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Evaluation of the Efficacy of a Candidate Turkey Dermatitis/Cellulitis Oil Emulsion Vaccine on Immune Response, Morbidity, and Mortality under Laboratory and Commercial Conditions

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Abstract

Alpha-toxigenic *Clostridium septicum* (CS), the cause of turkey cellulitis, results in devastating mortality with high costs for the industry. Various vaccinations have been evaluated to prevent this disease with moderate success. Ability of a CS bacterin-toxoid, in conjunction with adjuvants such as aluminum hydroxide, mannoslyated chitosan, or a water-in-oil Seppic Montanide 71 R VG adjuvant (OE) to induce immunity was evaluated in a 7-week study (Experiment 1). Poults (20/group) were vaccinated day-of-hatch, boosted at 5 weeks-of-age and compared to unvaccinated controls. Antibody levels were determined by ELISA for all experiments. In experiment 1, initial vaccination with the OE resulted in significantly ($P<0.05$) higher antibody levels at 5 weeks-of-age, and at 7 weeks-of-age OE resulted in numerically increased antibody levels compared to all vaccinated groups. Efficacy of the OE vaccine was then evaluated in two field trials (Experiment 2 and 3) with treatments including a non-vaccinated control group and a vaccinated group. Non-vaccinates were marked by removal of the dewclaw at the hatchery and comingled during growout (Experiment 2 and 3). Experiment 2 consisted of 3 houses: House 1 (HS1), House 2 (HS2), and House 3 (HS3). Mortality associated with cellulitis was recorded once the first case was observed. Blood samples were obtained at 8, 12, and 16 weeks-of-age. Antibody levels (S/P ratio) in vaccinated groups for weeks 12 and 16 were significantly higher ($P<0.05$) than the control groups for all 3 houses. In HS1 and HS2, low CS-associated mortality was observed and there was no significant difference in mortality/total (%) between control and vaccinated group. In HS3, control mortality/total (%) was significantly ($P<0.001$) higher than mortality in vaccinated turkeys. Experiment 3 consisted of 6 farms with 1-4 houses/farm. Vaccination significantly ($P<0.05$) reduced CS-related mortalities as compared to controls in 5 of 6 farms in experiment 3 and antibody titers were significantly ($P<0.05$) higher in vaccinated turkeys at 12 and 16 weeks for all 6 farms. Based on these results, W/O emulsion

vaccines, such as this alpha-toxin bacterin-toxiod with Montanide 71 R VG adjuvant, can be used to increase antibody titers and may reduce related mortalities in the field.

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To my mom and sister, thank you for always loving and believing in me no matter what the endeavor may be. To the Graham family, you all are intensely driven which motivates me to work harder each and every day.

Dedication

I would like to dedicate this thesis to my husband and my grandparents. Lucas, you inspire and challenge me daily. You believe in me regardless of the situation. Without your true love and friendship, I would not be where I am today. I love you more than you will ever know! Thank you, Nana and Papa, for being the most outstanding role models over the last 24 years. I couldn't have done any of this without you.

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Chapter I. Introduction

Turkey cellulitis, also referred to as clostridial dermatitis, is a problematic disease with prevalence escalating over the last two decades in the United States (Lighty et al., 2016). Peak incidence occurs in turkey flocks around 13-18 weeks-of-age (Clark et al., 2010). *Clostridium perfringens* and *Clostridium septicum* are opportunistic pathogens that have been isolated from cellulitis lesions (Thachil et al., 2010), however, *C. septicum* has been identified as the primary etiology responsible for cellulitis in commercial turkeys (Tellez et al., 2009). Controlling *C. septicum* is difficult due to the probability of the pathogen being a commensal organism within the gastrointestinal tract of healthy animals (Clark et al., 2010). The spores can also remain dormant in an unfavorable environment and then thrive when growth conditions are optimal (Clark et al., 2010). Immunosuppression or stressful conditions followed by challenge with *C. septicum*, a ubiquitous pathogen, can prompt disease (Clark et al., 2010). Development of clinical signs and mortalities related to cellulitis commonly result in the affected flock being treated with antibiotics (Clark et al., 2010). Proper management practices and antibiotic therapy have proven to reduce the incidence, but does not eliminate the disease.

