed that *C. septicum* was much more potent than *C. perfringens*. However, gross lesions produced by *C. perfringens* and *C. septicum* were almost identical. Surprisingly, the development of cellulitis lesions and mortality was markedly higher in 7-week-old birds than in 3-week-old birds. The results of our study demonstrated for the first time that both *C. perfringens* and *C. septicum* can multiply in the subcutaneous and muscle tissues and cause cellulitis lesions in turkeys. Our cellulitis disease model offers promise as a challenge model in the development of vaccines against cellulitis in turkeys. Both bivalent *C. perfringens* and *C. septicum* toxoid and *C. septicum* toxoid were found to be safe and offered complete protection against cellulitis following homologous challenge under experimental conditions. The use of these vaccines enabled us to reduce the mortality and antibiotic usage in preventing cellulitis in commercial turkeys. Multiple vaccinations or use of a day old vaccine followed by a booster dose probably will offer better protection than a single

vaccination at 6-weeks of age against cellulitis due to *C. perfringens* and *C. septicum* in turkeys.

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CHAPTER I

GENERAL INTRODUCTION

Over the last few years, cellulitis has become more prevalent in commercial turkeys especially in Minnesota, Wisconsin, Missouri and Virginia. Accompanied by high mortality and carcass condemnation in the processing plants, it often inflicts heavy economic loss for the turkey producers (Susan, 2003). Because of this, cellulitis is currently considered as a high priority research topic. The mortality is reported to be as high as 1-2% per week in the affected flocks.

A turkey health survey (Clark et al., 2010) of US veterinarians in turkey production ranked cellulitis at a score of 3.5; the survey ranked 34 current disease issues (1= no issue to 5 = severe problem) and had a survey response (reply) of 100% (n=19). During the 2007 Annual Convention of National Turkey Federation (NTF) in Tucson, Arizona, the attendees recommended that NTF host a series of meetings to further discuss turkey cellulitis. To help address this request, the Minnesota Turkey Growers Association held a meeting during the Midwest Poultry Federation Convention to further discuss this issue. The discussion focused on current research available and knowledge gaps and research needs. The meeting concluded with the attendees making several recommendations to the NTF Turkey cellulitis Task Force as it works toward better understanding the disease and developing a control strategy. In the year 2008, a Gold medal panel on cellulitis was held on December 16-17, Bloomington, MN, to invite suggestions for control measures for cellulitis in turkey production. Development of an effective vaccine against cellulitis was recognized as one of the urgent measures necessary to control cellulitis in commercial turkeys. The panel also suggested renaming the disease condition “Cellulitis” to “Clostridial dermatitis”.

Diagnostic laboratories have consistently isolated *Clostridium perfringens* and *Clostridium septicum* organisms from turkey cellulitis lesions. The mode of entry of these organisms is uncertain because in most cases of turkey cellulitis, there are no external wounds that would explain the entry of the organism into the bird. It appears that entry of the organisms into the bird is by oral route through contaminated feed.

It is widely accepted that natural outbreaks of cellulitis in turkeys are associated with proliferation of pathogens in the poultry environment. One can argue that a reasonable model of the disease should closely resemble the probable route of natural pathogen exposure, i.e.: oral. Necrotic enteritis is yet another disease in chickens caused by *C. perfringens*. Interestingly, efforts for consistent reproduction of necrotic enteritis by oral inoculation of *C. perfringens* has resulted in extremely variable results including severe clinical signs, subclinical NE and no lesions at all in exposed birds (Al-Sheikly et al., 1977; Chalmers et al., 2007; Pederson et al., 2008; Truscott et al., 1977). Such a significant lack of consistency in scientific data generated in different labs is puzzling indeed, particularly in view of a large number of claims identifying predisposing factors (Mcdevitt et al., 2006; Olkowski et al., 2006). Attempts to reproduce cellulitis in turkeys by injecting purified alpha toxin of *C. perfringens* subcutaneously were successful but injections with *C. perfringens* alone were not (Carr, 1996).

The incidence of *Clostridium perfringens*-associated diseases in poultry has increased significantly in the recent years because of the reduced use of antimicrobial growth promoters (van-Immerseel et al., 2004). It has become a common practice to use antimicrobial drugs in feed or water to control cellulitis in a preventative manner, but this practice is increasingly criticized or has been banned in some countries. Since cellulitis

cases in turkeys are also rising at an alarming rate, there is a need to investigate alternative approaches for its effective control.

Vaccines containing toxoids and killed bacteria have been successfully used in humans and animals against clostridial infections. Formalin-inactivated C. difficile toxoid was found to be highly safe and immunogenic against *C. difficile* infections in mice (Torres et al., 1995) and humans (Kotloff et al., 2001). For protection of birds affected with necrotic enteritis caused by *C. perfringens,* alpha toxin attracted the most attention in earlier studies but recent studies questioned the role of alpha toxins (Thompson et al., 2006).

Despite the importance of cellulitis in turkeys, there is very little known about the basis of immunity to this infection, although immunization is an obvious approach to control. The purposes of this study were therefore to examine the aspects of immunity to cellulitis in turkeys, specifically; to develop a challenge model to test any vaccines for cellulitis in turkeys and to test whether it is possible to immunize turkeys against cellulitis.

The following three main objectives are there in this dissertation research.

I. To characterize isolates of *Clostridium perfringens* and *Clostridium septicum* from cases of cellulitis in turkeys.

To achieve this objective the following experiments were carried out.

1. Laboratory characterizion of *C. perfringens* and *C. septicum* isolates using heat resistant spore count, hemolytic assay and mice lethal assay.

2. Conducted Multilocus sequence typing (MLST) to determine the genetic variation among *C. perfringens* and *C. septicum* isolated from cases of cellulitis in turkeys

3. Identified secretory components of *C. perfringens* and *C.septicum* using 2-DiGE analysis and MALDI-TOF mass spectrometry.

II. To study the role of *Clostridium perfringens* and *Clostridium septicum* in causing cellulitis in turkeys.

To achieve this objective the following experiments were carried out.

1. To conduct *in vivo* studies to examine C*lostridium perfringens* and *Clostridium septicum* in the cause of cellulitis in turkeys of different age groups.

III. To examine the use of *Clostridium perfringens* and/or *Clostridium septicum* inactivated vaccines to prevent cellulitis in turkeys.

To achieve this objective the following experiments were carried out.

1. Prepared and examined an experimental bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid for prevention of cellulitis in turkeys under experimental conditions.

2. Field tested the lab evaluated experimental *Clostridium perfringens* and *Clostridium septicum* toxoid for its use in minimizing losses in turkey flocks in Minnesota with existing cellulitis problem.

3. Prepared and examined an experimental *Clostridium septicum* toxoid for prevention of cellulitis in turkeys under experimental conditions.

4. Field tested the lab evaluated experimental *Clostridium septicum* bacterin toxoid for its use in minimizing losses in turkey flocks in Minnesota with existing cellulitis problem.

CHAPTER II

LITERATURE REVIEW

Over the last several years cellulitis has emerged as an economically important disease syndrome in turkeys. Accompanied by high mortality and carcass condemnation in the processing plants, cellulitis often inflicts heavy economic loss for the turkey producers (Olkowski et al., 1999; Susan., 2003). In recent years, cellulitis received more attention because of an alarming upward trend in the mortality in turkey grower flocks (Kumar et al., 1998). In a study conducted at a turkey processing plant in Ontario, Canada, 9% of the birds examined had severe cellulitis (St. Hilaire et al., 2003). Currently, cellulitis in turkeys is being diagnosed in Minnesota, Wisconsin, Missouri, Virginia and other turkey producing areas. In Minnesota, the mortality is reported to be as high as 1-2% per week in the affected flocks.

A. CELLULITIS

Cellulitis is described in turkeys as a condition characterized by inflammation of the skin and subcutaneous tissue with accumulation of focal yellow or yellow-brown exudate in subcutis of breast and tail areas (Carr et al., 1996; Kumar et al., 1998). Similar disease condition has been described in chickens with a different name “Gangrenous dermatitis” (van-Immerseel et al., 2004; Li et al., 2010b).

Only few references are available for clostridial infections in turkeys although the first case was reported as early as in 1939 (Fenstermacher et al., 1939). Suspected cases of Clostridial cellulitis have been observed in breeder toms following semen collection (Ficken et al., 1991). In turkeys, Clostridial cellulitis and mortality have been reported both in range and in breeder toms (Carr, 1996; Ficken et al., 1991) and also in hens (Carr et al., 1996). It was determined to have been initiated by infection of wounds that the hens

received during natural mating. The lesions associated with cellulitis usually appear at 13 to 16 weeks of age and persist until the birds are marketed (Carr et al., 1996).

Cellulitis in turkeys is characterized by edematous swelling of breast, neck, tail and legs, and accumulation of gaseous serosanguinous exudate within the subcutaneous tissue (Fenstermacher et al., 1939; Carr et al., 1996). In addition, the lesions are seen also on the back of the bird. Palpitation of the affected areas often reveals crepitation due to gas bubbles in the subcutis and musculature. On necropsy, one would find accumulation of bubbly, serosanguinous fluid in the subcutis. The affected tissues sometimes contain large amounts of gelatinous exudate. The underlying musculature may have a cooked appearance. The liver and spleen are often enlarged and may contain large necrotic infarcts. The kidneys are usually swollen (Carr, 1996). Interestingly, in most cases of cellulitis, there appears to be no obvious damage or trauma to the skin. Hence the role of any pathogen isolated from these lesions in the causation of the disease remains obscure (Olkowski et al., 1999). Not much information is available about the pathogenesis of cellulitis in turkeys.

In chickens, cellulitis conditions caused by *Clostridium septicum*, and *Clostridium perfringens* has been described previously as early as in 1965 (Saunders and Bickford, 1965). Severe outbreaks of cellulitis by *C. septicum* alone have also been reported in broiler chickens (Helfer et al., 1969; Willooghby et al., 1996). In chickens with gangrenous dermatitis, the wings, breast, abdomen and thighs are found to be commonly affected (Li et al., 2010b). It is frequently found in birds of 4 to 16 weeks of age. The disease is characterized by a sudden onset, an increase in mortality, gangrenous necrosis of the skin over the thighs and breast, and an accumulation of serosanguinous fluid in the

subcutaneous tissue of the breast and thighs (Gerdon et al., 1973; Hofacre et al., 1986). The skin in these areas appears blackened, moist, and devoid of feathers. There will be bloody fluid and emphysema in the subcutis. The underlying musculature is often tan or gray, emphysematous and may contain fluid between muscle bundles. Death occurs within hours (Ficken, 1991; Hofacre et al., 1986). Many cases of gangrenous dermatitis are believed to be a sequela to diseases that cause immunosuppression, such as infectious bursal disease, infectious anemia or avian adenovirus infection (Ficken, 1991).

B. ETIOLOGICAL AGENTS ATTRIBUTED TO CELLULITIS

Infections with *Clostridium perfringens*, and *Clostridium septicum* is reported to cause cellulitis in turkey breeder hens (Fenstermacher et al., 1939). Organisms isolated from the cellulitis lesions of turkeys in Minnesota in the recent years also primarily include *C. perfringens* type A and *C. septicum* either alone or in combination (personal communication, Brian McComb, 2009). The isolation of either *C. perfringens* type A or *C. septicum* or both from cases of cellulitis in chickens and turkeys has been emphasized in many reports (Carr, 1996; Hofacre et al., 1986). *Clostridium perfringens* has been cultured from gangrenous dermatitis lesions in broiler chickens (Saunders and Bickford, 1965) and is also identified as the organism responsible for the pathology associated with necrotic enteritis in broilers (Hofacre et al., 1986; McDevitt et al., 2006) as well as cellulitis in turkeys (Carr et al., 1996; Fenstermacher et al., 1939). Thus it is worth noting that *Clostridium perfringens* is associated with distinct disease manifestations in birds but the interesting observation here is that all three disease conditions appear distinct.

Occasionally *E. coli* were also isolated in low numbers from few cases of cellulitis in turkeys (Gomis et al., 2002).

The etiology of gangrenous dermatitis in broiler chickens is attributed to multiple bacterial pathogens. However, *Escherichia coli* are considered as one of the major pathogens causing gangrenous dermatitis in broiler chickens (Gomis et al., 1997; Leclerc et al., 2003; Messier et al., 1993). A unique subset of *E. coli* which is responsible for cellulitis in broiler chickens was identified later (Jeffrey et al., 2002). Concurrent infections with *C. septicum* and *Staphylococcus aureus* in cases with gangrenous dermatitis in 4-week-old broiler chickens has also been reported (Wilder et al., 2001). In addition, Frazier et al (1964) isolated *E. coli, Streptococcus fecalis,* Proteus sp., Bacillus sp., *Staphylococcus aureus* and *Clostridium septicum* from cases of gangrenous dermatitis in broiler chickens. Gangrenous dermatitis in broiler chickens has also been reported to be associated with *C. perfringens* Type A and *C. septicum* or with *C. novyi* either alone or in combination with *C. perfringens*, *C. septicum*, and *Staphylococcus aureus* (Susan, 2003).

Clostridium perfringens and *C. septicum* are both spore forming bacteria and their spores are highly stable and ubiquitously present in the environment. These pathogens are capable of producing a myriad of extracellular toxins and enzymes that degrade host tissues and are responsible for the necrotic lesions observed (Sawires et al., 2006; Smedley et al., 2004).

C. *CLOSTRIDIUM PERFRINGENS*

A group of anaerobic organisms affecting animals described as histotoxic clostridia are most commonly involved in gas gangrene, or myonecrosis secondary to

wound infections. This group includes: *C. perfringens*, *C. septicum*, *C. novyi*, *C. histiolyticum*, and *C. bifermentans* (Onderdonk et al., 1995). *Clostridium perfringens* is one of the most widespread of all pathogenic bacteria affecting humans and animals. Its main habitats are the soil and the intestinal tracts of animals and man. Armed with an arsenal of many potent extra-cellular toxins and enzymes, *C. perfringens* is recognized as the causative agent of human gas gangrene and food poisoning as well as several enterotoxemic diseases in livestock (Johansson et al., 2006).

Clostridium perfringens is found in the intestinal tract of healthy poultry as a normal inhabitant, usually in low numbers and has been isolated from processed carcasses as well as from the processing plants (Craven et al., 2001b; Martel et al., 2004). Hatcheries are identified as a potential source and reservoir for *C. perfringens* in integrated poultry operations (Craven et al., 2001a). Experimental inoculation of purified alpha toxin of *C. perfringens* produced lesions similar to cellulitis and also severe mortality in turkeys (Carr, 1996).

Necrotic enteritis and gangrenous dermatitis are among the most common Clostridial diseases observed in broiler chickens which are caused by *Clostridium perfringens*. In turkeys, clostridial cellulitis which is similar to gangrenous dermatitis in chickens is the major disease caused by *C. perfringens* type A and *C. septicum*. It is interesting to note that though both necrotic enteritis and gangrenous dermatitis in broilers are attributed to *C. perfringens* type A, both disease conditions are not yet reported in any flocks or in any farms at the same time (McDevitt et al., 2006). Moreover, the incidence of one or the other disease condition continues to be more pronounced in some well maintained farms than in others in spite of adopting strict control strategies. It

appears that the *C. perfringens* responsible for each disease conditions are different.

Clostridium perfringens isolates are grouped into types A through E based on their production of one of the four major lethal toxins; alpha, beta, epsilon, and iota (Sawires et al. 2006; Smedley et al., 2004). The growth of *C. perfringens* is restricted to the site of infection, whereas the alpha toxin produced is absorbed by circulatory system and causes massive intravascular hemolysis and destruction of capillary walls. All five types of *C. perfringens* produces alpha-toxin which is an enzyme phospholipase C also called as lecithinase (Karasawa et al., 2003; Smedley et al., 2004). In addition, *C. perfringens* produces an array of extracellular toxins including beta2 toxin, enterotoxin, perfringolysin, collagenase, lambda toxin, hyaluronidase, dnase, neuraminidase and urease (Sawires et al. 2006). Alpha toxin is markedly hemolytic and it is the only major toxin produced by *C. perfringens* type A (Titball et al., 1999). The sporulation process in *C. perfringens* is reported to be linked with alpha-toxin production (Varga et al., 2004).

Clostridium perfringens produces more than twenty different types of toxins and the toxin profile varies with the strain of bacteria (Meer et al., 1997; Shane et al., 2002). Among toxins, alpha toxin was believed to be the predominant toxin responsible for *C. perfringens* pathogenicity as well as protection. But recent studies indicate that Alpha toxin does not have any role in the pathogenesis of necrotic enteritis caused by *C. perfringens* nor do they protect birds as demonstrated with an alpha toxin mutant of *C. perfringens* (Keyburn et al., 2006). Thompson et al., (2006) demonstrated that immunogens other than alpha-toxin are important in protective immunity against *C. perfringens* infection in broiler chickens.

Multi locus sequence typing (MLST) analysis of 132 isolates of *C. perfringens* from different hosts identified 80 sequence types (ST) (Jost et al., 2006). Of all the loci examined, *plc* gene had the most alleleles of 37 (Jost et al., 2006).

Recently many hypothetical proteases and β2-toxins were found in virulent *C. perfringens* type A strains and that is believed to play a major role in eliciting a protective immune response against necrotic enteritis. A novel toxin NetB is now identified as a definitive virulence factor present in avian *C. perfringens* strains capable of causing necrotic enteritis in chickens (Keyburn et al., 2008). Another group of scientists recently demonstrated a hypothetical protein of 117 kDa suggestive of a protease found only in virulent *C. perfringens* type A causing necrotic enteritis (Kulkarni et al., 2006).. This protein is believed to play a major role in the development of necrotic enteritis and also in eliciting a protective immune response (Kulkarni et al., 2007). It is also shown that the β2-toxin gene (*cpb2*) found in isolates of *C. perfringens* cultured from avian hosts are atypical compared to those found in pigs (Jost et al., 2005).

D. *CLOSTRIDIUM SEPTICUM*

Clostridium septicum is an organism of considerable medical and veterinary importance. Little is known about the distribution and sources of *C. septicum* in poultry production facilities. *Clostridium septicum* has played an important role as an etiologic agent of traumatic gas gangrene and clostridium myonecrosis in humans (Smith-Slatas et al., 2006). In mammals, particularly ruminants, a condition known as malignant edema is caused by wound contamination with *Clostridium septicum*. *Clostridium septicum* also appears to be a significant contributor to the etiology of cellulitis in turkeys. However,

not much information is available regarding the influence or the disease causing potential of different strains of *C. septicum.*

Clostridium septicum produces four major toxins (alpha, beta, gamma and delta) that are responsible for tissue damage and toxemia (Timoney et al., 1998). These toxins include: the lethal necrotizing and hemolytic toxin (alpha-toxin); dnase (beta-toxin); hyaluronidase (gamma-toxin); and the thiol-activated toxin or septicolysin (delta toxin) (Cortinas et al., 1997). Other enzymes such as protease and neuraminidase are also produced by *C. septicum*. Alpha-toxin plays a major role in *C. septicum* infection and is also the major protective antigen. This toxin is produced as an inactive protoxin of approximately 48 KD and requires proteolytic processing for activation into cytolytically active protein. Hemolytic activities have been described for alpha-toxin and a thiol-activated cytolysin produced by *C. septicum*.

The alpha-toxin of *C. septicum* is not related to the alpha toxin of *C. perfringens* since it does not exhibit phospholipase C activity and does not cross-react immunologically with *C. perfringens* alpha-toxin (Ballard et al., 1992). For *in vitro* toxin production by *C. septicum* Brain heart infusion (BHI) medium is reported to be ideal (Ballard et al., 1992).

E. VACCINATION AGAINST CLOSTRIDIAL DISEASES

Clostridial vaccines containing toxoids and killed bacteria have been successfully used in humans and animals against clostridial infections. Toxoids against many Clostridial diseases like Black Quarter (*Clostridium chauvoei)* and tetanus (*C. tetani)* were found to be very safe and highly effective. Formalin-inactivated *C. difficile* toxoid

vaccine was found to be highly safe and immunogenic against *C. difficile* infections in humans (Kotloff et al., 2001). *Clostridium perfringens* infections have been successfully controlled in suckling piglets using a toxoid vaccine (Springer et al., 1999). Sheep challenged with *C. perfringens* toxoid was found to be protective against gas gangrene caused by *C. perfringens* (Boyd et al., 1972). A recombinant *C. perfringens* alpha toxoid is also found to be protective against Clostridial myonecrosis in mice studies (Neeson et al., 2007).

Given the key roles that some phospholipases play in the pathogenesis of many diseases, it is not surprising that inactivated forms of these secretory toxins have been considered as components of vaccines. The use of formaldehyde toxoids prepared from the secretory toxins present in the supernatant of the culture of *C. perfringens* has induced protection against necrotic enteritis in chickens. Immunity to necrotic enteritis in broiler chickens was found to be associated with the presence of antibodies against *C. perfringens* alpha toxin (Heier et al., 2001). In another study alpha toxin of *C. perfringens* (phospholipase C) was found to be protective against lethal infections caused by *C. perfringens* in mice (Stevens et al., 2004).

For protection of birds affected with necrotic enteritis caused by *C. perfringens,* alpha toxin attracted the most attention in earlier studies but recent studies questioned the role of alpha toxins (Thompson et al., 2006). Turkeys vaccinated with commercial *C. perfringens* toxoid made for necrotic enteritis (sheep vaccine) did not protect against cellulitis in turkeys (personal communication Brian McComb). It is conceivable that there are genomic differences in *C. perfringens* strains that affect their phenotypic expression of virulence and/or toxin production.

Genotyping methods will help us to distinguish bacterial strains. A multilocus sequence typing (MLST) of *C.perfringens* isolates from porcine origin identified three clonal complexes and eight sequence types by looking at the patterns of genetic polymorphism in the house-keeping genes (*colA, gyrA*, *plc*, *pfoS*, and *rplL*) (Jost et al. 2006).

Vaccination has long been used to protect cattle and sheep against *C. septicum* infection too. The difficulties in the preparation of *C. septicum* toxin cultures are well known. Cultures often produce lethal antigen only in low titre and, in addition, the immunogenicity of native toxin filtrates is weak, which results in poor antibody response in animals (Cortinas et al., 1997). Formalin inactivated *C. septicum* alpha toxoid was found to be protective against experimental challenge with *C. septicum* spores in mice (Amimoto et al., 2002). A polyvalent *C. perfringens, C. septicum* and Pasteurella bacterin was found to reduce mortality in gangrenous dermatitis affected broiler chickens (Gerdon et al., 1973).

In this respect, the *C. perfringens* and *C. septicum* alpha toxins attracted the most attention. However, recent studies (Kulkarni et al., 2006, Thompson et al., 2006) demonstrated that immunogens other than alpha-toxins are important in protective immunity against *C. perfringens* infection in broiler chickens.

CHAPTER III

CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* AND *CLOSTRIDIUM SEPTICUM* ISOLATES FROM CELLULITIS CASES

Summary

Twelve isolates each of *C. perfringens* and *C. septicum* were isolated from cellulitis lesions of turkeys from Minnesota. Characterization of these isolates were done through *in vitro* laboratory assays including heat resistant spore count, hemolysis assay and mice lethal studies as well as by genomic and proteomic studies. We observed variations in the heat resistant spore counts and hemolytic titers between the isolates examined. We also noticed differences in the MLD_{50} of *C. perfringens* and *C. septicum* isolates. *Clostridium perfringens* strains isolated from cellulitis cases were of toxino-type A by PCR. The phylogenetic tree constructed from *C. perfringens* isolates using MLST indicated clustering among *C. perfringens* from cellulitis cases. However, two major clusters were found among the *C. perfringens* isolates isolated from cellulitis cases. The phylogenetic tree constructed from *C. septicum* isolates indicated a high level of conservation present within the housekeeping gene fragments of *C. septicum.* The major secretory toxins we identified in *C. perfringens* isolates from cellulitis cases by MALDI-TOF mass spectrometry were phospholipase, collagenase, hyaluronidase, dnase, enolase, muramidase, pyruvate kinase and hypothetical proteins. We observed two distinct proteomic profiles for *C. perfringens* isolates. However, we observed only one type of proteomic profile for *C. septicum* isolates. The major secretory toxins we identified in *C. septicum* were alpha toxin, septicolysin, sialidase, Dnase, flagellin and hypothetical proteins. More sequence types were observed among *C. perfringens* than among *C. septicum* isolates. Our results indicate that *C. perfringens* isolates vary much in their toxin expression and appeared more diverse. *C septicum* isolates were found to be less diverse but more lethal than *C. perfringens isolates***.** These data indicate that *C. septicum*

isolates are more likely to cause cellulitis mortality in turkeys than *C. perfringens* however more studies are needed to substantiate this assertion.

INTRODUCTION

Clostridium perfringens and *Clostridium septicum* have been suspected in playing a role in causing cellulitis and mortality in turkey population. Their role has been suggested as early as 1939 (Fenstermacher et al., 1939) but conclusive studies were not undertaken. There are many reports about isolation of *C. perfringens* from cellulitis or gangrenous dermatitis cases in chickens (Helfer et al., 1969; Willooghby et al., 1996).

Organisms isolated from cellulitis lesions of turkeys in Minnesota during the years 2005- 2007 primarily include *C. perfringens* and *C. septicum* and either alone or in combination. *Clostridium perfringens* alone was reported to cause cellulitis in turkeys and experimental reproduction of the disease has been successful in adult turkeys (Carr et al., 1996). But experimental reproduction of cellulitis with *C. septicum* is not yet reported in chickens or turkeys. Though *Clostridium perfringens and C. septicum* were attributed as the primary agents causing cellulitis in turkeys based on the isolation results, the role of *C. perfringens* and *C. septicum* isolates in the development of cellulitis lesions was not studied in detail.

Clostridium is one of the most widespread of all pathogenic bacteria affecting humans and animals. Its main habitats are the soil and the intestinal tracts of animals and man. *Clostridium perfringens* is found in the intestinal tract of healthy poultry as a normal inhabitant, usually in low numbers (Songer, 1996). It has been isolated from processed carcasses as well as from the processing plants (Craven et al., 2001b; Martel et al., 2004). Hatcheries were identified as a potential source and reservoir for *C. perfringens* in integrated poultry operations (Craven et al., 2001a).

Clostridium septicum is an organism of considerable medical and veterinary importance. But little is known about the distribution and sources of *C. septicum* in poultry production facilities.

The objective of this study was to characterize *Clostridium perfringens* and *Clostridium septicum* isolates from cellulitis cases in turkeys. Characterization of the isolates were done through *in vitro* laboratory studies like hemolysis assay and mice lethal studies as well as by genomic and proteomic studies. This enabled us to select the isolates for developing a disease model as well as for vaccine production.

MATERIALS AND METHODS

1. Source of Bacteria

Clostridial isolates included in this study originated from field cases of cellulitis in turkeys either submitted to our laboratory from different turkey farms of a turkey company in Minnesota or were collected by us during farm visits from 2007-2008.

2. Bacterial Isolation and Identification

Samples were collected in 0.1% Bacto peptone (Becton, Dickinson and Company, Sparks, MD) using sterile swabs from cellulitis lesions during necropsy of fresh birds. Isolation of *Clostridium perfringens* was done in cooked meat medium (Jong et al., 2002) at 37 C for 24 h under anaerobic conditions in Mitsubhisi® anaerobic boxes (Mitsubhisi gas chemical co, NY, USA). Oxygen absorbing gaspaks (Anaeropack-Anaero, Mitsubhisi gas chemical co, NY, USA) were used in anaerobic boxes to maintain strict anaerobic conditions. Subsequent subculturing was done on anaerobic sheep blood agar plates

(Oxoid, NY, USA) or tryptose sulphite cycloserine (TSC) agar with egg yolk (Merck, Darmstadt, Germany) agar plates (Oxoid, NY, USA). They were examined for their morphology, staining, and cultural characteristics. Specicity of *C. perfringens* colonies was ascertained by their growth on tryptose sulphite cycloserine (TSC) agar with egg yolk (Merck, Darmstadt, Germany) and reverse CAMP test.

Isolation of *C. septicum* was made in cooked meat medium and brain heart infusion (BHI) broth (Oxoid, Ogdensburg, NY, USA) under anaerobic conditions. Subsequent subculturing was done on anaerobic sheep blood agar plates (Oxoid, NY, USA) or Phenyl ether alcohol (PEA) agar plates (Oxoid, NY, USA). They were examined for their morphology, staining, and cultural characteristics.

Suspected *Clostridium perfringens* and *Clostridium septicum* isolates was further identified by biochemical tests using API 20A, (Gubash, 1980; Buchanan, 1982) and PCR. *Clostridium perfringens* was confirmed by 16S rDNA based PCR (Wang et al., 1994) and was genotyped using multiplex PCR (Meer et al., 1997) and alpha-toxin PCR (Kalender et al., 2005). *Clostridium septicum* was confirmed by alpha-toxin PCR (Sasaki et al., 2001). Twelve isolates each of *Clostridium perfringens* type A (UMNCP 1 through UMNCP 12) and *Clostridiun septicum* (UMNCS 101 through UMNCP 112) were included in this study after confirming their identity.

For making the spore culture, *Clostridium perfringens* inoculated blood agar plates was incubated at 37 C for 24 h under anaerobic conditions in Mitsubhisi® anaerobic boxes (Mitsubhisi gas chemical co, NY, USA) to make the preculture stock. One individual colony was picked and transferred to 10ml of pre-reduced Fluid

thioglycolate (FTG) medium (Difco) and incubated at 37 C for 24 h under anaerobic conditions to make the preculture stock.

Sporulation properties of *C. perfringens* isolates were examined as previously described (de-Jong et al 2002; Shih et al., 1996). Briefly, the isolates were cultured in 10 ml pre-reduced FTG tubes for 24 h at 37 C and subsequently they were subcultured in tryptose sulphite cycloserine (TSC) agar with egg yolk (Merck, Darmstadt, Germany). Inoculated plates were incubated anaerobically for 24 h. One individual colony was inoculated into a preculture in FTG, which was incubated for 24 h and subsequently subcultured into Duncan and Strong (DS) sporulation media (Duncan and Strong, 1968). A 5 ml of 24h growth in FTG media was transferred to 30 ml DS media and incubated under anaerobic condition for 24 h at 37C for maximum spore yield. Spore cultures of *Clostridium septicum* were prepared by inoculating into fresh BHI and incubated under anaerobic condition at 37C for 18 h and 25C for 6 h (Cortinas et al., 1997; McCourt et al., 2005).

3. Estimation of heat resistant spore counts

Heat resistant spores from isolates of *Clostridium perfringens* and *C. septicum* were estimated as described before (de Jong et al., 2002). *Clostridium perfringens* and *Clostridium septicum* spore cultures were heated for 20 min at 70C in a water bath and immediately cooled in ice water. Ten-fold serial dilutions were made from these spore cultures in normal saline solution and pour plated into TSC agar plate for *C. perfringens* and Phenylethyl alcohol blood agar (PEA) plates for *C. septicum*. The number of discrete

colonies was then counted after 12 h of anaerobic incubation at 37C to assess the titer of viable spores present. The experiment was repeated four times.

4. Estimation of Toxin production *in vitro* by Hemolytic assay

Hemolytic activity for *C. perfringens and C. septicum* cultures was determined by a microtiter assay as previously described (Ballard et al., 1992; Titball et al., 1989). Briefly, 3 ml of sheep blood was centrifuged and the sedimented RBCs were washed 3 times with normal saline and a 1% RBC suspension in PBS without Calcium and Magnesium was made. The culture supernatants were diluted two-fold serially across the microtiter plate in 100 µl of PBS without Calcium and Magnesium. One-hundred µl of 1% sheep RBC suspension was added into all the wells and the microtiter plate was incubated at 37C for 1h. The highest dilution of the *C. perfringens* or *C. septicum* culture supernatant producing visible hemolysis was considered as the toxin titer. The experiment was repeated four times.

5. *In vivo* studies on the biological effect of Clostridia and their toxins in mice

Twelve isolates each of *C. perfringens* and *C. septicum* were subjected to the mice assay. All *in vivo* experiments involving Clostridia were conducted with prior approved protocols from Institutional Animal Care and Usage Committee (IACUC) and Institutional Biosafety Committee (IBC) of University of Minnesota and the procedures were performed accordingly.

The 24 h spore cultures of both *Clostridium perfringens* and *Clostridium septicum* were tested in mice for the evaluation of the potency of Clostridial toxins present in the

spore culture. The biological activity of both *C. perfringens* and *C. septicum* toxins present in the culture were assessed by determining the 50% minimum lethal dose (MLD_{50}) in mice. MLD_{50} is described as the minimum amount of toxin required to cause mortality in half of the mice population and is expressed in milligrams. The protein content in the culture supernatants was quantitated using a Pierce BCA protein Assay kit (Thermo scientific, IL, USA).

The MLD_{50} was determined as follows. Briefly, six serial dilutions from 100 ul of each spore culture was made in sterile saline. Six mice (Swiss Webster outbred females 18 to 24 g) per group were injected intraperitoneally with 200ul of serially diluted *C. perfringens* or *C. septicum* culture supernatants. Six mice served as sham-inoculated controls and received an equal volume of sterile broth. The effect of Clostridial toxins in mice includes marked respiratory distress, severe convulsions and death. Time to death was monitored for each mouse to obtain an estimate of lethal fractions. The number of survivors was recorded for each group after 48 h. The MLD_{50} was calculated by the method of Reed and Muench (Reed et al., 1938). The experiment was repeated only once.

6. Multilocus sequence typing (MLST)

All twelve isolates each of *C. perfringens* and *C. septicum* isolated from clinical cases of cellulitis were subjected to MLST. *Clostridium perfringens* and *C. septicum* strains were grown on anaerobic sheep blood agar plates (Oxoid, Ltd). The plates were incubated anaerobically at 37°C for 24 h using the AnaeroPack System^{TM} (Mitsubishi Gas Chemical America, Inc., New York, NY). *Clostridium perfringens* and *C. septicum* DNA for PCR was prepared by boiling. Briefly, bacterial cells were scraped from plates,

resuspended in water and subjected to boiling for 20 min. Cell debris was removed by centrifugation at 13,100g for 5 min and the supernatant was used as template DNA for PCR.

Seven housekeeping loci and the alpha toxin gene were selected for the characterization of *C. perfringens* isolates by MLST as described before (Jost et al., 2006). They were ddlA (D-alanine-D-alanine ligase), dut (deoxyuridine-triphosphatase), glpK (glycerol kinase), gmk (deoxyguanylate kinase), recA (recombinase), sod (superoxide dismutase), tpi (triose phosphate isomerase) and plc (alpha toxin). The choice of these genes was based on their use in MLST schemes before (Jost et al., 2006). The *C. perfringens* primers used in this study are shown in Table 1.

Similarly, isolates of *C. septicum* were also subjected to PCR amplifications with Platinum Taq DNA Polymerase (Invitrogen Life Technologies) using oligonucleotide primer sequences for the house-keeping genes csa (alpha toxin), col A (collagenase), recA (recombinase), ddl (D-alanine-D-alanine ligase), gyr A (DNA gyrase subunit A), dnaK (dna kinase), groEL (GroEL protein) and glpK (glycerol kinase) as previously described (Neumann et al., 2009). The *C. septicum* primers used in this study are showed in Table 2.

PCR amplification of DNA was performed using Taq DNA Polymerase (Platinum PCR Supermix, Invitrogen) in a reaction buffer containing 1.65mM MgCl2, 0.22mM dNTPs and 1.5µM of each oligonucleotide primer. PCR was performed using 35 cycles, with one cycle consisting of 1 min at 94C, 1 min at 50C and 1 min at 72C. Final extension step was at 72C for 5 min. The PCR products were then purified using Qiaquick PCR purification kit (Qiagen). The purified PCR products in duplicate were

submitted to Biomedical Genomic Center, University of Minnesota for Sanger sequencing. The DNA sequences were edited using Sequencer version 4.1.2 (Gene Codes, Ann Arbor, MI) and added to an alignment containing the genes extracted from published genome sequences of other *C. perfringens* isolates.

A phylogenetic analysis was conducted using software MEGA 4.1 http://www.megasoftware.net/ mega41.html). The sequence quality of all the Trace files in a chromatogram was read and poor quality sequences were trimmed. The sequence files were renamed according to the genes and isolate names. The sequences were then aligned using Mega 4.1 software and Polymorphisms were recorded. We included sequence types from reference *C. perfringens* strains SM101, ATCC13124, Strain13 which originated from food poisoning, human gas gangrene and soil respectively and *C. septicum* ATCC12464 in the dendrogram to determine the phylogenetic relationship between them. No information is available regarding the origin of *C. septicum* ATCC12464 strain.

Two-parameter distances were computed and used to generate phylogenetic trees using neighbour-joining method. The statistical reliability of internal branches was assessed from 500 bootstrap pseudoreplicates. Sequence types (STs) were assigned on the basis of unique allelic profiles.

7. Two-dimensional gel electrophoresis (2-DiGE) analysis and MALDI-TOF mass spectrometry.

All twelve isolates of *C. perfringens* and *C. septicum* mentioned in the previous chapter were used in this study. The *Clostridium perfringens* culture from fluid

thioglycolate Medium (Difco^{TM}) after anaerobic incubation for 24 h at 37°C were transferred to DS medium to enhance toxin production. The *Clostridium septicum* were cultured in BHI medium (Difco^{TM}) after anaerobic incubation for 24 h at 37°C. The culture supernatants were treated with protease inhibitors and dialyzed by use of 10-kDa cutoff zeba filter columns (Millipore Inc., Billerica, MA) to obtain secreted proteins. The protein content in the culture supernatants was quantitated using a Pierce BCA protein Assay kit (Thermo scientific, IL, USA)

One hundred micrograms of protein sample was subjected to Immobilized pH gradient (IPG) Isoelectric focusing (IEF) in the 1st Dimension. Readystrip IPG strips (Bio-rad laboratories, Hercules, CA) 11 cm of PH range 3-10 were used in this study. This separated proteins by their charge (pI). For SDS second dimension 8-16% Criterion precast gels (Bio-rad laboratories, CA) were used. The resulting gel was then Deep Purple dye (Thermo scientific, Rockford, IL) stained for visualizing the protein spots. The gels were then scanned using an Eagle eye spectrophotometer to obtain image files. The protein spots were identified, spot picked and trypsin digested before subjecting to MALDI-TOF mass spectrometry analysis. Two sets of spots were picked for each isolate and identified by MALDI-TOF mass spectrometry analysis.