Chapter II. Literature Review

Clostridium septicum and other related Clostridial species

Clostridium septicum is a Gram-positive, anaerobic spore forming bacillus that produces four toxins: alpha-toxin, beta-toxin, gamma-toxin, and delta-toxin (Hatheway 1990) with alpha-toxin as the most necrotizing (Tweten et al., 2001). Bacterial toxin production can vary based on temperature, time, pH, and availability of nutrients (Lalitha et al., 2005). In turkeys, *C. septicum* infection is associated with turkey cellulitis (Tellez et al., 2009; Thachil et al., 2013). Areas affected include the breast, tail, and thigh area where gas produced by the actively replicating anaerobic bacteria accumulates at the site of infection (Clark et al., 2010). In early observed cases, *C. perfringens* Type A was isolated from lesions containing gelatinous fluid under the skin with the musculature having a dark or greenish appearance (Carr et al., 1995). Other etiologies, including *C. perfringens*, have been categorized as a pathogen associated with turkey cellulitis (Gomis et al., 2001; Gornatti-Churria et al., 2018), however, *C. septicum* has been proven to be the principal causative agent (Tellez et al., 2009) and the accumulation of *C. septicum* alpha toxin is responsible for accompanying clinical signs (Kennedy et al., 2005).

Following subcutaneous challenge with 0.5, 1, 2, or 3mL of *C. perfringens* or *C. septicum* spore culture, *C. septicum* spore challenge group had more associated mortalities within 48h in both 3-week and 7-week old turkeys than the *C. perfringens* spore challenge group (Thachil et al., 2010). Gross macroscopic lesions on breast and tail area were observed in both challenge groups although more severe lesions were detected in the *C. septicum* challenge group (Thachil et al., 2010). Hemorrhage and edema was associated with both the dermis and subcutis, with heterophil infiltration more pronounced in the *C. septicum* 1mL challenge group and gas formation identified heavily in the *C. septicum* 3mL challenge group (Thachil et al., 2010). In

turkeys challenged with the higher doses of *C. septicum*, notable cellular necrosis and myonecrosis was observed. Within these necrotic regions, there were high numbers of rod shaped bacteria and low amounts of inflammatory cells (Thachil et al., 2010). There was an inverse relationship noted between heterophil infiltration and actively replicating bacteria within the tissue of *C. septicum* infected turkeys, the group that showed classical cellulitis mortality response and clinical signs (Thachil et al., 2010). To investigate the incidence and relationship between *C. septicum* in broilers and turkeys, 109 *C. septicum* isolates were recovered from both turkey and broiler flocks that had a history of dermatitis or cellulitis (Neumann et al., 2009). Out of the analyzed sequences, only one sequence showed commonality between the broiler and turkey strain suggesting isolates show specificity (Neumann et al., 2009).

In broiler chickens, gangrenous dermatitis is caused by both *C. perfringens* and *C. septicum* around 6 weeks-of-age (Li et al., 2010). Microscopic evaluation of gangrenous dermatitis lesions reveal gas and fluid accumulation, necrosis and heterophil infiltration (Li et al., 2010). Gangrenous dermatitis lesions observed in the breast, abdomen and thigh area were heavily discolored with emphysema and serosanguinous fluid (Li et al., 2010; Lee et al., 2012). Intestinal observations reveal severe hemorrhaging thus increasing the permeability of the intestinal epithelial barrier, allowing potentially allowing for enteric translocation of clostridial pathogens to the submucosa, and potentially systemically (Li et al., 2010). To identify causative pathogens associated with gangrenous dermatitis after immunocompromising broilers with an infectious bursal disease virus vaccination at 14 days, *Staphylococcus aureus* and *C. septicum* isolates, alone or in combination, were administered intramuscularly or subcutaneously to 4-week-old broilers (Wilder et al., 2001). Elevated mortality was observed in the *C. septicum* and *S. aureus* challenge groups. However, *C. septicum* alone was not responsible for dermatitis

associated lesions in this experiment (Wilder et al., 2001). Li et al., (2010) identified that gangrenous dermatitis affected broilers had significantly higher *C. perfringens* serum antibodies than the non-infected broilers. Interestingly, there was no difference in *C. septicum* serum antibodies or *Eimeria spp.* antibodies between the infected and non-infected group, although antibody levels were high in both (Li et al., 2010). This indicates that *C. perfringens* may be the etiology responsible for gangrenous dermatitis associated lesions and mortalities. In another study, serum samples were collected from non-affected and gangrenous cellulitis affected broilers at 35 days-of-age where the non-affected broilers had higher antibody levels to *C. perfringens* alpha-toxin and netB than the broilers with clinical signs (Lee et al., 2012). The authors hypothesized that the non-affected broilers had elevated antibodies levels which protected them from acquiring the disease (Lee et al., 2012).