Statistical analysis: Statistical analysis was performed using SAS software (SAS Language Version 9.2, SAS Institute Inc, Cary, NC). Pearson correlation coefficient was used to determine whether there is any correlation between spore count, hemolytic titers and $MLD_{50.}$ A P value of less than 0.05 was considered significant.

RESULTS

1. Bacterial Isolation and Identification

Clostridium perfringens appeared as large smooth convex colonies having a double-zone of β-hemolysis on anaerobic sheep blood agar. They were positive for reverse CAMP test and were identified as *Clostridium perfringens* based on API-20A testing. All the twelve *Clostridium perfringens* isolates were identified as *Clostridium perfringens* type A based on multiplex PCR and alpha-toxin PCR.

Clostridium septicum produced a thick swarming growth with a narrow zone of β-hemolysis on anaerobic sheep blood agar. All the twelve *Clostridium septicum* isolates were confirmed using API 20A test and alpha-toxin PCR for *C. septicum*.

2. Estimation of heat resistant spore counts:

We observed a variation in the heat resistant spore counts between the isolates examined (Tables 3 and 4). Among twelve isolates of *Clostridium perfringens*, the average spore count ranged from 1.4×10^8 to 5.2×10^6 spores/ml. Two isolates UMNCP 01 and UMNCP 04, recorded a maximum spore count of 1.4×10^8 and 6.4×10^7 spores/ml respectively. Among the *C. septicum* isolates examined, the spore count ranged from 4.8×10^7 to 2.6×10^5 spores/ml. The isolates UMNCS 106 and UMNCS 107 had the highest spore count of 4.8×10^7 spores/ml and 8.2×10^6 spores/ml respectively.

3. Estimation of Toxin production *in vitro* by Hemolytic assay

We found differences in the hemolytic titers for *C. perfringens* spore cultures. They ranged from 512 to 4 (Tables 3 and 4). The hemolytic titer for *C. septicum* spore

cultures ranged from 256 to 64. *Clostridium perfringens* isolate UMNCP 01 and UMNCP 04 had the highest titer of 512. The hemolytic titers for *C. septicum* spore cultures were 256 and 128 for isolates UMNCS 106 and UMNCS 107, respectively. *Clostridium septicum* isolate UMNCS 106 had the highest titer of 256.

4. *In vivo* studies on the biological effect of Clostridia and their toxins in mice

Mice assay studies revealed a difference between *C. perfringens* and *C. septicum* isolates in their MLD_{50} values (Tables 3 and 4). The MLD_{50} of most potent *C. perfringens* spore cultures were found to be 2.12 mg for UMNCP 01 and 2.75 mg for UMNCP 04. For most *C. septicum* isolates (UMNCS 106 and UMNCS 107) the MLD_{50} was 0.024 mg and 0.031 mg, respectively.

Among *C. perfringens* strains examined, correlation was found between heat resistant spore count and hemolytic titers ($P = 0.0004$) but the correlation was less between hemolytic titers and MLD_{50} values ($P = 0.04$). There was no correlation observed between spore count and MLD_{50} values ($P = 0.11$).

Among *C. septicum* strains examined, correlation was found between heat resistant spore count and hemolytic titers ($P = 0.003$) but the correlation was less between hemolytic titers and MLD_{50} values ($P = 0.02$). There was no correlation observed between spore count and MLD_{50} values ($P = 0.06$).

5. To determine the genetic relationship among *C. perfringens* and *Clostridium septicum* isolates from cases of cellulitis in turkeys using Multilocus sequence typing (MLST)

Clostridium perfringens strains isolated from cellulitis cases were of toxino-type A by PCR. Seven housekeeping genes and one toxin gene were analyzed for characterization of both *C. perfringens* and *C. septicum* isolates by MLST. MLST analysis of 12 *C. perfringens* isolates identified 11 sequence types. Two major clusters were found among the *C. perfringens* isolates examined (Figure 1). All the *C. perfringens* isolates examined fell into a single cluster except UMNCP 8 and UMNCP 9 isolates which clustered with *C. perfringens* strain 13.

Some of the *C. perfringens* isolates like UMN CP 05 and UMNCP06 with less hemolytic titers fell into a cluster by itself. Besides that no such correlation between toxin production and genetic relatedness was observed. Members in both clusters varied considerably in their hemolytic toxin titer representing no direct connection between the expression of hemolytic toxin and phylogenetic relationship. Among the eight loci examined *plc* locus had the maximum number of alleles (8).

MLST analysis of 12 *C. septicum* isolates yielded only two sequence types (ST). All the *C. septicum* isolates examined fell into a single cluster along with ATCC strain 12464 except UMNCS9 isolate (Figure 2). Two polymorphic sites were identified in the amplified fragment of gyrA.

6. To identify the secretory components of *C. perfringens* and *Clostridium septicum* using 2-DiGE analysis and MALDI-TOF mass spectrometry.

The major secretory toxins we identified in *C perfringens* isolates by MALDI-TOF mass spectrometry were phospholipase, collagenase, hyaluronidase, Dnase, enolase, muramidase, pyruvate kinase and hypothetical proteins. We observed two distinct proteomic profiles for *C. perfringens* isolates (Figure 3). We observed only one type of proteomic profile for *C. septicum* isolates (Figure 4). The major secretory toxins we identified in *C. septicum* isolates were alpha toxin, septicolysin, sialidase, Dnase, Transferrin, flagellin and Gelsolin precursor (actin depolymerizing factor).

DISCUSSION

In chickens, a condition similar to cellulitis, often referred to as gangrenous dermatitis, is found to be caused by a different set of bacterial pathogens like *Escherichia coli* (Frazier, 1964; Kumor et al., 1998; McDevitt et al., 2006), *Clostridium perfringens* and *Clostridium novyi* (Reed et al., 1938; Smith-Slatas et al., 2006), *Clostridium septicum* (Gomis et al., 1997; Wilder et al; 2001) and *Staphylococcus aureus* (Wang et al., 1994). *Escherichia coli, Streptococcus fecalis,* Proteus sp., Bacillus sp., *Staphylococcus aureus* and *Clostridium septicum* were isolated from gangrenous dermatitis cases in broiler chickens (Fenstermacher et al., 1939). However organisms isolated from the cellulitis lesions of turkeys in Minnesota in the recent years primarily include *C. septicum* and *C. perfringens* either alone or in combination.

Most of the isolates of *C. perfringens* and *C.septicum* that produced higher spore counts also showed higher hemolytic titers. This is in agreement with the earlier findings

that alpha-toxin production is associated with sporulation in *Clostridium perfringens* (Varga et al., 2004). However, Cortinas et al., 1997, reported an absence of correlation between hemolytic titers and cell growth with few *C. septicum* isolates tested. We observed no correlation between spore counts and MLD_{50} values either in *C. perfringens* or *C.septicum* isolates. This could be due to the differences in the production of non-hemolytic toxins. The low hemolytic activity could be due to the action of endogenous proteases on the hemolytic toxins produced by *C. septicum* (Cortinas et al., 1997).

Though the spore count and hemolytic effects of *C. perfringens* were found to be higher than *C. septicum in vitro*, the lethal effects in mice as well as in turkeys were not comparable. The marked hemolytic activity of *C. perfringens* isolate may be due to the secretion of alpha-toxin (phospholipase C) which is markedly hemolytic (Timoney et al., 1998). Hemolytic activities have also been described for the alpha-toxin produced by *C. septicum.* However, the alpha-toxin of *C. septicum* is not related to the alpha-toxin of *C. perfringens* since it does not exhibit phospholipase C activity and does not cross-react immunologically with *C. perfringens* alpha-toxin (Amimoto et al., 2002). The mortality studies in mice showed that *C. septicum* was more toxic than *C. perfringens* isolates.

We observed differences in the toxicity and biological effects of different strains of *C. perfringens* and *C. septicum* either *in vitro* or *in vivo* in mice. Differences in the toxin production ability as well as disease causing potential between Clostridial isolates have been reported before. In an experimental infection with Clostridia, all the five isolates of *C. perfringens* and three of four of *C. septicum* isolates did not produce any cellulitis lesions in 10-week-old turkey breeder hens when given intravenously (Tellez et al., 2009) whereas only one isolate of *C. septicum* caused disease.

Most of the *C. perfringens* isolates examined fell in a single cluster indicating a clonal relationship. Among the eight loci examined *plc* locus had the maximum number of alleles (8) corroborating the fact that *plc* is highly diverse in nature genetically (Chalmers et al., 2008). The phylogenetic tree constructed from *C. perfringens* isolates indicated a high level of diversity within the housekeeping gene fragments of *C. perfringens*. Our findings are in agreement with previous reports (Jost et al., 2006). However, the dN/dS ratio indicated that most of these polymorphisms found in the eight genes examined resulted from synonymous substitutions. This indicates that no functional change occurred in any of the final protein products.

The data generated by MLST indicate that there is considerable genetic diversity among *C. perfringens* isolates. Some of the *C. perfringens* isolates like UMNCP05 and UMNCP06 with less hemolytic titers fell into a cluster by itself. *Clostridium perfringens* isolates UMN CP05 and UMNCP06 were found to have the least hemolytic titers. Besides that no such correlation between toxin production and genetic relatedness was observed. Members in both clusters varied considerably in their hemolytic toxin titer representing no direct connection between the expression of hemolytic toxin and phylogenetic relationship.

UMNCP 8 and UMNCP 9 isolates were found to be clustered with reference isolate *C. perfringens* strain 13. The latter is reported to be a natural isolate from the soil and pathological studies have shown that this strain can establish experimental gas gangrene (myonecrosis) in a murine model (Shimizu et al., 2001; Rood, 1998). *Clostridium perfringens* strain ATCC13124 which is a human gas gangrene isolate and SM101, a human food poisoning isolate fell apart in a distant cluster indicating no

phylogenetic relationship with any of the turkey isolates examined. There are studies which reveal that *C. perfringens* from porcine origin and NE cases were clonal (Chalmers et al., 2008). However, there were no reports regarding *C. perfringens* isolates from cellulitis cases in turkeys.

The present study indicates that *C. perfringens* isolates from cellulitis cases showed substantial clonality. All the reference strains fall in a subset of its own. This indicates that *C. perfringens* from cellulitis cases showed no ancesteral relationship with reference strains like ATCC 13124 and SM 101.

In summary, MLST results indicate that there is considerable genetic diversity among *C. perfringens* isolates from cellulitis cases and that the MLST scheme can be applied for typing of *C. perfringens* isolates causing cellulitis. MLST analysis of *C. septicum* isolates from cellulitis cases appeared highly clonal. The diversity observed among isolates of *C. septicum* in this study is considerably less than has been reported for similar analyses performed on *C. perfringens* (Jost et al., 2006) and *C. septicum* (Neumann et al., 2010). One possible explanation for this is that our analysis included primarily isolates from cellulitis cases in turkeys whereas these other two studies examined isolates from many different animal hosts and disease presentations.

The major secretory toxins in *C. perfringens* isolates were phospholipase, collagenase, hyaluronidase, dnase, muramidase and hypothetical proteins. Hypothetical proteins were observed mostly in profiles of C. perfringens isolates having highest hemolytic titers. The use of it as a biomarker for cellulitis causing *C. perfringens* isolates needs to be further investigated. *Clostridium perfringens* is reported to produce different types of toxins and the toxin profile varies with the strain of bacteria (Sawires et al. 2006;

Smedley et al., 2004). *C. perfringens* produces extracellular toxins including beta2 toxin, enterotoxin, perfringolysin, collagenase, lambda toxin, hyaluronidase, dnase, neuraminidase and urease (Sawires et al. 2006). Our results suggest involvement of different toxins of *C. perfringens* in pathogenesis of cellulitis in turkeys.

Clostridium perfringens isolate UMNCP01 that appeared most potent differed in their secretory protein profile from less potent isolate like UMNCP06. The role of hypothetical protein 1232 needs to be further investigated. The functional annotation of this protein is suggestive of a protease. The proteomic profiles of all *C. septicum* isolates appeared identical.

The major secretory toxins in *C. septicum* isolates were alpha toxin, septicolysin, sialidase, Dnase, flagellin and Gelson precursor (actin depolymerizing factor). The Gelson precursor (actin depolymerizing factor) might have some role in the pathogenesis of cellulitis, but more studies are warranted to confirm this assertion. Since *C. septicum* genome is not completely sequenced, not much information is available for the development of a recombinant vaccine at this point. Unlike *C. perfringens* we observed only one type of proteomic profile for *C. septicum* isolates. These results support our findings in the MLST analysis as well as previous reports (Neumann et al., 2010) that genomes of *C. septicum* poultry isolates are highly conserved.

TABLES

Table 1. Oligonucleotide primers used for *C. perfringens* MLST (Jost et al., 2006)

ene	Primer	Sequence (5^1–3^1)	Amplicon size (bp)
plc	Forward	ATATGAATGGCAAAGAGGAAAC	544
	Reverse	AGTTTTTCCATCCTTTGTTTTG	
ddlA	Forward	ATAATGGGGGATCATCAGTTGC	429
	Reverse	TTATTCCTGCTGCACTTTTAGG	
dut	Forward	TTAAGTATTTTGATAACGCAAC	441
	Reverse	CTGTAGTACCAAATCCACCACG	
glpK	Forward	TGGGTTGAGCATGATCCAATGG	547
	Reverse	CACCTTTTGCTCCAAGGTTTGC	
gmk	Forward	TAAGGGAACTATTTGTAAAGCC	475
	Reverse	TACTGCATCTTCTACATTATCG	
recA	Forward	GCTATAGATGTTTTAGTTGTGG	475
	Reverse	CTCCATATGAGAACCAAGCTCC	
sod	Forward	GATGCTTTAGAGCCATCAATAG	475
	Reverse	AATAATAAGCATGTTCCCAAAC	
tpi	Forward	AAATGTGAAGTTGTTGTTTGCC	451
	Reverse	CATTAGCTTGGTCTGAAGTAGC	

Table 2. Oligonucleotide primers used for *C. septicum* MLST (Neumann et al., 2009)

Gene	Primer	Sequence (5^1–3^1)	Amplicon size (bp)
colA	Forward	GCTTCAGCATATGAACTAGTTAAGGG	562
	Reverse	CTGGATGTCCTTCGTCTAAAGGCT	
csa	Forward	TGATTGCAATTCAGTGTGCGGCAG	632
	Reverse	GCTTGTCTCAACTTTACTGTCAGC	
ddl	Forward	GCTTTGCATGGTAAATTTGGTGAAGATGG	597
	Reverse	AGGAGTCCTATCCTCTGTAACTATCA	
dnaK	Forward	GCAGTTATGGAAGGTGGAGAACCA	744
	Reverse	ACCAGTTGCATCTGCAGTGATGAG	
glpK	Forward	CCCAATGGAAATTTGGGCAAGCCA	606
	Reverse	GCTGCTTGTTGGTCTCCAGCAATA	
groEL	Forward	GGGAGCACAGTTAGTGAAGGAAGT	630
	Reverse	CCTGGTGCCTTTACTGCAACACAT	
gyrA	Forward	GGTGATAATGCAGCGGCAATGAGA	792
	Reverse	GATATGAGCTCTTGCTTCAGCC	
recA	Forward	TGCAGAGCATGCCTTAGATCC	600
	Reverse	CCATATGAGAACCAAGCTCCAC	

Table 3. Heat resistant spore count, hemolytic titers and MLD_{50} of various isolates of *C. perfringens*

	Isolate ID#	source	HR-spore count (per ml)	Hemolytic titer	MLD_{50} (mg)
1	UMNCP 01	skin lesion	14×10^7	512	2.12
2	UMNCP 02	skin lesion	0.84×10^7	224	4.86
3	UMNCP 03	skin lesion	3.4×10^7	96	3.92
4	UMNCP 04	skin lesion	6.4×10^7	512	2.75
5	UMNCP 05	skin lesion	2.6×10^7	4	8.50
6	UMNCP 06	skin lesion	5.1×10^7	28	8.45
7	UMNCP 07	liver	5.8×10^7	144	5.64
8	UMNCP 08	skin lesion	0.52×10^7	84	6.32
9	UMNCP 09	liver	4.6×10^7	96	5.68
10	UMNCP 10	skin lesion	3.5×10^7	56	6.34
11	UMNCP 11	skin lesion	2.8×10^7	84	7.52
12	UMNCP 12	skin lesion	1.8×10^7	112	3.68

Table 4. Heat resistant spore count, hemolytic titers and MLD_{50} of various isolates of *C. septicum*

	Isolate ID#	source	HR-spore count (per ml)	hemolytic titer	MLD_{50} (mg)
1	UMNCS 101	skin lesion	$3 .9 \times 10^6$	96	0.12
2	UMNCS 102	skin lesion	2.4×10^6	40	0.082
3	UMNCS 103	skin lesion	4×10^6	112	0.14
4	UMNCS 104	skin lesion	3.9×10^6	96	0.126
5	UMNCS 105	skin lesion	4.5×10^6	40	0.158
6	UMNCS 106	skin lesion	48×10^6	256	0.024
7	UMNCS 107	skin lesion	8.2×10^6	128	0.031
8	UMNCS 108	skin lesion	30×10^6	28	0.264
9	UMNCS 109	skin lesion	0.26×10^6	96	0. 236
10	UMNCS 110	liver	5.1×10^6	80	0.24
11	UMNCS 111	skin lesion	1.7×10^6	28	0.15
12	UMNCS 112	skin lesion	6.4×10^6	112	0.168

FIGURES

Figure 1. Phylogenetic tree constructed by Neighbor-Joining method from concatenated sequences of various *C. perfringens* isolates from cellulitis cases and reference isolate Strain 13, ATCC13124 and SM101.

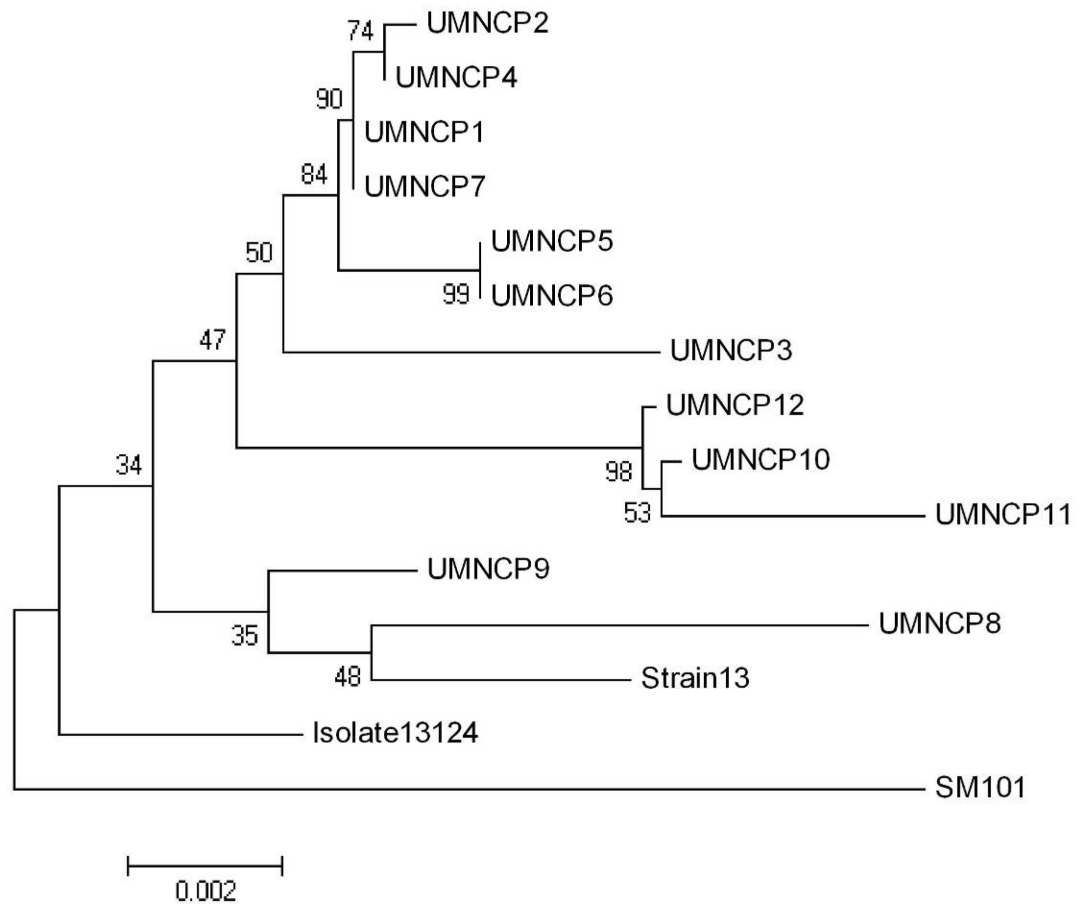


Figure 2. Phylogenetic tree constructed by Neighbor-Joining method from concatenated sequences of various *C.septicum* isolates from cellulitis cases and reference isolate ATCC12434.

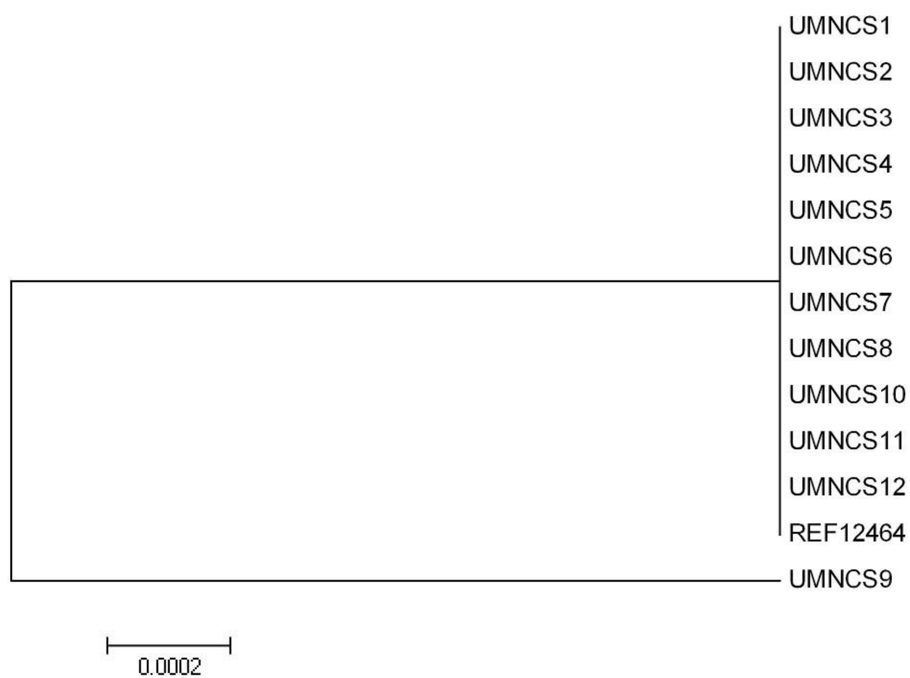


Figure 3. Two distinct proteomic profiles of *C. perfringens* UMNCP01 and UMNCP 06 isolates after 2-DiGE.

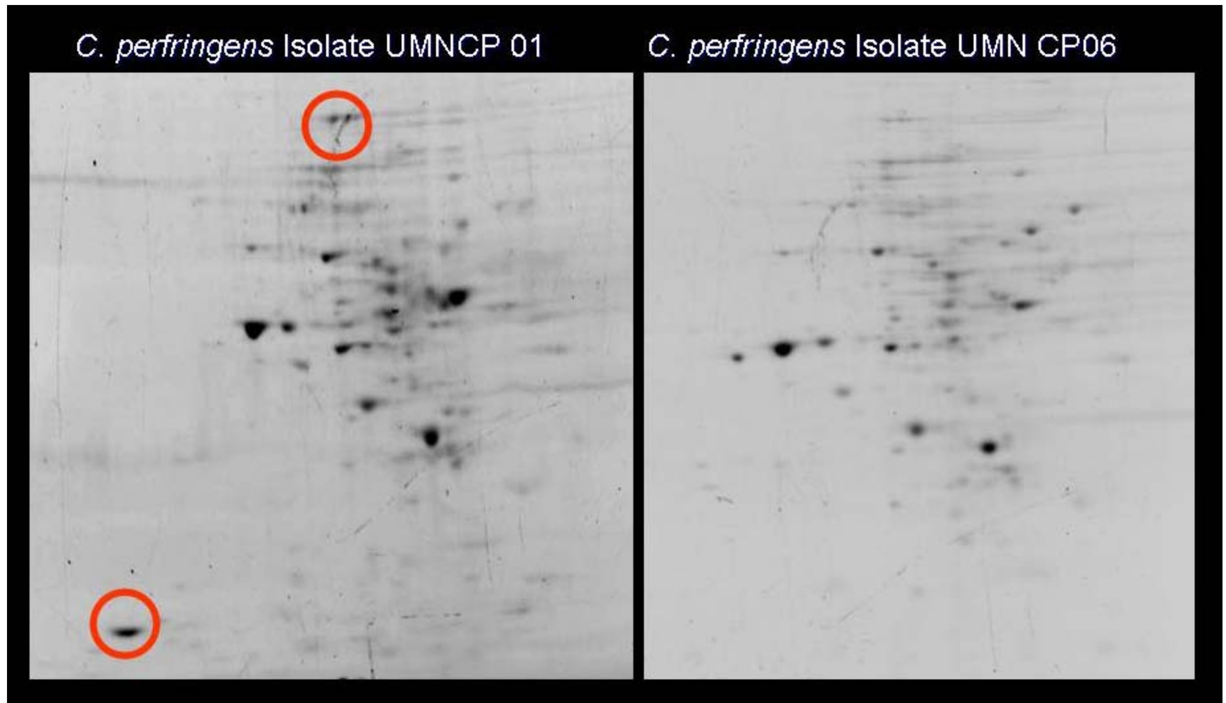
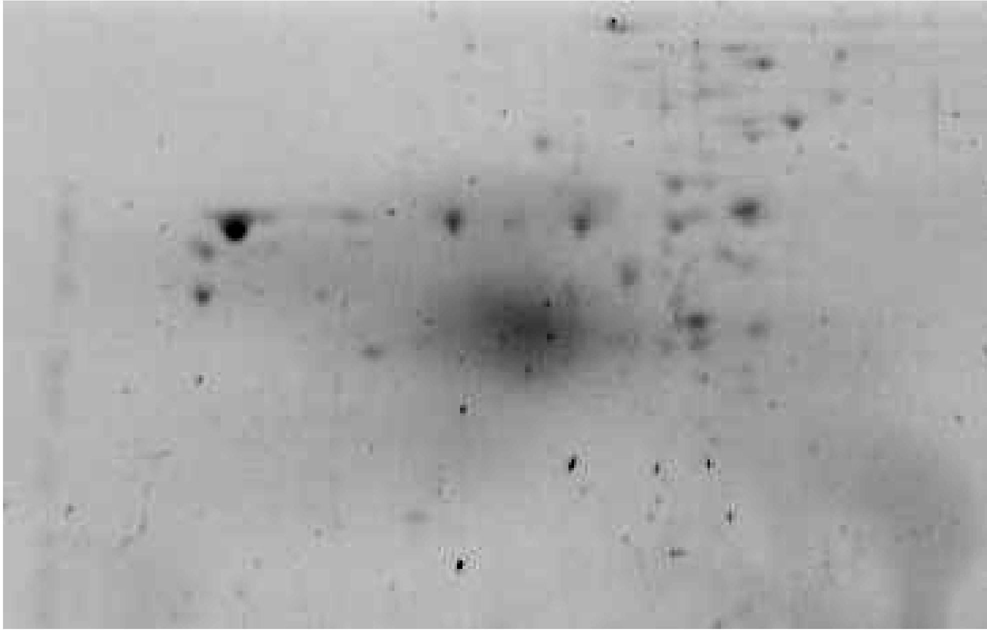


Figure 4. Proteomic profile of of *C. septicum* after 2-DiGE



CHAPTER IV

ROLE OF *CLOSTRIDIUM PERFRINGENS* AND *CLOSTRIDIUM SEPTICUM* IN CELLULITIS IN TURKEYS

Summary

Clostridium perfringens and *Clostridium septicum* are widespread in the poultry environment and those happened to be the primary agents we isolated from cellulitis cases in turkeys. The objective of this study was to examine the role of *Clostridium perfringens* and *Clostridium septicum* in the experimental development of cellulitis lesions in turkeys. The study was conducted in turkeys of 3-weeks of age and 7-weeks of age. A higher mortality was observed in turkey poults given *C. septicum* culture as compared to birds given *C. perfringens* culture in both age groups of birds. These observations were comparable to our mice assay results where *C. septicum* culture was found to be more lethal to mice even at lower doses when compared with the *C. perfringens* culture. However, mortality and cellulitis lesions were more pronounced in birds of 7-weeks of age than in birds of 3-weeks of age. Surprisingly, in 7-week-old birds, the development of fulminant cellulitis lesions and mortality was markedly higher than in 3-week old birds. Administration of either 1 ml of *C. septicum* or 3 ml of *C. perfringens* produced a higher percentage of cellulitis mortality in 7-week-old birds. The cellulitis lesion development was also more prominent in 7-week old birds than in 3-week old birds. More over, there were no *C. perfringens* or *C. septicum* noticed microscopically in the muscle tissues examined from lesions in 3-week old birds. In 7-week old birds died of cellulitis, there were numerous multiple patches of muscles in which the fibers were fragmented because of bacterial multiplication giving the areas a "moth eaten" appearance. There were several areas in the subcutaneous tissues and muscles that were infiltrated by large numbers of inflammatory cells. Multiplication of either *C. perfringens* or *C. septicum* in the muscle tissues of cellulitis affected birds has not

been reported before. Numerous thick, rod-shaped bacteria were also present in the areas of inflammation in the subcutaneous tissues and muscle fibers clearly indicating the multiplication of bacteria within these regions and subsequent damage due to the enormous amounts of toxins liberated. Thus, *C. septicum* appears to be more potent in causing severe cellulitis lesions and higher mortality in turkeys although the role of *C. perfringens* in causing fatal cellulitis cannot be ignored. Our results indicate that both agents may contribute to the outcome of the disease. Our cellulitis disease model offer promise to use it as a challenge model in the development of vaccines against cellulitis in turkeys.

INTRODUCTION

Clostridium perfringens and *Clostridium septicum* have been suspected in playing a role in causing cellulitis and mortality in turkeys. Organisms isolated from the cellulitis lesions of turkeys in Minnesota primarily include *C. perfringens and C. septicum* either alone or in combination. The isolation of *C. perfringens* from cellulitis cases of chickens and turkeys has been emphasized in many reports (Carr, 1996; Hofacre et al., 1986). Little is known about the pathogenic potential of *C. perfringens and C. septicum* as disease reproduction is often difficult. *Clostridium perfringens* was reported to cause cellulitis in turkeys and experimental reproduction of the disease has been successful in adult turkeys (Carr et al., 1996). Detailed studies had not been under taken with *C. septicum* so far. Recent reports claim *C. septicum* as the primary agent causing cellulitis in turkeys based on the isolation results (Tellez et al., 2009), however the role of *C. septicum* isolates in the development of cellulitis lesions was not studied in detail.

Clostridium perfringens and *Clostridium septicum* are widespread in the poultry environment. *Clostridium perfringens* is even found in the intestinal tract of healthy poultry as a normal inhabitant, usually in low numbers (Songer, 1996). However, little is known about the distribution and sources of *C. septicum* in poultry production facilities.

The age range at which cellulitis is seen in turkeys appears to be varied. In the field, one can observe cellulitis cases even in birds as young as 7-weeks of age. Since there is no consistency, we planned on examining birds of two different age groups: at 3-weeks age and at 7-weeks of age. The younger age was first examined to see whether we could reproduce the disease in this age group assuming that if this can be done it would reduce housing, ease of handling considerations. The objective of this study was to

examine the roles of *Clostridium perfringens* and *Clostridium septicum* in the experimental development of cellulitis lesions in turkeys.

MATERIALS AND METHODS

Bacteria: The spore culture preparations of *C. perfringens* (UMNCP 01) and *C. septicum* (UMNCS 106) which had the highest spore count, hemolytic activity and MLD_{50} were used to study their effects in turkey poults. *Clostridium perfringens* culture containing 6.4 x 10^7 heat resistant spores/ml and *C. septicum* (UMNCS 106) culture containing 8.2 x 10^6 heat resistant spores/ml were used in this study. The MLD_{50} /ml of *C. perfringens* (UMNCP 01) spore culture was found to be 2.12 mg and *C. septicum* (UMNCS 106) 0.024 mg.

Effect of *C. perfringens* and *C. septicum* in three-week-old turkeys

A dose-response study was conducted to examine the lethal effects of *C. perfringens* and *C. septicum* spore cultures following subcutaneous inoculation and to examine the development of any cellulitis in 3-week-old turkey poults. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) and the procedures were performed in accordance with the requirements. The experimental birds were kept and reared in Research Animal Resources (RAR) isolation facilities of University of Minnesota.

Experimental design

The studies were conducted with seventy-two, 3-week-old commercial Nicholas White turkey poults obtained from farms with no history of cellulitis. Two treatments

were done by subcutaneous injection with either *C. perfringens* or *C. septicum* spore culture to assess the biological effect.

Briefly, the birds were randomly divided into two groups, I and II, of thirty six birds each. Each group was further divided into six subgroups of six birds each. Birds in each subgroup in group I were given different doses (0.5 ml, 0.75 ml, 1 ml, 2 ml and 3 ml) of *C. perfringens* culture alone subcutaneously on the breast region. Birds from group II, were given different doses (0.5 ml, 0.75 ml, 1 ml, 2 ml and 3 ml) of *C. septicum* culture alone subcutaneously in the breast region. One subgroup consisting of six birds each in groups I and II served as sham-inoculated controls where 3ml of sterile anaerobic culture media was injected subcutaneously on the breast region. The dead birds at 24 h and 48 h were necropsied and tissue samples from cellulitis lesions were examined for bacterial isolation and histopathology.

Effect of *C. perfringens* and *C. septicum* in seven-week-old turkeys

A dose-response study was conducted to examine the development of cellulitis in 7-week-old turkey poults. From most field observations, seven weeks of age appears to be the earliest age at which turkeys were found to be developing cellulitis. In this study, we used the same *C. perfringens* and *C. septicum* spore cultures that we used to test the 3-week-old poults. The inoculum contained either *C. perfringens* or *C. septicum* spore cultures.

A study was conducted with 36, seven-week-old birds. Different doses of 0.5 ml, 0.75 ml, 1 ml, 2 ml and 3 ml each of a preparation containing either *C. perfringens* or *C. septicum* spore culture were inoculated into three birds in each subgroup. The route of

inoculation in seven-week-old turkey poults was subcutaneously on the breast region. The dead birds at 12 h, 24 h and 48 h were necropsied and tissue samples from cellulitis lesions were examined for bacterial isolation and histopathology.

RESULTS

Effect of *C. perfringens* and *C. septicum* in three-week-old turkeys

The mortality observed in birds injected with spore cultures of *C. perfringens* or *C. septicum* preparations is presented in Table 1. Higher mortality was observed with *C. septicum* culture than with *C. perfringens*. Among birds inoculated with cultures from *C. septicum* there was mortality even at lower doses (2 ml and 1 ml). In group of birds given *C. perfringens* culture, mortality was seen only in those given a higher dose (3 ml). These observations were comparable to our mice assay where the *C. septicum* culture was found to be more lethal to mice even at lower doses when compared with the *C. perfringens* culture.

A visual inspection revealed no blisters or vesicles in any part of the body externally. The breast, thigh muscles and internal organs appeared hyperemic in birds. There was little subcutaneous accumulation of inflammatory frothy fluid at the inoculated area or anywhere else in the subcutaneous tissues.

Histopathology: Histopathology of the subcutaneous and muscle tissues from dead birds revealed few microscopic lesions and inflammatory changes. These birds revealed few localized areas of hemorrhage in the dermis and subcutis. The subcutis and deep dermis were infiltrated by few heterophils and macrophages. A few thick rod-shaped bacteria

were visible in the inflamed subcutaneous area. The underlying muscle tissues did not exhibit any inflammatory changes or presence of bacteria in any of the birds.

Effect of *C. perfringens* and *C. septicum* in seven-week-old turkeys

The mortality observed in birds injected with *C. perfringens* and *C. septicum* in seven-week-old birds is shown in Table 2. We observed 100% mortality within 12 h when either 2 ml or 3 ml of *C. septicum* spore culture was inoculated. The mortality was 66 % even in the one ml inoculated group. No mortality was observed when either 0.75 ml or 0.5 ml of the same inoculum was injected. However, in the *C. perfringens* inoculated group, mortality was observed only with 3 ml of inoculation.

Cellulitis lesions developed mainly on the breast region and extended to the abdomen and tail regions of the birds in both *C. perfringens* and *C. septicum* inoculated groups (Figure 1A). Blisters and vesicles were also noticed on the breast region in both groups (Figure 1B). In the *C. perfringens* treated group, 3 ml inoculation was required to produce consistent lesions. There was subcutaneous accumulation of a frothy viscous inflammatory fluid in cellulitis lesions in all the dead birds in both *C. perfringens* as well as *C. septicum* inoculated birds (Figure 1C). However blisters and vesicles were more prominent and extensive with birds given 2 ml or 3 ml of spore culture of *C. septicum*. In birds given 2 ml of spore culture of *C. septicum*, the lesions were noticed on the breast region as well as on the abdomen and tail regions. The presence of frothy sanguinous subcutaneous exudate in the subcutaneous tissue was more apparent in *C. septicum* group inoculated with 2 ml as compared to the 3 ml of *C. septicum* inoculated group or the 3 ml of *C. perfringens* inoculated group. Palpitation of the affected areas often revealed

crepitation due to gas bubbles in the subcutis and musculature in all the dead birds. The breast muscle and internal organs were hyperemic in birds given higher doses (1 ml, 2 ml and 3 ml) of *C. septicum*.