In ruminants, blackleg is caused by *C. chauvoei,* an anaerobic, spore-forming, bacillus which is generally present in the environment that primarily affects cattle (Useh et al., 2006; Uzal et al., 2012) and sheep (Useh et al., 2006). It is widely believed that *C. chauvoei* spores reside in tissues and germinate at the site of an injury (Useh et al., 2006). The pH and oxygen availability within damaged muscle continues to change due to replication of *C. chauvoei,* creating an environment where the pathogen can efficiently propagate (Useh et al., 2006). *C. chauvoei* produces gamma toxins, beta-toxins, hemolysins, and neuraminidases (Useh et al., 2003). *C. septicum* has also been isolated from blackleg lesions in cattle but differs from *C. chauvoei* based on the presence of edema which is generally not apparent with *C. chauvoei* infections (Hatheway, 1990). To prevent blackleg, cattle are vaccinated annually with formalin inactivated bacterins and if an infection occurs, animals are typically treated with penicillin (Useh et al., 2006).

Opportunistically pathogenic clostridial species are nearly ubiquitous in areas where commercial poultry or livestock are raised. As many or all of these species have a primary niche of amplification within the gastrointestinal tract, either ingestion of spores or sporulation of living vegetative cells within the digesta provide a nearly constant source for potential translocation across the enteric epithelial barrier. When these spores, perhaps carried by the circulation, are present in damaged tissues with relative anoxia, they may then have the potential to germinate, replicate, and generate local toxin-associated necrosis, thereby increasing the ability of these organisms to anaerobically continue to grow. These exotoxins, such as alpha toxin produced by *C. septicum*, can also be responsible for initial lesions and ultimately mortality related to localized tissue diseases.

Clostridium septicum Pathogenesis

As described above, turkey cellulitis is caused by the accumulation of Clostridia, specifically *C. septicum,* in lesions leading to edema and inflammation (Clark et al., 2010). Lesions containing fluid and gas caused by *C. perfringens* and *C. septicum* are often similar, but mortality is more frequent in *C. septicum* infected turkeys (Thachil et al., 2010). Pathogens can enter circulation by translocating through the intestinal barrier, through broken skin or lesions, or by oral inoculation followed by subsequent physical trauma or gut leakage (Clark et al., 2010). With little known about the mechanisms of this disease, isolation and identification of the causative agent was imperative to further understand the pathogen most commonly associated with turkey cellulitis. In 2009, *C. septicum* was determined to be the most common pathogen related to turkey cellulitis in commercially produced turkeys (Tellez et al., 2009).

C. septicum alpha-toxin is structurally similar to *Aeromonas hydrophila* aerolysin (Ballard et al., 1995) being a pore-forming cytolysin where this specific toxin attaches to glycosylphosylinositol (GPI) protein receptors (Gordon et al., 1999; Kennedy et al., 2005). This attachment and pore formation results in cell lysis. Kennedy et al. (2005) determined that the primary virulent attribute of *C. septicum* is related to alpha-toxin activation and production. Pore forming toxins, such as *C. septicum* alpha toxin, affect the permeability of the host cell membrane by attaching and creating a pore (Popoff et al., 2014) leading to necrosis or apoptosis (Bischofberger et al., 2012). Pore size ranges from 1.3-1.6 nm in diameter (Knapp et al., 2009). Alterations in permeability caused by pore formation disrupt several cellular interactions, including osmotic pressure and ion regulation within the cytosol. When exposed to certain pore forming proteins, pores are created in low numbers on the membrane and can be repaired via calcium dependent mechanisms (Babiychuk et al., 2011). This mechanism has not been evaluated for *C. septicum* alpha toxin although the probability of membrane repair occurring after encounter with pore forming toxins is minimal due to the rapid nature of pore formation (Bischofberger et al., 2012), such as with *C. septicum* alpha toxin. Increased pore formation associated with *C. septicum* alpha toxin may hinder the cell's ability to self-repair due to the accumulation of toxin.