Clostridium perfringens and *C. septicum* were reisolated from cellulitis lesions of dead birds. All birds which were given 1 ml, 0.75 ml, and 0.5 ml of *C. perfringens* or 0.75 ml, and 0.5 ml of *C. septicum* survived without showing any signs of cellulitis. In these birds, a visual inflammatory swelling was noticed at the site of inoculation in 24 and 48 h, which started subsiding by 96 h.

Histopathology: Histopathology of subcutaneous and muscle tissues from birds that died of *C. perfringens* infection showed edema in the dermis. The dermal capillaries were engorged with blood. There was marked fibrin exudation and acute hemorrhage in the dermis and subcutis. The subcutis and deep dermis were infiltrated by large numbers of heterophils and macrophages. Subcutaneous tissues contained large clear spaces consistent with appearance of gas bubbles. Moderate numbers of thick rod-shaped bacteria were visible in the inflamed area (Figure 1D). Many of the bacteria had terminal spores. Some of the underlying muscle fibers had lost cellular detail (Figure 1F). There were patches of muscle in which the fibers were fragmented giving the areas a “moth eaten” appearance. There were a few areas in the muscle that were infiltrated by inflammatory cells. Thick rod-shaped bacteria were present in the areas of inflammation in the muscle fibers clearly indicating the multiplication of bacteria within the muscle fibers and subsequent damage possibly due to the toxins liberated.

In the *C. septicum* inoculated group, subcutaneous and muscle tissue examination revealed microscopic lesions and inflammatory changes in birds given higher doses (1ml, 2 ml and 3 ml) of *C. septicum*. These birds also showed edema in the dermis. The dermal capillaries also were engorged with blood. There was marked fibrin exudation and acute hemorrhage in the dermis and subcutis. In the 1 ml *C. septicum* inoculated group, the subcutis and deep dermis were infiltrated by numerous heterophils and macrophages where few to moderate bacteria were present (Figure 1E). However, in the 3 ml inoculated group, the subcutaneous tissues contained large clear spaces consistent with appearance of gas bubbles. Numerous rod-shaped bacteria were also present in the inflamed area but the heterophils and macrophages were absent in areas where active bacterial multiplication was going on.

In all the birds given higher doses (1ml, 2 ml and 3 ml) of *C. septicum*, the underlying muscle fibers had lost considerable cellular detail (Figure 1G). There were numerous multiple patches of muscle in which the fibers were fragmented giving the areas a “moth eaten” appearance (Figure 1H). There were several areas in the muscle that were infiltrated by large numbers of inflammatory cells. Numerous thick, rod-shaped bacteria were also present in the areas of inflammation in the muscle fibers clearly indicating the multiplication of bacteria within the muscle fibers and subsequent damage possibly due to the toxins liberated.

DISCUSSION

We were able to successfully reproduce cellulitis lesions and mortality in turkeys. Both *Clostridium perfringens* and *Clostridium septicum* were found to cause similar

cellulitis lesions in seven-week-old turkeys. In a recent report of an experimental infection with Clostridia, all five isolates of *C. perfringens* and three isolates of *C. septicum* out of four isolates tested did not produce mortality in 10-week-old turkey breeder hens when given intravenously (Tellez et al., 2009). The reported mortality with the *C. septicum* challenge was 78.5 % in a group of fourteen poults..

It is widely presumed that the natural outbreaks of cellulitis in turkeys are associated with a proliferation of Clostridial pathogens in the poultry environment. One can argue that a reasonable model of the disease should closely resemble the probable route of natural pathogen exposure, i.e.: oral. Necrotic enteritis is yet another disease in chickens caused by *C. perfringens*. Interestingly, efforts for consistent reproduction of NE by oral inoculation of *C. perfringens* in different labs has resulted in extremely variable results ranging from severe clinical signs, subclinical NE in exposed birds to no lesions at all (Aiello et al., 2003, Carr et al., 1996; Olkowski et al., 1999 and Titball et al., 1999). Such a significant lack of consistency in scientific data generated in different labs is puzzling in view of a large number of claims identifying predisposing factors (Martel et al., 2004; Ninomiya et al., 1994).

Attempts to reproduce cellulitis in turkeys by injecting purified alpha-toxin of *C. perfringens* subcutaneously have been successful but injections with *C. perfringens* alone have not been successful (Carr, 1996). Moreover, oral challenges have not been successful with either *C. perfringens* or *C. septicum* in our repeated earlier attempts. This directed us to investigate this disease in turkeys through subcutaneous inoculations of varying amounts of spore cultures of *C. perfringens* and *C. septicum.*

The mortality studies in mice as well as in three-week old birds showed that *C. septicum* spores cultures were more toxic than *C. perfringens* spore cultures. *C. perfringens* spore cultures produced mortality only at higher doses of 3 ml whereas *C. septicum* caused mortality even at the lower dose of 1 ml in turkeys.

Surprisingly, in seven-week-old birds, the development of fulminant cellulitis and mortality was markedly higher than in three-week old birds. Administration of either 1 ml of *C. septicum* or 3 ml of *C. perfringens* produced a higher percentage of mortality in seven-week-old birds. The cellulitis lesion development was also more prominent in seven-week old birds than in three-week old birds. More over, there were no *C. perfringens* or *C. septicum* noticed in the muscle tissues examined from lesions in three-week old birds. Considering the fact that a seven-week-old poult will weigh approximately four times that of a three-week-old poult, it appears that the younger birds are least susceptible to Clostridial cellulitis than the older birds. This finding also correlates with the potential age of onset of cellulitis one may notice in turkeys in the field.

The gross lesions produced by *C. perfringens* and *C. septicum* were almost identical. However, the fulminant nature and the extent of lesions in the muscle tissue were more pronounced in the *C. septicum* inoculated birds than the *C. perfringens* inoculated birds. The gross and microscopical lesions obtained after administration of spore cultures of either *C. perfringens* or *C. septicum* in seven-week-old birds were comparable to the cellulitis lesions reported either with *C. perfringens* experimental infection or field observations reported by Carr et al., 1996. However, multiplication of

either *C. perfringens or C. septicum* in the muscle tissues of cellulitis affected birds has not been reported before.

In conclusion, *C. septicum* appears to be more potent in causing severe cellulitis lesions and higher mortality in turkeys although the role of *C. perfringens* in causing fatal cellulitis cannot be ignored. It could be possible that both agents may contribute to the outcome of the disease. Our cellulitis disease model offer promise to use it as a challenge model in the development of vaccines against cellulitis in turkeys.

TABLES

Table 1: Mortality in three-week old birds following subcutaneous injection with either *C. perfringens* or *C. septicum* spore culture.

	Group I [A] (*C. perfringens*)					Group II [A] (*C. septicum*)				
	3ml	2ml	1ml	0.75ml	0.5ml	3ml	2ml	1ml	0.75ml	0.5ml
24h	1	0	0	0	0	3	2	1	0	0
48h	1	0	0	0	0	1	0	0	0	0
Total (%)	2(33)	0(0)	0(0)	0(0)	0(0)	4(66)	2(33)	1(16)	0(0)	0(0)

[A] Each dose was inoculated in 6 birds each (n=6)

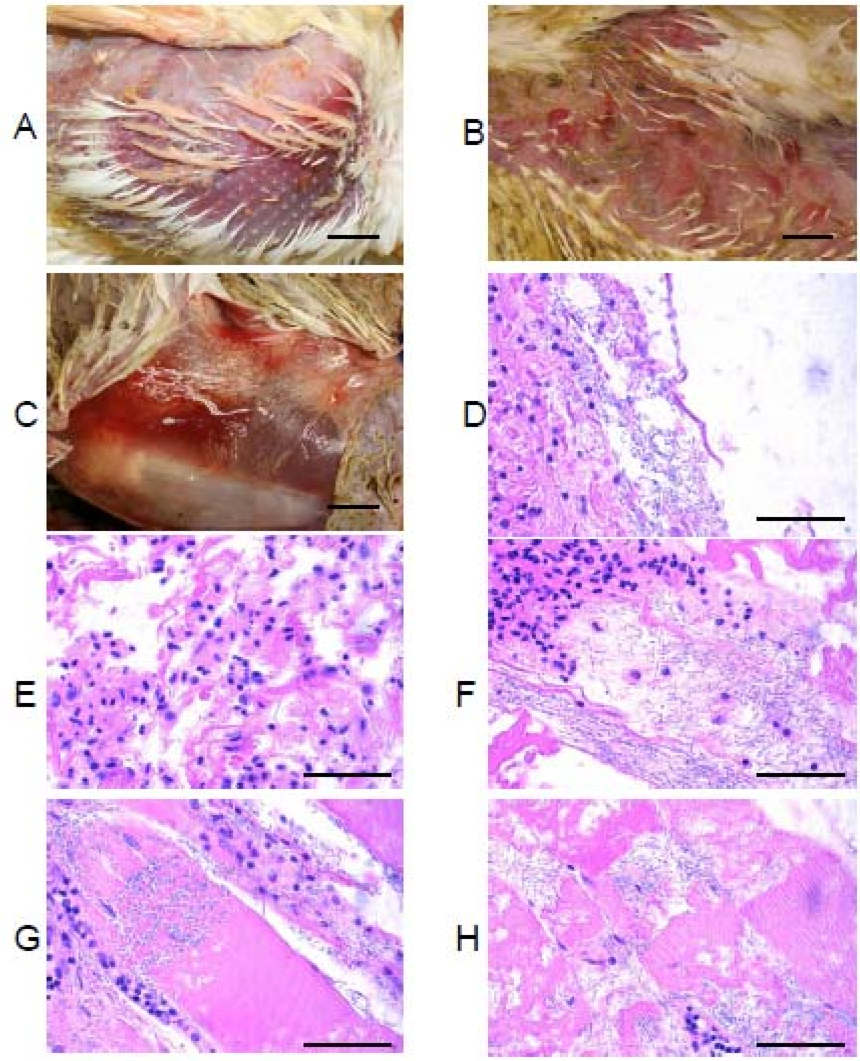
Table 2: Mortality in seven-week old birds following subcutaneous injection with either *C. perfringens* or *C. septicum* spore culture.

	Group I [A] (*C. perfringens*)					Group II [A] (*C. septicum*)				
	3ml	2ml	1ml	0.75ml	0.5ml	3ml	2ml	1ml	0.75ml	0.5ml
12h	1	0	0	0	0	3	0	0	0	0
24h	1	0	0	0	0	0	2	0	0	0
48h	0	0	0	0	0	0	1	1	0	0
Total (%)	2(66)	0(0)	0(0)	0(0)	0(0)	3(100)	3(100)	1(33)	0(0)	0(0)

[A] Each dose was inoculated in 3 birds each (n=3)

FIGURES

Figure 1. Gross lesions and histopathologic changes of subcutaneous and muscle tissue in cellulitis in turkeys. Sections were stained with H& E. (A) Inflammation extending from the breast region to abdomen and tail regions in a bird inoculated with 3 ml of *C. perfringens* spore culture. Bar = 1cm. (B) Vesicles and blisters extending on the breast region in a bird inoculated with 2 ml of *C. septicum* spore culture. Bar = 1 cm. (C) The presence of froathy sanguinous exudate in the subcutaneous tissue in a bird inoculated with 3 ml of *C. perfringens* spore culture. Bar = 1 cm. (D) Subcutaneous tissue showing presence of moderate number of thick rod-shaped bacteria in a bird inoculated with 3 ml of *C. perfringens* spore culture. Bar = 100 µm.(E) Subcutaneous tissue showing presence of thick rod-shaped bacteria in a bird inoculated with 1 ml of *C. septicum* spore culture. Bar = 100 µm. (F) Breast muscle studded with thick rod-shaped bacteria in a bird inoculated with 3 ml of *C. perfringens* spore culture. Bar = 50 µm. (G) Breast muscle studded with thick rod-shaped bacteria in a bird inoculated with 2 ml of *C. septicum* spore culture. Bar = 50 µm. (H) Breast muscle with multiple patches of muscle tissue in which the fibers were fragmented giving a “moth eaten” appearance in a bird inoculated with 3 ml of *C. septicum* spore culture. Bar = 50 µm.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CHAPTER V

LABORATORY STUDIES ON A BIVALENT VACCINE CONTAINING *CLOSTRIDIUM PERFRINGENS* AND *CLOSTRIDIUM SEPTICUM* TOXOID TO CONTROL CELLULITIS IN TURKEYS

Summary

Clostridium perfringens and *Clostridium septicum* were recognized as the causative agents for cellulitis in turkeys. The objective of this study was to develop and to evaluate the use of a bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid to control cellulitis in commercial Nicholas White turkeys. A bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid was prepared and tested in six-week-old commercial Nicholas White turkey poults under laboratory conditions for its safety and efficacy. Vaccinated birds were then exposed to a homologous challenge. The mortality and lesion development in vaccinated and unvaccinated controls were recorded and compared. Blood samples from birds in both groups were examined to detect antibody response to *C. perfringens* and *C. septicum* toxoid using ELISA. All the birds challenged with *C. perfringens* and *C. septicum* in non-vaccinated group developed severe cellulitis lesions and died within 24 h of challenge. There were several areas in the subcutaneous tissues and muscles that were infiltrated by large numbers of inflammatory cells. Numerous thick, rod-shaped bacteria were also present in the areas of inflammation in the subcutaneous tissues and muscle fibers indicating the multiplication of bacteria. The bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid developed was found to be completely safe and protective against homologous challenge. It produced antibodies which appeared protective. Two doses of vaccine was found to be more effective in protection than a single dose of the same preparation. The bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid could now be tested in turkeys in the field to reduce losses due to cellulitis in turkeys.

INTRODUCTION

The prevalence and severity of cellulitis has increased over the last several years, since the time it was first reported in 1939. This could be due to increasing regulations in the use of in-feed antibiotic growth promoters (AGP) and restriction in antibiotic usage (Li et al., 2010b). Currently, cellulitis in turkeys has been diagnosed in Minnesota, Wisconsin, Missouri, Virginia, and other turkey producing areas (Clark et al., 2010).

Turkey industry in Minnesota and elsewhere is in need of an effective vaccine to control cellulitis in their production facilities. The objective of this study was to develop a *Clostridium perfringens* and *Clostridium septicum* formalin-inactivated toxoid (FIT) vaccine for cellulitis in turkeys.

Clostridial vaccines containing toxoids and killed bacteria have been successfully used in humans and animals against various clostridial infections. Sheep challenged with *C. perfringens* toxoid were found to be protected against gas gangrene caused by *C. perfringens* (Boyd et al., 1972).

In this study, we have examined the effect of immunization of turkeys with an experimental bivalent *C. perfringens* and *C. septicum* toxoid in an attempt to control cellulitis in turkeys under laboratory and field conditions.

MATERIAL AND METHODS

Preparation of a bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid

Based on our previous studies (Chapter 3) we selected *C. perfringens* (UMNCP 1) and *C. septicum* (UMNCS 106) to make a bivalent toxoid against cellulitis in turkeys. The stock cultures of these isolates were grown in DS sporulation media and BHI (Oxoid,

Ogdensburg, NY) respectively for 24 h. At the end of 24 h, aliquots from each culture were tested in mice for the toxin content. The MLD_{50} in mice for *C. perfringens and C. septicum* was found to be 2.12 mg and 0.024 mg toxin units respectively. The cultures were then treated with 0.5 % formalin and incubated at 37 C for 18 h for inactivation of the toxin. The complete inactivation of the anaculture was confirmed by subculturing a sample on anaerobic sheep blood agar plates and TSC agar plates anaerobically. Failure of any growth was taken as complete inactivation of bacteria. The inactivation of the toxins was also confirmed by hemolysis assay using sheep RBC.

The bivalent toxoid was mixed with vaccine grade mineral oil Dakreol-6VR (Pennsylvania Refining Company, Butler, PA) as adjuvant and Arlacel-A (Sigma Chemicals) as an emulsifier. Arlacel-A was used at the rate of 10 % (vol/vol) in the oil component of toxoid. Briefly, for every 1,000 ml of the experimental bivalent toxoid vaccine, the aqueous component contained bacterin-toxoid of *C. perfringens* (500 ml) and *C. septicum* (7.81 ml). The oil component contained mineral oil (472.97 ml) and Arlacel-A (19.22 ml). A water-in-oil emulsion was prepared after slowly mixing the oil component in a blender to which the aqueous component was added over a period of 5 min. The final toxoid preparation was adjusted to contain 2 MLD_{50} toxin units per ml each of inactivated *C. perfringens and C. septicum* preparation.

Laboratory evaluation of bivalent toxoid in turkey poults.

The experimental bivalent toxoid was first tested in six-week-old turkey poults for its safety and efficacy. The birds were inoculated subcutaneously with either of the two

doses: a 1ml dose containing inactivated 2 MLD_{50} toxin units or a 2ml dose containing inactivated 4 MLD_{50} toxin units of the toxoid preparation.

Briefly, forty eight six-week-old commercial Nicholas White turkey poults obtained from a source with no history of cellulitis were divided into four groups (groups I to IV) of twelve birds each. Birds in Group I were inoculated with 1 ml dose and birds in group II were inoculated with 2ml dose each of the toxoid preparation subcutaneously at the wing web. The birds in group III and IV were treated as non-vaccinated controls. All the birds were monitored twice daily for any adverse effects of the toxoid. Any birds showing severe pain and distress were to be euthanized in a carbon dioxide chamber. On fourteenth day post-vaccination, half of the birds from group I and II were given a booster dose of corresponding experimental toxoid at the same dose as the first vaccine. The blood from all the birds was collected at 0, 14 and 28 days post- one-vaccination and on 7-days-post booster vaccination for serological examination.

After 28-days post-vaccination, six birds each from groups I and II which received one vaccination and six birds from non-vaccinated controls (group III) were challenged with subcutaneous inoculation of 1 ml each of *C. perfringens* and *C. septicum* culture containing 1.4×10^8 and 4.8×10^7spores/ml respectively on the breast region. Similarly, after 14-days post- booster vaccination, remaining six birds each from groups I and II and six birds from non-vaccinated controls (group III) were challenged .The challenge dose we used in this experiment was the one we optimized in our previous studies. The blood from all the birds were examined for sero-conversion to the vaccine using an ELISA test. All the birds at the end of the study were euthanized in a carbon dioxide chamber as per AVMA guidelines for euthanasia (2007).

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), and the procedures were performed in accordance with the requirements. The animals were reared at Research Animal Resources (RAR) isolation facilities at the University of Minnesota (Saint Paul, MN).

Statistical Analysis: Statistical analysis was performed by SAS software (SAS Language Version 9.2, SAS Institute Inc, Cary, NC). The mortality data following challenge was analyzed using Fishers exact test. The serological data was analyzed using GLM procedure for repeated measures analysis of variance where a P value of 0.05 was considered as significant.

RESULTS

The use of the experimental bivalent toxoid was found to be safe and no adverse effects were noticed in any of the birds at the site of inoculation with either administration of a 1 ml or 2 ml dose. All the birds challenged with *C. perfringens* and *C. septicum* in non-vaccinated group (group III) developed severe cellulitis lesions (Figure 1A- 1F) and died within 24 h of challenge (Table 1).

A single administration of experimental bivalent toxoid at a 2 ml dose per bird protected all birds post-challenge. No mortality or cellulitis lesion development was observed in birds twice vaccinated and challenged in the vaccinated groups. Birds once vaccinated with 1ml dose per bird lost one bird due to cellulitis within 48 h post-challenge.

The serum antibody titers (OD values) obtained for *C. perfringens* and *C. septicum* ELISA are shown in Tables 2 and 3 respectively. A significant rise in *C.*

perfringens and *C. septicum* antibody titers were noticed in the vaccinated birds on 14 and 28 days post one-vaccination or two vaccinations with either doses of the vaccine.

DISCUSSION

Subcutaneous inoculation of *C. perfringens* and *C. septicum* cultures consistently produced cellulitis lesions and mortality in naïve turkeys in this study as in our previous studies. The cellulitis lesions produced with (Figure 7A-F) incoculation of *C. perfringens* and *C. septicum* cultures (Figure 7A-F) were comparable with the lesions produced before with either *C. perfringens* or *C. septicum* cultures separately.

The experimental bivalent *C. perfringens* and *C. septicum* toxoid which we developed was found to be completely safe and effective in reducing cellulitis lesions and mortality in turkeys under laboratory conditions. There are a number of reports on the use of Clostridial vaccines. Clostridial vaccines against *C. perfringens* and *C. septicum* containing toxoids and killed bacteria have been successfully used before against clostridial infections in humans and animals (Boyd et al., 1972). Similarly, a multicomponent *C. perfringens, C. septicum* and Pasteurella bacterin was reported to reduce mortality in gangrenous dermatitis affected broiler chickens (Gerdon et al., 1973).

Serum ELISA results showed significant antibody response against *C. perfringens* as well as *C septicum* in vaccinated birds. Antibody response against *C. septicum* bacterin/toxoid preparation was reported before in immunized turkeys under experimental conditions (Tellez et al., 2009). In our laboratory studies, the use of the toxoid was found to protect against cellulitis in all the vaccinated birds when compared

with non-vaccinated birds. Better protection and high serum antibody titers were noticed in birds administered either 2 doses of 1 ml vaccination or one dose of 2 ml vaccination. Our results offer promise for the use of *C. perfringens* and *C. septicum* toxoid successfully to mitigate cellulitis cases in turkeys in the field.

In summary, the experimental bivalent *C. perfringens* and *C. septicum* toxoid we developed offered a complete protection against cellulitis following homologous challenge under experimental conditions. Use of a use of a higher concentration (2ml dose) of the toxoid or a booster vaccination may offer a better protection against cellulitis due to *C. perfringens* and *C. septicum* in turkeys.

TABLES

Table 1: Mortality following challenge with *C. perfringens* and *C. septicum* in vaccinated and non-vaccinated birds (n=6).

	Non-vaccinated	Vaccinated	
		@ 1ml dose	@ 2ml dose
Once vaccinated	6/6[a]	1/6[a]	0/6[b]
Twice vaccinated	6/6[a]	0/6[a]	0/6[b]

[a,b] Fraction of birds within a row with no common superscript are considered significantly different (P<0.001).

Table 2: *C. perfringens* alpha-toxin ELISA serum antibody titer (OD values) following vaccination with *C. perfringens* and *C. septicum* toxoid in vaccinated and non-vaccinated birds.

	Post-one-vaccination OD Values			Post-two-vaccinations OD Values
GROUPS	0dpv	14dpv	28dpv	7dp2v
Vac @ 1ml dose	0.3296 (0.07)^{a} *	0.3597(0.06)^{b}	0.4682(0.13)^{b}	0.5963(0.13)^{c}
Vac @ 2ml dose	0.3307 (0.06)^{a}	0.34984 (0.05)^{b}	0.5474(0.10)^{c}	0.6006(0.20)^{c}
Non-vaccinated	0.3155 (0.06)^{a}	0.3386 (0.08)^{a}	0.3212(0.08)^{a}	0.3404 (0.07^{a})

* Values in paranthesis indicate standard deviation

dpv = days-post-vaccination.

^{abcd} Means within a row with no common superscript are considered significantly different ($P < 0.05$). 0.4682(0.13)^{b}

Table 3: *C. septicum* ELISA serum antibody titer (OD values) following vaccination with *C. perfringens* and *C. septicum* toxoid in vaccinated and non-vaccinated birds.

	Post-one-vaccination OD Values			Post-two-vaccinations OD Values
	0dpv	14dpv	28dpv	7dp2v
Vac @ 1ml dose	0.2701(0.07)[a] *	0.3309 (0.06)[a]	0.4095(0.12)[b]	0.5658(0.19)[c]
Vac @ 2ml dose	0.2880 (0.08)[a]	0.3441 (0.07)[a]	0.4984(0.16)[b]	0.644(0.15)[c]
Non-vaccinated	0.2889 (0.07)[a]	0.2465 (0.08)[a]	0.2884(0.07)[a]	0.2689(0.09)[a]

* Values in paranthesis indicate standard deviation

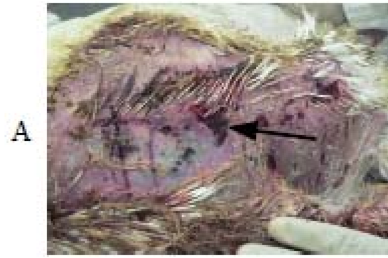
dpv = days-post-vaccination.

[abcd] Means within a row with no common superscript are considered significantly different ($P < 0.05$).

FIGURES

Figure 1. Gross lesions and histopathologic changes of subcutaneous and muscle tissue in cellulitis in turkeys from *C. perfringens* and *C. septicum* challenge. Sections were stained with H & E. (A) Blisters and vesicles extending from the breast region to abdomen and tail regions in a bird inoculated with *C. perfringens* and *C. septicum* spore culture. (B) The presence of froathy sanguinous subcutaneous exudate in the subcutaneous tissue in a bird inoculated with *C. perfringens* and *C. septicum* spore culture. (C) Histopathology section showing presence of moderate number of thick rod-shaped bacteria in the subcutaneous tissue in a bird inoculated with *C. perfringens* and *C. septicum* spore culture. Bar = 100 µm (D) Histopathology section showing presence of multiple patches of muscle tissue in which the fibers were fragmented giving the areas a "moth eaten" appearance in a bird inoculated with *C. perfringens* and *C. septicum* spore culture Bar = 50 µm. (E) A nonvaccinated bird showing cellulitis lesions on the breast region post-inoculation of *C. perfringens* and *C. septicum.* (F) A vaccinated bird showing absence of any lesions on the breast region at 5-day-post inoculation of *C. perfringens* and *C. septicum* toxoid.

A



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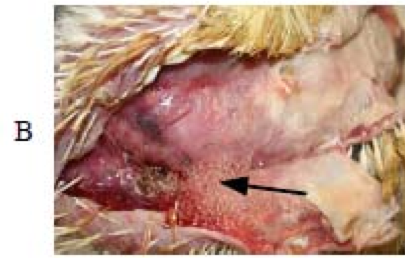

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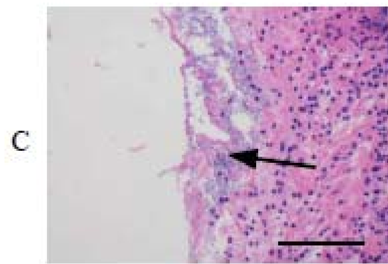

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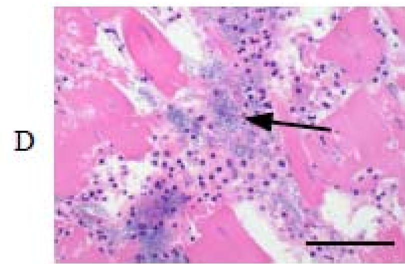
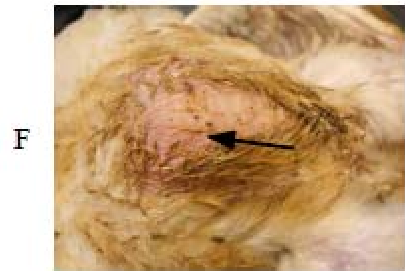


E

F



CHAPTER VI

FIELD TRIALS WITH BIVALENT *CLOSTRIDIUM PERFRINGENS* AND *CLOSTRIDIUM SEPTICUM* TOXOID

Summary

Cellulitis has emerged as a major problem in the turkey industry over the last few years. *Clostridium perfringens* and *Clostridium septicum* were identified as the causative agents for cellulitis in turkeys. The objective of this study was to field test the bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid we developed to reduce cellulitis mortality and antibiotic usage in commercial turkeys. The bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid was evaluated for its use in the field in a flock of commercial turkeys consisting of 16,000 birds where 8000 birds were vaccinated and an equal number were kept as non-vaccinated controls. The birds were vaccinated at six-weeks of age. Cellulitis related mortality in both groups was recorded and compared. Blood samples from birds in both groups were examined to detect antibody response to *C. perfringens* and *C. septicum* toxoid using ELISA. The use of bivalent *Clostridium perfringens* and *Clostridium septicum* toxoid developed was found to be safe and effective. It produced antibodies which appeared protective and did significantly reduce antibiotic usage to control cellulitis as well as mortality. Though the toxoid did not offered complete protection in the field the use of a booster dose may help in better protection against cellulitis in turkeys.

INTRODUCTION

With permission from the Board of animal health and University of Minnesota and consent from a leading commercial turkey producer in Minnesota, a field trial was conducted in a cellulitis endemic farm to test the efficacy of our experimental bivalent *C. perfringens and C. septicum* toxoid.

MATERIALS AND METHODS

Experimental design: The commercial turkey brooder barn we selected typically broods 16,000 turkey poults. All the birds were male poults that were vaccinated for Newcastle (3d & 3wk) and hemorrhagic enteritis (HE) (4wk). One half of the birds (n=8,000) at the age of 6-weeks were selected randomly from a single brooder barn for vaccination. One milliliter dose of the vaccine was administered subcutaneously at the neck region using a vaccine applicator.

Following vaccination, all the vaccinated and non-vaccinated groups were transferred to grower farms where there was a consistent history of cellulitis. The vaccinated and non-vaccinated birds were kept separated in the same barn by putting a firm wire-mesh separation in the middle of each barn. The birds were likely to be exposed to Clostridia found in the poultry environment (natural challenge). A therapeutic antibiotic penicillin G sodium usage in water was in place as a precautionary measure as and when mortality rose above 0.1% per day. Daily mortality and packs of penicillin (one pack of penicillin contains 1.0 billion I.U. (601.2 g) of Penicillin G Potassium) used to control mortality was recorded in both vaccinated and non-vaccinated birds from 13 to 22 weeks of age. In the farm, there is routine monitoring of all sick, moribund and dead

birds twice daily and those identified sick and moribund were euthanized. The results were compared to detect any observable differences in the usage of antibiotics or occurrence of cellulitis related mortality between vaccinated and non-vaccinated birds.

Following field vaccination, five birds each at 10-weeks of age from vaccinated and non-vaccinated groups from each farms were transferred to RAR facilities at University of Minnesota. Blood was collected for ELISA and all the birds were challenged as mentioned before. The cellulitis development and mortality between the groups was recorded. All the birds at the end of the study were euthanized in a carbon dioxide chamber.

Statistical analysis: Statistical analysis was performed using SAS software (SAS Language Version 9.2, SAS Institute Inc, Cary, NC). The mortality data following challenge with spore culture was analyzed using Fishers exact test. A survival analysis of maximum likelihood estimates using proportional hazards model (PH-REG procedure) was conducted to analyze the mortality rates in vaccinated and non-vaccinated groups. A Pearson's Chi square test was used to compare the use of antibiotics between vaccinated and non-vaccinated groups. The serological data was analyzed using GLM procedure for repeated measures analysis of variance where a P value of 0.05 was considered as significant.

RESULTS

Field trials with bivalent toxoid in commercial turkeys

Mortality in non-vaccinated and vaccinated birds are shown in Table 1 and Figure 1. There were significant differences in the mortality (P<0.001) between non-vaccinated group and vaccinated group. The use of this experimental bivalent toxoid reduced mortality from 9.4 % to 7.4 %. This accounts to 21% reduction in cellulitis related mortality in vaccinated birds when compared to non-vaccinated birds. A Kaplan Meier survival curve comparing the survival distribution function of non-vaccinated and vaccinated birds was shown in Figure 2. The Hazard ratio was found to be 1.3 for the non-vaccinated group over vaccinated group of birds.

The need for use of antibiotic penicillin was significantly reduced because of vaccination (P<0.0001) from 547 packs in non-vaccinated birds to 361 packs in vaccinated birds (Figure 3). This accounts for a 35% reduction in the use of antibiotics in vaccinated birds when compared with non-vaccinated birds.

Laboratory challenge studies with bivalent *C. perfringens and C. septicum* vaccinated and non vaccinated birds from the field.

When we challenged ten birds transferred from vaccinated and non-vaccinated groups from field trials at the university, we observed a mortality of 80% (8/10) in 24 h in non-vaccinated birds at 95% confidence level with a confidence interval of 55-100%. The two birds which survived did show signs of cellulitis lesions at 2-6 days but the lesions subsided in few days. All birds from vaccinated group resisted challenge and did not develop any signs of cellulitis lesions or mortality and were found to be completely

protected. Vaccinated birds had *C. perfringens* alpha toxin ELISA serum antibody titer of 0.4861 +/- 0.09 and non-vaccinated birds had an antibody titer of 0.3945 +/- 0.10. The *C. septicum* ELISA serum antibody titers were 0.4582 +/- 0.11 and 0.3654 +/- 0.13 respectively.

DISCUSSION

In our laboratory studies, better protection were seen in birds administered either 2 doses of 1 ml vaccination or one dose of 2 ml vaccination. However, administration of a volume of greater than 1 ml was not tried under field conditions due to difficulties in labor and handling.

When a single administration at 1ml dose of our experimental bivalent *C. perfringens* and *C. septicum* toxoid was used under field conditions, it was found to be protective and it reduced number of cellulitis cases, antibiotic usage and mortality in commercial turkeys. The toxoid significantly reduced the mortality by 21 % in vaccinated birds when compared with non-vaccinated birds. A 21 % reduction in mortality is highly significant, considering the fact that the disease appears in adult birds and the normal mortality rate at this age group is negligible in commercial settings**.** The reason for a partial protection was assumed to be due to insufficient antigen concentration in a single dose of the vaccine. Being a toxoid, multiple vaccinations may be required for prolonged protection.

The use of *C. septicum* bacterin/toxoid preparation elicited antibody response against *C. septicum* in immunized turkeys under experimental conditions. Tellez et al.,

2009 also reported similar findings in vaccinated turkeys however no challenge studies were conducted to demonstrate protection.

The incidence of *C. perfringens* and *C. septicum* associated diseases in poultry has increased significantly in the recent years because of the reduced use of antimicrobial growth promoters (van Immerseel et al., 2004). Cellulitis is commonly controlled in a preventative manner now by incorporation of antimicrobial drugs in the feed or water, but this practice is increasingly criticized or has been banned in some countries. Since cellulitis cases in turkeys are also rising at an alarming rate, there is a need to investigate alternative biologic approaches for its effective control. We were able to reduce the dose and duration of antibiotic Penicillin G sodium usage significantly in turkeys where the vaccine was used to control cellulitis and mortality when compared with non-vaccinated controls.

It is widely accepted that the natural outbreaks of cellulitis in turkeys are associated with proliferation of pathogens in the poultry environment. Under field conditions, the commercial poults are reared on deep litter systems and the litter is not replaced for every flocks. Build up of Clostridial load may occur during this time. A greater environmental load may help these Clostridial spores to persist and also may increase the chance of fecal-oral route of spread over time. Under such scenarios, adverse effects of greater severity may be expected in the field when birds were exposed to extremely high dose of Clostridial challenge than under experimental conditions. However additional studies are required to know the effect of Clostridial load in the litter on the incidence of cellulitis cases under field and experimental conditions.

With single vaccination in two farms, in antibiotic cost alone the industry saved approximately 4278 dollars considering the fact that each pack of penicillin costs 23

dollars. When reduction in mortality is also taken into account, the average savings per barn could be more than six thousand dollars. Our results establish the fact that *C. perfringens* and *C. septicum* toxoids could be used successfully to mitigate cellulitis cases in turkeys in the field.

In summary, the experimental bivalent *C. perfringens* and *C. septicum* toxoid we developed offered a complete protection against cellulitis following homologous challenge under experimental conditions. The same vaccine enabled us to reduce the mortality and use of antibiotic treatment in preventing cellulitis in commercial turkeys. Multiple vaccinations as well as use of a higher concentration of antigens or a more refined toxoid may offer a better protection against cellulitis due to *C. perfringens* and *C. septicum* in turkeys.

TABLES

Table 1. Comparison of mortality and antibiotic usage post-vaccination with *C. perfringens* and *C. septicum* toxoid in non-vaccinated and vaccinated birds from 13-weeks to 22-weeks of age.

	Non-vaccinated group	Vaccinated group
Number of birds	8,000	8,000
Mortality	748	595 [a]
% Mortality	9.4	7.4 [a]
Total penicillin usage*	547 packs	361 packs [a]
Penicillin usage days	59	31[a]

[a] indicates $P < 0.05$

* one pack of penicillin contains 1.0 billion I.U. (601.2 g) Penicillin G Potassium

Figure 1. Mortality following vaccination with a bivalent *C perfringens* and *C. septicum* toxoid in non-vaccinated (line) and vaccinated birds (broken line) from 13-weeks of age until 22-weeks of age.