Kennedy et al., (2009) analyzed high mobility group box 1 (HMGB1), a protein that binds to receptor for advanced glycation end products (RAGE) instigating an inflammatory cascade that signals necrosis in adjacent cells (Scaffidi et al., 2002), for protein expression within the nucleus and the cytoplasm to determine if *C. septicum* alpha toxin induces HMGB1 expression in a target cell. A murine myoblast cell line was used with treatments consisting of a non-treated control, a group treated with 0.1µg/mL alpha toxin, and a group treated with 1µg/mL

alpha toxin (Kennedy et al., 2009). Translocation of HMGB1 into the cytoplasm only occurred in the 1µg/mL treated group indicating that *C. septicum* alpha toxin at that level initiates necrosis within cells induced by Ca^{2+} level changes within the cell (Kennedy et al., 2009). Necrosis associated with *C. septicum* infections is due to HMGB1 expression by damaged cells, ionic changes caused by pore formation, and other regulatory changes within the cell (Kennedy et al., 2009). Although lethality of *C. septicum* alpha toxin has been evaluated, the primary route of transmission or portal of entry for turkey cellulitis is still being investigated.

Outside-In Theory

Clostridium septicum is the primary etiology causing turkey cellulitis (Tellez et al., 2009) though the portal of entry is not fully understood. This bacterium could potentially enter the host via puncture wounds or scratches (Clark et al., 2010) suggesting that the pathogen could potentially enter from the "outside". In an experiment, day-of-hatch poults were subcutaneously injected in the breast area with *C. septicum* supernatant, neat *C. septicum* culture, or a conjunction of supernatant and convalescent antiserum (Tellez et al., 2009). Supernatant alone induced classical cellulitis clinical signs within 2h of inoculation (Tellez et al., 2009). No mortalities occurred 24h post-inoculation in the *C. septicum* supernatant only group. Supernatant with various dilutions of the convalescent antisera did not result in any lesions or mortality. However, injection with the *C. septicum* neat culture resulted in 78.5% mortality. Each affected poult had inflammation of the lungs, heart, and peritoneum similarly to observed cellulitis cases. Since morbidities, but no mortalities occurred in the supernatant only group, Tellez et al. suggested that the toxin produced by *C. septicum* may not be responsible for associated mortalities. However, subcutaneous challenge with neat *C. septicum* culture was resulted in high

mortality which may indicate the bacteria were replicating quickly thus producing high concentrations of exotoxins.

Thachil et al., (2013) investigated immune response post-vaccination with a *C. septicum* bacterin-toxoid oil emulsion vaccine with a subsequent challenge in the breast area in commercial turkeys. In this study, 24 hours post challenge in the breast area, there was 100% mortality in the control group (Thachil et al., 2013) providing further evidence that *C. septicum* can enter through openings in the skin. Reproduction of disease and mortality via subcutaneous challenge indicates *C. septicum* could enter through a puncture or wound, such as observed with a needle during challenge. In the field, this may be via scratches, abrasions, or cuts on the animal.

Inside-Out Theory

Clostridial species are ubiquitous; therefore, disease may not be apparent until stress induces intestinal inflammation ultimately resulting in gut leakage allowing the passage of the pathogen into circulation (Gornatti-Churria et al., 2018). Once in circulation, cells may proceed to the site of damage or bruising where replication and toxin production likely occur (Clark et al., 2010). Braxy in sheep and calves, or inflammation of the abomasum caused by a *C. septicum* infection, often results in septicemia and high mortality rates (Songer 1996). Intestinal barrier failure allows for the propagation of diseases related to *C. septicum*, such as cellulitis.

Cellulitis has not been successfully recreated with an oral challenge model. An experiment was conducted to determine the effect of dexamethasone treatment, an immunosuppressant, on mortality associated with *C. perfringens* and *C. septicum* challenge (Thachil et al., 2014). In this experiment, oral challenge with *C. perfringens* or *C. septicum* at both ~10^7 and 10^9 cfu/bird did not cause related mortalities in the control or dexamethasone

treated group. However, subcutaneous challenge with low dose of *C. perfringens* resulted in 0% mortality in both groups while the high dose caused 42% mortality in the positive control group and 100% mortality in the dexamethasone treated group (Thachil et al., 2014). A subcutaneous challenge of the low dose *C. septicum* resulted in 16% morality in the controls and 83% mortality in the dexamethasone group (Thachil et al., 2014). The high dose *C. septicum* subcutaneous challenge caused 100% mortality in both the control group and dexamethasone treated group. Dexamethasone treatment in conjunction with the oral challenge had no effect on mortality (Thachil et al., 2014) providing more evidence for the "outside-in" theory. Disease may not have been observed in the orally challenged group because these animals were not punctured while the subcutaneously injected turkeys were subjected to dermal puncture at challenge. A replicate experiment should be conducted to determine if oral challenge followed by sham subcutaneous challenge (no organism) induces cellulitis. This would provide more information for the argument of "outside-in" versus "inside-out".