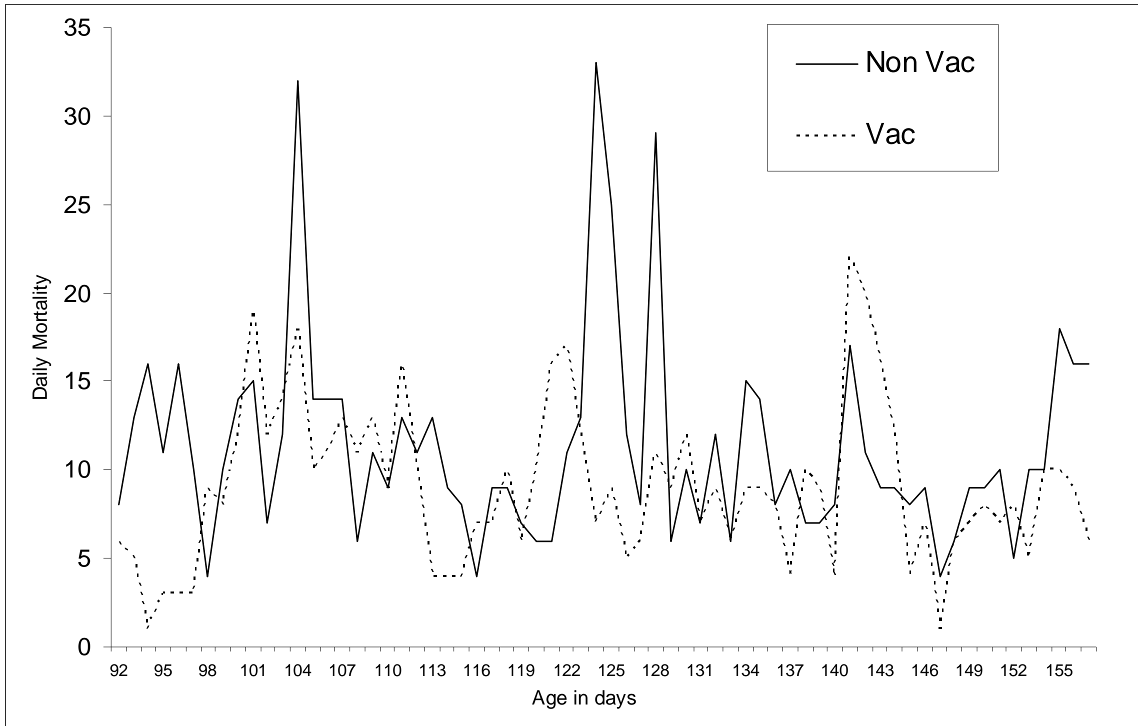


Figure 2: Kaplan Meier survival curve showing the survival distribution function following vaccination with bivalent *C perfringens* and *C. septicum* toxoid in non-vaccinated (broken line) and vaccinated birds (line) from 13-weeks of age until 22-weeks of age.

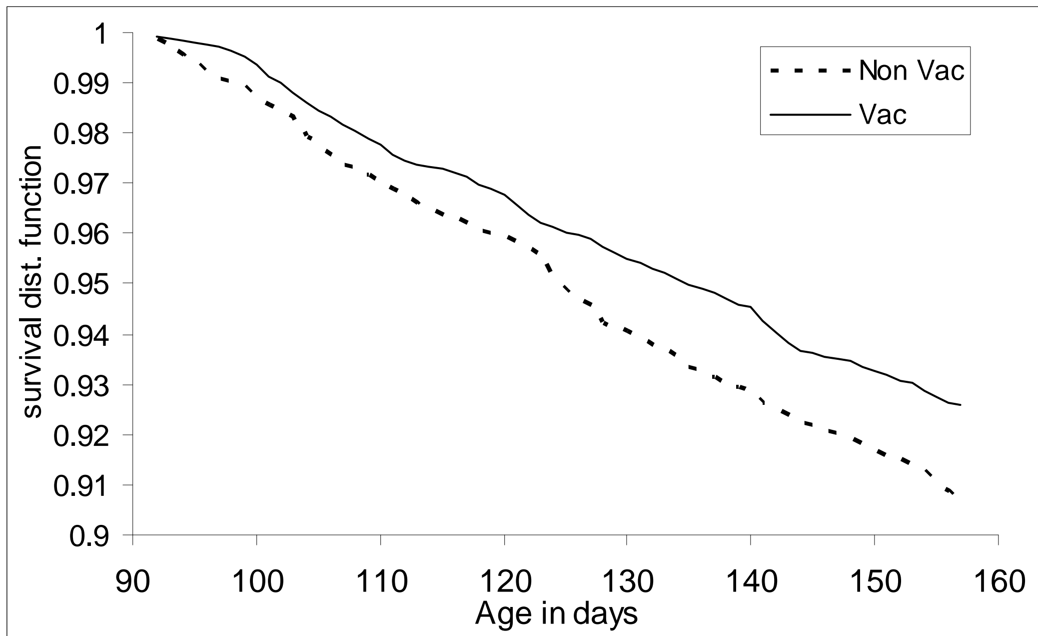
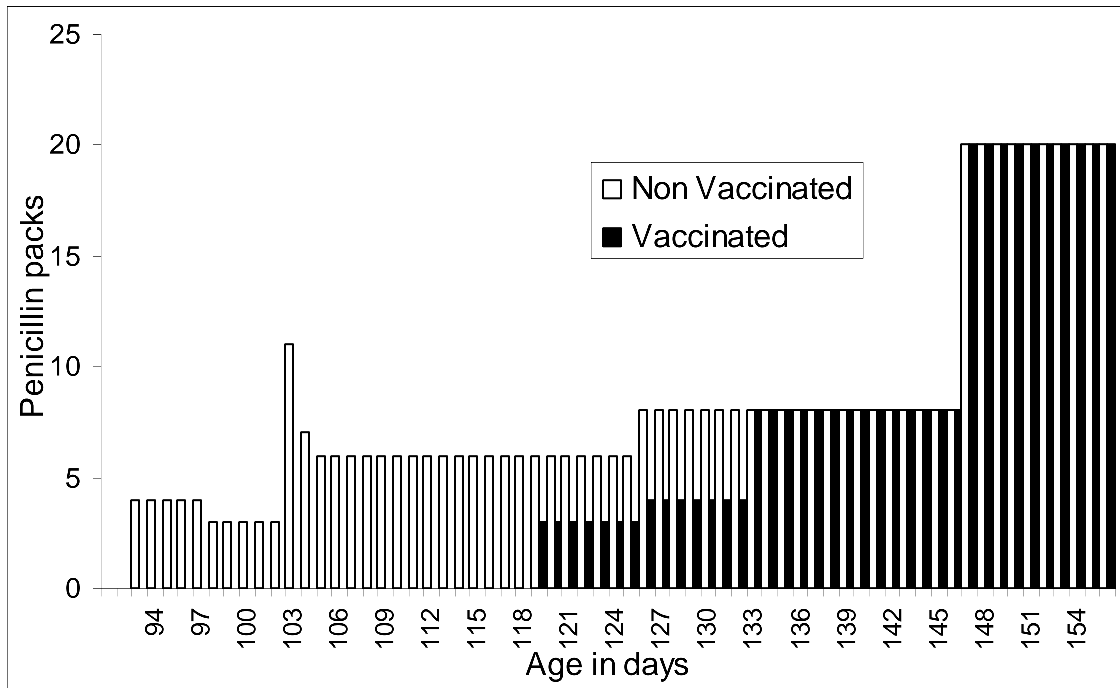


Figure 3. Antibiotic usage following vaccination with bivalent *C perfringens* and *C. septicum* toxoid in non-vaccinated (black bars) and vaccinated birds (white bars) 13-weeks of age until 22-weeks of age.



CHAPTER VII

LABORATORY STUDIES ON *CLOSTRIDIUM SEPTICUM* TOXOID TO CONTROL CELLULITIS IN TURKEYS

Summary

Isolation results from cellulitis cases from various diagnostic laboratories across the state indicated an increased isolation rate of *Clostridium septicum* alone from cellulitis cases in the recent years. More over, results from our study also suggested increased potency for *Clostridium septicum* to cause cellulitis in turkeys than *Clostridium perfringens.* The objective of this study was to develop and to evaluate the use of a *Clostridium septicum* anaculture toxoid alone to control cellulitis in commercial turkeys. A *Clostridium septicum* toxoid was prepared and tested in six-week-old commercial Nicholas White turkey poults under laboratory conditions for its safety and efficacy. Vaccinated birds were exposed to a homologous challenge. The mortality and lesion development in vaccinated and unvaccinated controls were recorded and compared. Blood samples from birds in both groups were examined to detect antibody response to *C. septicum* toxoid using ELISA. The birds challenged with *C. septicum* in vaccinated group did not show any cellulitis lesions or mortality where as birds in the non-vaccinated group (group III) developed severe cellulitis lesions and died within 24 h of challenge. Better protection were seen in birds administered either 2 doses of 1ml vaccination or one dose of 2ml vaccination. The *Clostridium septicum* toxoid developed was found to be completely safe and effective against homologous challenge. It produced antibodies which appeared protective. The *Clostridium septicum* toxoid developed could now be tested in the field to reduce losses due to cellulitis in turkeys.

INTRODUCTION

Over the years we noticed a change in the isolation pattern of Clostridial isolates from cellulitis cases in turkeys. Data from diagnostic labs as wells as from turkey field veterinarians (personel communication) indicated an increased isolation of *C. septicum* as against *C. perfringens* from cellulitis cases. Our experimental data showed greater potency for *C. septicum* than *C. perfringens* to cause cellulitis in turkeys. Recent studies (Tellez et al., 2009) have drawn attention to *C. septicum* and their role in causing cellulitis.

The objective of this study was to develop and test a *Clostridium septicum* alone toxoid in the changing circumstances for cellulitis in turkeys. In this study, we have examined the effect of immunization of turkeys with an experimental *C. septicum* toxoid vaccine in an attempt to control cellulitis in turkeys under laboratory and commercial conditions.

MATERIAL AND METHODS

Bacterial isolates and growth conditions: Based on our previous studies with Clostridial isolates for their spore and toxin production and mice lethal assay, we selected *C. septicum* (UMNCS 106) isolate for vaccine production. It was grown in BHI at 37 C for 18 h and 25 C for 6 h. The cultures were then treated with 0.5 % formalin and incubated at 37 C for 18 h for inactivation of the toxin. The complete inactivation was confirmed by subculturing a sample on anaerobic sheep blood agar plates anaerobically. Failure of any growth was taken as complete inactivation of bacteria. The inactivation of the toxins was also confirmed by hemolysis assay using sheep RBC.

Preparation of experimental formalin-inactivated toxoid (FIT)

An experimental toxoid was prepared using formalin-inactivated *C. septicum* culture and a vaccine grade mineral oil Dakreol-6VR as adjuvant and Arlacel-A as an emulsifier. Arlacel-A was used at the rate of 10 % (vol/vol) in the oil component of toxoid. Briefly, for every 1,000 ml of the experimental bivalent toxoid vaccine, the aqueous component contained bacterin-toxoid of *C. septicum* (500 ml). The oil component contained mineral oil (450 ml) and Arlacel-A (50 ml). A water-in-oil emulsion was prepared after slowly mixing the oil component in a blender to which the aqueous component was added over a period of 5 minutes. The final toxoid preparation was adjusted to contain 128 MLD_{50} toxin units per ml of inactivated *C. septicum* preparation. The experimental birds were then vaccinated at different doses to evaluate the efficacy of the vaccine at each dose rate. The different dose rates examined are 1ml (128 MLD 50 toxin units) and 2ml (256 MLD 50 toxin units).

Laboratory evaluation of *C. septicum* toxoid in turkey poults.

The experimental bivalent toxoid was first tested in six-week-old turkey poults for its safety and efficacy. The birds were inoculated subcutaneously with either of the two doses: a 1 ml dose containing inactivated 128 MLD_{50} toxin units or a 2 ml dose containing inactivated 256 MLD_{50} toxin units of the toxoid preparation.

Briefly, ninety six, six-week-old commercial Nicholas White turkey poults obtained from a source with no history of cellulitis were divided into four groups (groups I to IV) of twenty four birds each. Birds in Group I were inoculated with 1ml dose and birds in group II were inoculated with 2ml dose each of the toxoid preparation

subcutaneously at the wing web. The birds in group III and IV were treated as non-vaccinated controls. All the birds were monitored twice daily to see for any adverse effects of the toxoid. Any birds showing severe pain and distress were planned to be euthanized in a carbon dioxide chamber. On fourteenth day post-vaccination, half of the birds from group I and II were given a booster dose of corresponding experimental toxoid at the same dose as the first vaccine. The blood from all the birds was collected at 0, 14 and 28 days post- one-vaccination and on 7-days-post booster vaccination for serological examination.

After 28-days post-vaccination, twelve birds each from groups I and II which received one vaccination and twelve birds from non-vaccinated controls (group III) were challenged with subcutaneous inoculation of 2 ml of *C. septicum* culture containing 4.8 x 10^7spores/ml respectively on the breast region. Similarly, after 14-days post- booster vaccination, remaining twelve birds each from groups I and II and six birds from non-vaccinated controls (group III) were challenged. The sera from all the birds were examined for sero-conversion to the vaccine using an ELISA test. All the birds at the end of the study were euthanized in a carbon dioxide chamber as per AVMA guidelines for euthanasia (2007).

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), and the procedures were performed in accordance with the requirements. The animals were reared at Research Animal Resources (RAR) isolation facilities at the University of Minnesota (Saint Paul, MN).

RESULTS

Laboratory evaluation of *C. septicum* toxoid in turkey poults.

No adverse effects were noticed in any of the birds at the site of inoculation of experimental bivalent toxoid with either administration of 1ml or 2ml dose of the toxoid. All the birds challenged with *C. septicum* in non-vaccinated group (group III) developed severe cellulitis lesions and died within 24 h of challenge (Table 1).

Groups given 1ml dose rate (128 MLD 50 units) per bird lost one bird with in 48 h of inoculation. Two vaccinations with a low dose (1 ml) of toxoid were as protective as one or two doses of high dose (2 ml) of toxoid.

A single administration of experimental bivalent toxoid at a 2ml dose per bird protected all birds post-challenge. No mortality or cellulitis lesion development was observed in birds twice vaccinated and challenged in the vaccinated groups. Birds once vaccinated with 1 ml dose per bird lost one bird due to cellulitis within 48 h post-challenge.

The serum antibody titer (OD values) obtained by ELISA is shown in Table 2. A significant rise in *C. septicum* antibody titers were noticed in the vaccinated birds on 14 and 28 days-post-one-vaccination or two with either doses of the vaccine.

DISCUSSION

Subcutaneous inoculation of *C. septicum* cultures consistently produced cellulitis lesions and mortality in naïve turkeys in this study as in our previous studies. Similar descriptions of lesions caused by *C. perfringens* (Carr et al., 1996) have been reported before but not with *C. septicum* in turkeys.

Serum ELISA results showed significant antibody response against *C perfringens* as well as *C septicum* in vaccinated birds. Antibody response against *C. septicum* bacterin/toxoid preparation was reported before in immunized turkeys under experimental conditions (Tellez et al., 2009). The experimental *C. septicum* toxoid which we developed was found to be completely safe and effective in reducing cellulitis lesions and mortality in turkeys under laboratory conditions. Better protection and higher serum antibody titers were seen in birds administered either 2 doses of 1 ml vaccination or one dose of 2 ml vaccination. Our results offer promise for the use of *C. septicum* toxoid successfully to mitigate cellulitis cases in turkeys in the field.

In summary, the experimental *C. septicum* toxoid we developed offered complete protection against cellulitis following homologous challenge under experimental conditions. Use of a higher concentration (2ml dose) of the toxoid or a booster vaccination may offer better protection against cellulitis due to *C. septicum* in turkeys.

TABLES

Table 1: Mortality following challenge with *C. septicum* in *C. septicum* vaccinated and non-vaccinated birds (n=12).

	Non-vaccinated	Vaccinated	
		@ 1 ml dose	@ 2 ml dose
Once vaccinated	12/12[b]	1/12[a]	0/12[a]
Twice vaccinated	12/12[a]	0/12[a]	0/12[a]

[a,b] Fraction of birds within a row with no common superscript are considered significantly different ($P<0.001$).

Table 2: *C. septicum* ELISA serum antibody titer (OD values) following vaccination with *C. septicum* toxoid in vaccinated and non-vaccinated birds (n=12).

	Post-one-vaccination OD Values			Post-two-vaccinations OD Values
	0dpv	14dpv	28dpv	7dp2v
Vac @ 1 ml dose	0.2459 (0.13)*[a]	0.6574 (0.24)[b]	0.704(0.10)[b]	0.9217(015)[c]
Vac @ 2 ml dose	0.2691(0.09)[a]	0.7645 (0.23)[b]	0.7254(0.09)[b]	1.0686(0.14)[c]
Non-vaccinated	0.2708 (0.54)[a]	0.2182 (0.17)[a]	0.2814(0.05)[a]	0.2689(0.05)[a]

* values in paranthesis indicate standard deviation; dpv = days-post-vaccination.

[abcd] Means within a row with no common superscript are considered significantly different ($P<0.05$).

CHAPTER VIII

FIELD TRIAL WITH *CLOSTRIDIUM SEPTICUM* TOXOID

Summary

Cellulitis continued to be a major problem in the turkey industry in the recent years irrespective of all the control measures adopted in the field. Cellulitis due to *Clostridium septicum* in turkeys increased at an alarming rate. The objective of this study was to field test the *C. septicum* toxoid we developed to reduce cellulitis mortality and antibiotic usage in commercial Nicholas White turkeys. The *Clostridium septicum* toxoid was evaluated for its use in the field in a flock of commercial turkeys consisting of 16,000 birds where 8000 birds were vaccinated and an equal number were kept as unvaccinated controls. The birds were vaccinated at six-weeks of age. The mortality in both vaccinated and unvaccinated groups was recorded and compared. Blood samples from birds in both groups were examined to detect antibody response to *C. septicum* toxoid using ELISA. In another field trial, we vaccinated 2000 birds in a barn consisting of 8000 birds and both groups were kept together in same barns. Cellulitis mortality was recorded in vaccinated and non-vaccinated birds. The use of *Clostridium septicum* toxoid developed was found to be safe and effective in both trials. Vaccination produced antibodies and did significantly reduce antibiotic usage to control cellulitis as well as mortality. Though a single vaccination with *C. septicum* toxoid did not offered complete protection in the field the use of multiple vaccinations may elicit a better antibody response and protection against cellulitis in turkeys. The results of our study offer promise in reducing antibiotic usage and also to reduce mortality due to cellulitis in turkeys due to *C. septicum*.

INTRODUCTION

With permission from the Board of animal health and University of Minnesota and consent from a leading commercial turkey producer, field trials were planned to test the efficacy of our experimental bivalent toxoid. We conducted two field studies under two commercial settings.

MATERIAL AND METHODS

Experimental design for Study I: The experimental design in this study was similar to the previous vaccination trial explained in Chapter IV. Briefly, Four thousand poults at the age of 6-weeks were vaccinated in a flock of 8,000 Nicholas White turkey poults in a brooder barn with experimental bivalent toxoid with 1x dose. All the birds were male poults that have been vaccinated for Newcastle (3d & 3wk) and hemorrhagic enteritis (HE) (4wk). One half of the birds were selected randomly from a single brooder barn for vaccination. The vaccine was administered subcutaneously at the neck region using a vaccine applicator.

Following vaccination, 2000 birds each from vaccinated and non-vaccinated group were transferred to two different grower Farms (Farm I and Farm II) where there was a consistent history of cellulitis. The vaccinated and non-vaccinated birds were separated by a wire-mesh and subjected to similar management conditions. The birds were likely to be exposed to Clostridia found in the poultry environment (natural challenge). A therapeutic antibiotic penicillin G sodium usage in water was in place as a precautionary measure as and when cellulitis related mortality rose above 0.1% per day. Daily mortality and packs of penicillin (one pack of penicillin contains 1.0 billion I.U.