Experimental Vaccines

Toxoid vaccinations for various Clostridial diseases in humans (Kotloff et al., 2000) and livestock (Hammer et al., 2007) have been effective. Blackleg in cattle and ruminant animals caused by *Clostridium chauvoei* is controlled frequently with vaccines (Useh et al., 2003). A variety of toxoid vaccines are administered to prevent blackleg consisting of seven *Clostridium* isolates, although *C. chauvoei* and *C. septicum* are the two that calves are required to be vaccinated for (Uzal et al., 2012). *C. chauvoei* spores within the environment can be ingested, spread throughout circulation and reside in muscular tissue (Useh et al., 2006.). Once an injury occurs around where the spores are located, blackleg symptoms and mortality ensue (Useh et al., 2006). However, a toxoid vaccine that provides optimal protection without antibiotic treatment

under commercial conditions has not been discovered for turkey cellulitis. Experimental vaccinations for turkey cellulitis have included inactivated *C. perfringens* and *C. septicum* cells and toxins alone and in combination (Tellez et al., 2009; Thachil et al., 2012; Thachil et al., 2013). The antigen can be administered as an inactivated bacterin-toxoid or toxoid which will stimulate an immune response, specifically antibody production, but not cause infection in the animal. A *C. perfringens* and *C. septicum* toxoid vaccine subcutaneously administered at 6 weeks-of-age significantly reduced mortality and penicillin usage, and significantly increased serum antibody levels to *C. septicum* and *C. perfringens* alpha-toxin in commercial turkeys (Thachil et al., 2012). The mortality percentage in the nonvaccinated group was 9.4% and 7.4% in the vaccinated group indicating that the vaccination is providing immune protection and preventing mortalities related to cellulitis (Thachil et al., 2012). There were 547 packs of penicillin used over 59 days in the nonvaccinated group compared to 361 packs of penicillin used over 31 days in the vaccinated group (Thachil et al., 2012). Vaccination reduced penicillin usage days by 50% over the 22- week period although vaccination did not eliminate the need for penicillin treatment. Although the *C. perfringens* and *C. septicum* toxoid vaccination provided significant protection, this vaccination alone cannot fully control or eliminate this disease.

Although not proven, the possible mode of action for oil emulsion vaccines may be the ability of creating a depot effect at the site of injection where the antigen is slowly released and presented to the immune system (Aucouturier et al., 2001). Thachil et al., (2013) conducted an experiment utilizing a *C. septicum* toxoid oil emulsion vaccine. Turkeys were vaccinated with a 1mL dose (1.5g of toxoid) or 2mL dose (3g of toxoid) at 6 weeks-of-age (Thachil et al., 2013). A group of turkeys were boosted at 14 days post-vaccination. Antibody levels were significantly different between both vaccinated groups and the control groups at both doses as expected. Two

field studies were conducted to test the effects of the *C. septicum* bacterin-toxoid. In field study 1 and 2, turkeys were vaccinated with the 1mL dose at 6 weeks-of-age. Cellulitis associated mortality was reported at 12.1% for the control group and 10.5% for the vaccinated group. The vaccinated turkeys had significantly less mortalities reported and were administered penicillin 40 days less than the control. In field study 2, mortality in the control group was 1.68% versus 0.87% for the vaccinated group (Thachil et al., 2013). This indicates that this *C. septicum* bacterin-toxoid has protective effects against cellulitis in the field, but does not fully eliminate the use of antibiotics or mortality occurrence.

A *C. septicum* bacterin-toxoid vaccine has been evaluated using aluminum hydroxide as an adjuvant (Tellez et al., 2010). To test the ability of inactivated *C. septicum* vaccine, 10-week-old turkeys were vaccinated with a formalin inactivated *C. septicum* bacterin-toxoid with aluminum hydroxide included as the adjuvant (Tellez et. al, 2009). Vaccinated turkeys had significantly higher *C. septicum* antibody levels than the nonvaccinated turkeys (Tellez et al., 2009). Vaccination with *C. septicum* bacterin-toxoid induces an immune response regardless of the adjuvant although protection may vary greatly between the adjuvants used.

Lancto et al. (2014) evaluated the efficacy of a noncytolytic *C. septicum* alpha toxin to provide protection against *C. septicum* challenge. Challenge related mortalities were significantly lower in the noncytolytic *C. septicum* alpha toxin group when compared to non-vaccinated controls and there were numerically fewer mortalities within the *C. septicum* alpha toxin-vaccinated group, although not significant (Lancto et al., 2014). The authors suggested that the safety concerns and cost to produce the recombinant vaccine are lower than for the bacterin-toxoid vaccine and even indicate that a water administration with a vector expressing alpha toxin

antigen would be a viable vaccine alternative to the bacterin-toxoid preparation (Lanco et., 2014).

Prevention and Treatment Methods

Antibiotics, including penicillin and lincomycin, and/or iodine are administered in the drinking water (Lighty et al., 2016) at onset of cellulitis-related mortalities and are provided until cellulitis lesions and mortalities are absent for at least 72 hours (Clark et al., 2010). Currently, the only effective treatment for turkey cellulitis is antibiotic therapy. Few alternative treatment and prevention approaches have been investigated. Dexamethasone, a synthetic glucocorticoid, causes immunosuppression in turkeys (Huff et al., 2013; Thachil et al., 2014) and increases intestinal leakage and bacterial translocation to the liver in chickens (Vicuna et al., 2015). It has also been shown to increase the incidence of turkey cellulitis mortalities (Huff et al., 2013; Huff et al., 2014) To evaluate the effects of yeast extract in the feed or vitamin D in the drinking water at reducing the incidence of cellulitis associated with stress, turkeys were administered dexamethasone intramuscularly in conjunction with yeast extract or vitamin D supplementation (Huff et al., 2014). No cellulitis-related mortalities occurred in the yeast extract treated group however, 47% of mortalities in the vitamin D treated group had characteristic cellulitis lesions (Huff et al., 2014). Administration of yeast extract in the feed at the late stages of production reduced incidence of cellulitis breakout in a flock although further studies need to be conducted to determine if yeast extract supplementation can prevent cellulitis in a challenge model.

Direct-fed microbials are probiotics included in the diet that are beneficial to the host (Lee et al., 2010). The addition of a commercially available direct-fed microbial significantly reduced the presence of *C. perfringens* in the ceca and in the feces compared to a non-treated group (Rahimi et al., 2011). Commercially, for cellulitis prevention, selected *Bacillus* isolates

have been included in the diet, although this does not fully avert the disease (Clark et al., 2010). Periodic inclusion of direct-fed microbial probiotics would provide flocks with beneficial bacteria that may prevent colonization of potentially harmful bacteria, such as *C. septicum.*

In 2008, a meeting was held by collaborators in the industry to discuss and assemble prevention measures for producers to ultimately minimize the risk of disease and mortality associated with turkey cellulitis (Clark et al., 2010). A few recommendations included continuous education for farmers and others involved with turkey production, antibiotic treatment (dose, timing, type), immediate removal of any mortalities, and improved management practices to reduce the impact of environmental related factors (Clark et al., 2010). In the United States, many commercial turkey producers continuously reuse litter and the build-up of used litter is correlated with turkey cellulitis (Clark et al., 2010). In Europe, turkey cellulitis is not an apparent threat to the turkey industry due to the all-in, all-out type of production systems, including complete cleanout and disinfection, that has been adopted (Clark et al., 2010). Reusing litter in the US, especially after a flock with high incidence of cellulitis, possibly subjects subsequent flocks to a challenge with the pathogenic organism that is present in the environment. Similarly, to the experimental breast area challenge, the flocks raised on reused litter may be naturally challenged through cuts or scratches on the skin. Complete removal of used litter and replacement with fresh litter may be necessary to reduce disease related to litter quality. A study conducted comparing control farms with high incidence farms determined that elevated soil pH, high humidity levels within the barn, and the presence of a litter composting pile 200 feet from the barn increased the occurrence of turkey cellulitis (APHIS, 2012). Barn management practices can be improved, but these practices are difficult to implement across the United States.