(601.2 g) of Penicillin G Potassium) used to control cellulitis related mortality was recorded in both vaccinated and non-vaccinated birds from 10-weeks of age until 22-weeks of age. In the farm, there is routine monitoring of all the sick, moribund birds and dead birds twice daily and those identified sick and moribund were euthanized. The results were compared to detect any observable differences in the occurrence of mortality between vaccinated and non-vaccinated birds.

Ten birds at 10-weeks of age from vaccinated and non-vaccinated groups from the field vaccinated trials were transferred to RAR facilities at University of Minnesota. All the birds were challenged as mentioned before. The cellulitis development and mortality between the groups was recorded. All the birds at the end of the study were euthanized in a carbon dioxide chamber.

Statistical analysis was performed by SAS software (version 9.2)[e]. The mortality data following challenge with spore culture was analyzed using Fishers exact test. A survival analysis of maximum likelihood estimates using proportional hazards model (PH-REG procedure) was conducted to analyze the mortality rates in vaccinated and non-vaccinated groups. A Pearson's Chi square test was used to compare the use of antibiotics between vaccinated and non-vaccinated groups. The serological data was analyzed using GLM procedure for repeated measures analysis of variance where a P value of 0.05 was considered as significant.

Experimental design for Study II: In the second experimental model, the vaccinated birds and non vaccinated birds were kept together to eliminate any bias towards exposure to Clostridia from the environment with in the barn. Briefly, 4,000 poults at the age of 6-

weeks were vaccinated in a flock of 20,000 Nicholas White turkey poults in a brooder barn with experimental bivalent toxoid with 1x dose. All the birds were male poults that have been vaccinated for Newcastle (3d & 3wk) and hemorrhagic enteritis (HE) (4wk). The vaccinated birds used were toe clipped for identification purpose. The vaccine was administered subcutaneously at the neck region using a vaccine applicator.

Following vaccination, 2000 birds each from vaccinated and 8000 birds each from non-vaccinated group were transferred to two different grower Farms (Farm I and Farm II) where there was a consistent history of cellulitis. The vaccinated and non-vaccinated birds were kept together and subjected to similar management conditions. The birds were likely to be exposed to Clostridia found in the poultry environment (natural challenge). A therapeutic antibiotic penicillin G sodium usage in water was in place as a precautionary measure as and when the mortality due to cellulitis rose above 0.1% per day. Daily mortality with visible cellulitis lesions were recorded in both vaccinated and non-vaccinated birds until the birds were marketed. In the farm, there was routine monitoring of all the sick, moribund birds and dead birds twice daily and those identified sick and moribund were euthanized. The results were compared to detect any observable differences in the occurrence of mortality between vaccinated and non-vaccinated birds.

RESULTS

The mortality due to cellulitis in vaccinated and non-vaccinated birds are shown in Table 1 and Figure 1. There were significant differences in the mortality ($P<0.005$) in the vaccinated group from non vaccinated groups. The use of experimental *C. septicum* toxoid reduced cellulitis-related mortality from 12.1 % to 10.5 %. This accounts for a 14%

reduction in cellulitis-related mortality in vaccinated birds when compared with non-vaccinated birds.The Hazard ratio was found to be 1.14 for the non-vaccinated group over vaccinated group of birds. A Kaplan Meier survival curve comparing the survival distribution function of non-vaccinated and vaccinated birds was shown in Figure 2. No significant differences was noticed in terms of mortality between the farms (P=0.6343).

The need for use of antibiotic penicillin was significantly reduced because of vaccination (P<0.001) from 204 packs in non-vaccinated birds to 84 packs in vaccinated birds. This accounts for a 59% reduction in the use of antibiotics in vaccinated birds when compared with non-vaccinated birds. The days required to use peniciilin to control cellulitis was also reduced from 68 days in non-vaccinated group to to 28 days in vaccinated group (Figure 3).

In the second study, *C. septicum* toxoid significantly reduced cellulitis mortality in vaccinated birds from 1.68 % to 0.87 %, which accounts approximately 50% reduction in mortality (Table 2).

Challenge studies with experimental formalin-inactivated toxoid (FIT) vaccinated birds against *Clostridium septicum*.

Ten birds each from vaccinated and nonvaccinated groups from the field vaccinated trials were transferred to RAR facilities at University of Minnesota. The blood was collected at 3 weeks post vaccination from the vaccinated and the nonvaccinated birds and all the birds were challenged. Challenge inoculum and dose used was the same as in our previous studies. We observed a mortality of 100% (10/10) in 24 h in non-vaccinated birds. All birds from vaccinated group resisted challenge and did not develop any signs of

cellulitis lesions or mortality and were found to be completely protected. Vaccinated birds had *C. septicum* ELISA serum antibody titer of 0.6870 +/- 0.12 and non-vaccinated birds had an antibody titer of 0.3626 +/- 0.09.

DISCUSSION

When a single administration at 1ml dose of our experimental bivalent or *C. septicum* toxoid was used under field conditions, it was found to be protective and it did reduce the number of cellulitis cases, antibiotic usage and mortality due to cellulitis in commercial turkeys.

The toxoid significantly reduced the mortality by 14% in vaccinated birds when compared with non-vaccinated birds. A 14% reduction in mortality is highly significant, considering the fact that the disease appears in adult birds and the normal mortality rate at this age group is negligible in commercial settings**.** The reason for a partial protection was assumed to be due to single vaccination or an insufficient antigen concentration in a single dose of the vaccine.

The use of *C. septicum* bacterin/toxoid preparation was reported to elicit antibody response against *C. septicum* in immunized turkeys under experimental conditions (Tellez et al., 2009). Our results establish the fact that *C. septicum* toxoids could be used successfully to mitigate cellulitis cases in turkeys in the field. However, being a toxoid multiple vaccinations are required to boost the immunity for longer periods until the birds are marketed. The use of a formalin-inactivated *C. difficile* toxoid in humans (Kotloff et al., 2001) as well as use of a formalin inactivated *C. septicum* alpha toxoid in mice (Amimoto et al., 2002) were found to be protective but the immunity was short lived. In

another study, a multicomponent *C. perfringens, C. septicum* and Pasteurella bacterin was reported to reduce mortality in gangrenous dermatitis affected broiler chickens however no information is available about the duration of immunity (Gerdon, 1973).

With single vaccination in two farms, in antibiotic cost alone the industry saved approximately 2760 dollars considering the fact that each pack of penicillin costs 23 dollars. When reduction in mortality is also taken into account, the average savings per barn could be around five thousand dollars.

In summary, the experimental *C. septicum* toxoid we developed offered complete protection against cellulitis following homologous challenge under experimental conditions. The same vaccine was found to be effective in reducing mortality and use of antibiotic treatment in preventing cellulitis in commercial turkeys. In the future, use of multiple vaccinations as well as use of day-old-vaccination has to be considered for reducing further losses due to cellulitis in commercial turkey production.

TABLES

Table 1. Comparison of cellulitis related mortality and antibiotic usage post-vaccination with *C. septicum* toxoid in non-vaccinated and vaccinated birds from 10-weeks of age to 22-weeks of age.

	Non-vaccinated group	Vaccinated group
Number of birds	8,000	8,000
Mortality	970	840 [a]
% Mortality	12.1	10.5 [a]
Penicillin usage *	204 packs	84 packs [a]
Penicillin usage days	68	28 [a]

[a] indicates $P < 0.05$

* one pack of penicillin contains 1.0 billion I.U. (601.2 g) Penicillin G Potassium

Table 2. Comparison of cellulitis related mortality in *C. septicum* toxoid vaccinated and non-vaccinated birds from 7-weeks to 19-weeks of age.

	Non-vaccinated group	Vaccinated group
Number of birds	16,000	4,000
Cellulitis Mortality	268	35 [a]
% Cellulitis Mortality	1.68 %	0.87 % [a]

[a] indicates $P < 0.05$

FIGURES

Figure 1. Mortality following vaccination with *C. septicum* toxoid in non-vaccinated (line) and vaccinated birds (broken line) from 10-weeks of age until 22-weeks of age.

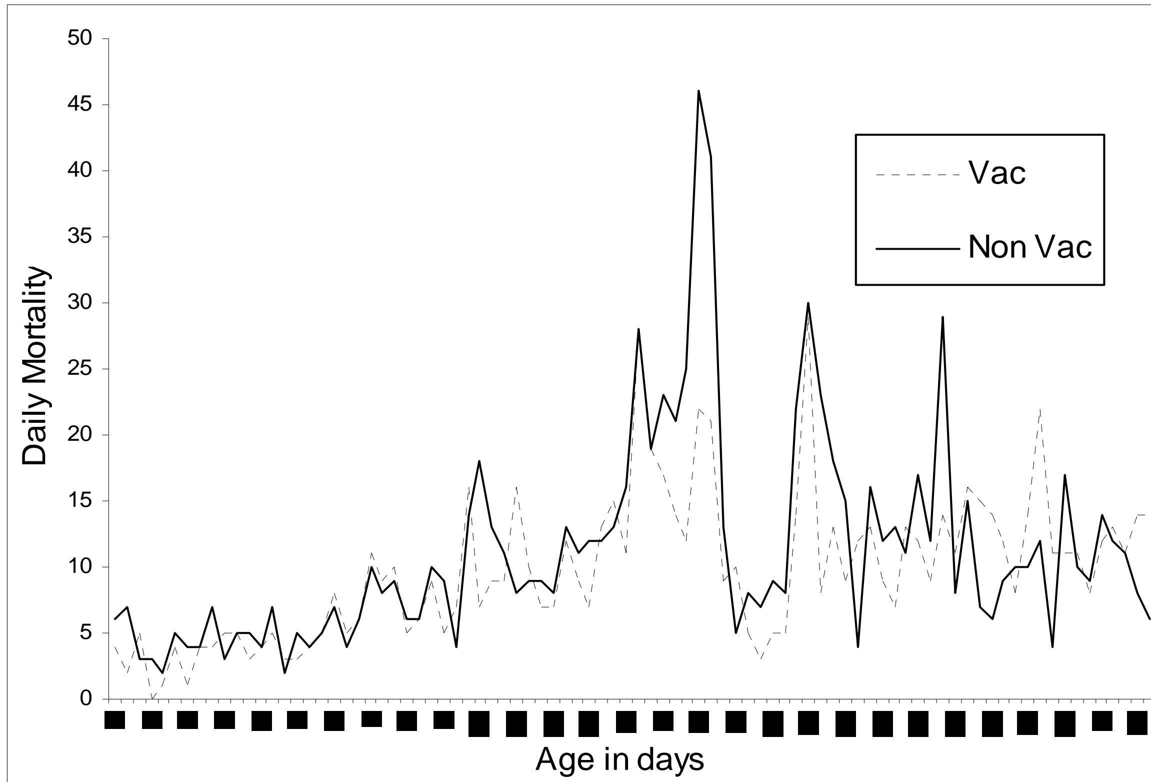


Figure 2. Kaplan Meier survival curve showing the survival distribution function following vaccination with *C. septicum* toxoid in non-vaccinated (broken line) and vaccinated birds (line) from 10-weeks of age until 22-weeks of age.

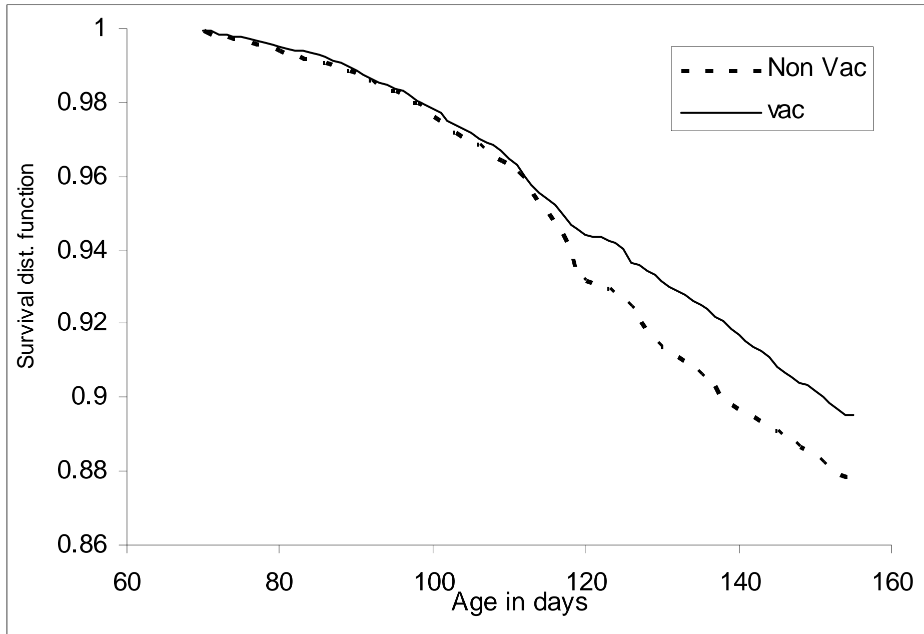
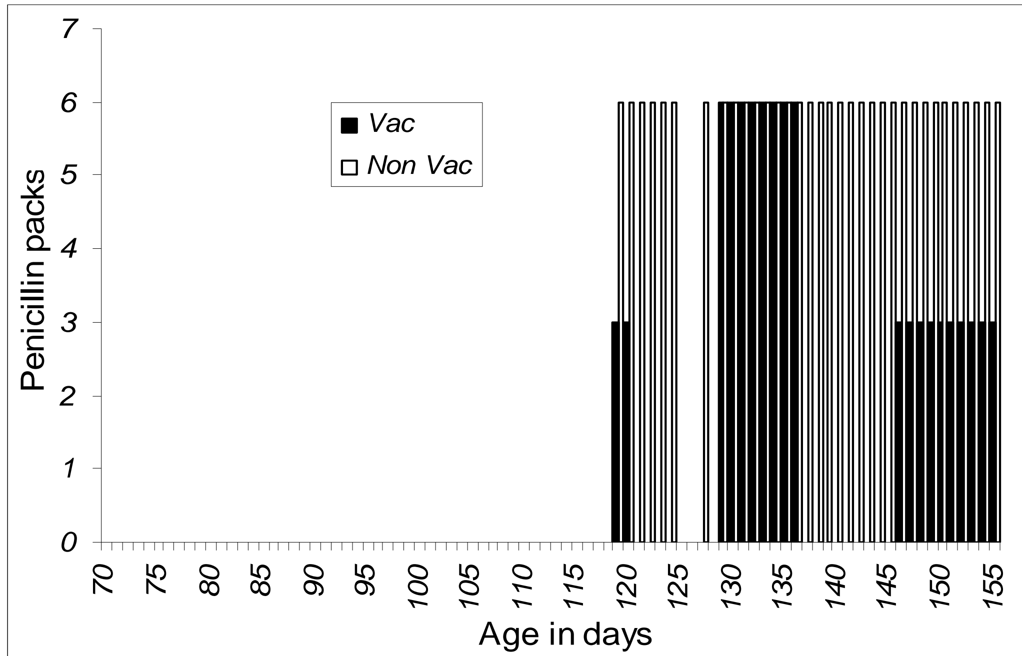


Figure 3. Antibiotic usage following vaccination with *C. septicum* toxoid in non-vaccinated (black bar) and vaccinated birds (white bar) from 10-weeks of age until 22-weeks of age.



CHAPTER IX

CONCLUSIONS

Cellulitis has continued to cause extensive losses in turkey production in USA due to severe mortality, carcass condemnation and treatment costs. *Clostridium perfringens* and *Clostridium septicum* are considered as the primary causative agents of cellulitis in turkeys. The results of this study emphasized the role of these two agents in causing cellulitis in turkeys. Cellulitis lesions and mortality in turkeys were successfully reproduced in turkeys with either *Clostridium perfringens* or *Clostridium septicum* isolated from cellulitis cases. *Clostridium perfringens* spore cultures produced mortality only at higher doses whereas *C. septicum* caused mortality even at the lower dose in turkeys. This necessitates the need to reduce exposure to both *Clostridium perfringens* and *Clostridium septicum* to control cellulitis in commercial turkey production.

Clostridium perfringens and *Clostridium septicum* isolates were found to vary in their abilities to produce spores as well as toxins. We observed differences in the toxicity and biological effects of different strains of *C. perfringens* and *C. septicum* either *in vitro*, or *in vivo* in mice as well as in turkeys. Though the spore count and hemolytic effects of *C. perfringens* were found to be higher than *C. septicum in vitro*, the lethal effects in mice as well as in turkeys were not comparable. *Clostridium septicum* was found to be more potent than *Clostridium perfringens* in causing cellulitis in turkeys. Control measures could now be targeted more on *C. septicum* to reduce losses in turkey production. The development of fulminant cellulitis lesions and mortality was markedly higher in seven-week-old birds than in three-week old birds. The phylogenetic tree constructed from *C. septicum* isolates indicated a high level of conservation present within the genome of *C. septicum.* These results will enable us in selecting a suitable vaccine candidate for the control of cellulitis.

The major secretory toxins we identified in *C. perfringens* isolates from cellulitis cases by MALDI-TOF mass spectrometry were phospholipase, collagenase, hyaluronidase, Dnase, enolase, muramidase, pyruvate kinase and hypothetical proteins. We observed two distinct proteomic profiles for *C. perfringens* isolates and the role of hypothetical proteins in cellulitis need to be further investigated. We observed only one type of proteomic profile for *C. septicum* isolates. The major secretory toxins we identified in *C. septicum* were alpha toxin, septicolysin, sialidase, Dnase, flagellin and hypothetical proteins.

The gross and microscopical lesions obtained after administration of spore cultures of either *C. perfringens* or *C. septicum* in seven-week-old birds were comparable to the cellulitis lesions reported before either with *C. perfringens* experimental infection or field observations. However, multiplication of either *C. perfringens or C. septicum* in the muscle tissues of cellulitis affected birds has not been reported before. *C. septicum* appears to be more potent in causing severe cellulitis lesions and higher mortality in turkeys although the role of *C. perfringens* in causing fatal cellulitis cannot be ignored. Our cellulitis disease model offer promise to use it as a challenge model in the development of vaccines against cellulitis in turkeys.

The experimental bivalent *C. perfringens* and *C. septicum* toxoid as well as *C. septicum* toxoid we developed offered complete protection against cellulitis following homologous challenge under experimental conditions. The use of these vaccines was found to be effective in reducing mortality and use of antibiotic treatment due to cellulitis significantly in commercial turkeys.

On future directions, an immediate follow up of the present study would be to

develop a day-old bivalent or a *C. septicum* toxoid. A vaccination protocol with a day-old vaccination followed by a booster at six-weeks of age may elicit better protection against cellulitis due to *C. perfringens* and *C. septicum* in turkeys. Multiple vaccinations as well as use of a higher concentration of antigens need to be considered. This approach will eventually help us to control cellulitis in turkey flocks of Minnesota.

CHAPTER X

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ROLE OF *CLOSTRIDIUM PERFRINGENS* AND *CLOSTRIDIUM SEPTICUM* IN CAUSING TURKEY CELLULITIS

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