Conclusion

Clostridia are ubiquitous and are able to reside in the environment for extended periods of time. Commercial turkeys are frequently exposed to *C. septicum,* the pathogenic etiology linked to turkey cellultis, most frequently by injury to the dermis where this anaerobic, highly toxigenic rod replicates rapidly at the site of entry. In livestock, clostridial-related diseases can be prevented with vaccines and controlled with antibiotics. However, numerous vaccinations are difficult for commercial turkey producers because of the large labor cost attributed to handling each turkey. Experimental cellulitis vaccines have been evaluated with moderate success due to the need of intermittent treatment with antibiotics even after vaccination. Evaluating the efficacy of multiple adjuvants with a *C. septicum* bacterin-toxoid can provide implications for which vaccine to use in the field.

References

Aucouturier, J., L. Dupuis, and V. Ganne. 2001. Adjuvants designed for veterinary and human vaccines. Vaccine 19:2666–72.

Babiychuk, E. B., K. Monastyrskaya, S. Potez, and A. Draeger. 2011. Blebbing confers resistance against cell lysis. Cell Death Differ. 18:80–9.

Ballard, J., J. Crabtree, B. A. Roe, and R. K. Tweten. 1995. The primary structure of Clostridium septicum alpha-toxin exhibits similarity with that of Aeromonas hydrophila aerolysin. Infect. Immun. 63:340–4.

Bischofberger, M., I. Iacovache, and F. G. Van Der Goot. 2012. Pathogenic pore-forming proteins: function and host response. Cell host \& microbe 12:266–275.

Carr, D., D. Shaw, D. A. Halvorson, B. Rings, and D. Roepke. 1996. Excessive mortality in market-age turkeys associated with dermatitis. Avian diseases:736–741.

Clark, S., R. Porter, B. McComb, R. Lipper, S. Olson, S. Nohner, and H. L. Shivaprasad. 2010. Clostridial dermatitis and dermatitis: an emerging disease of turkeys. Avian Dis. 54:788–94.

Gomis, S., K. Amoako, M. Ngeleka, L. Belanger, B. Althouse, L. Kumor, E. Waters, S. Stephens, C. Riddell, A. Potter, and others. 2002. Histopathologic and bacteriologic evaluations of dermatitis detected in legs and caudal abdominal regions of turkeys. Avian diseases 46:192–197.

Gordon, V. M., K. L. Nelson, J. T. Buckley, V. L. Stevens, R. K. Tweten, P. C. Elwood, and S. H. Leppla. 1999. Clostridium septicum alpha toxin uses glycosylphosphatidylinositol-anchored protein receptors. Journal of Biological Chemistry 274:27274–27280.

Gornatti-Churria, C. D., M. Crispo, H. Shivaprasad, and F. A. Uzal. 2017. Gangrenous dermatitis in chickens and turkeys. Journal of Veterinary Diagnostic Investigation:1040638717742435.

Hammer, J. M., M. Fuhrman, and M. Walz. 2008. Serological evaluation of a Clostridium perfringens type A toxoid in a commercial swine herd. Journal of swine health and production 16:37–40.

Hatheway, C. L. 1990. Toxigenic clostridia. Clinical microbiology reviews 3:66–98.

Huff, G., W. Huff, and N. Rath. 2013. Dexamethasone immunosuppression resulting in turkey clostridial dermatitis: a retrospective analysis of seven studies, 1998-2009. Avian diseases 57:730–736.

Huff, G., W. Huff, and N. Rath. 2014. Effects of vitamin D and yeast extract supplementation on turkey mortality and clostridial dermatitis incidence in a dexamethasone immunosuppression model. Avian diseases 58:572–578.

Kennedy, C. L., E. O. Krejany, L. F. Young, J. R. O'Connor, M. M. Awad, R. L. Boyd, J. J. Emmins, D. Lyras, and J. I. Rood. 2005. The alpha-toxin of Clostridium septicum is essential for virulence. Mol. Microbiol. 57:1357–66.

Kennedy, C. L., D. J. Smith, D. Lyras, A. Chakravorty, and J. I. Rood. 2009b. Programmed cellular necrosis mediated by the pore-forming alpha-toxin from Clostridium septicum. PLoS pathogens 5:e1000516.

Knapp, O., E. Maier, S. B. Mkaddem, R. Benz, M. Bens, A. Chenal, B. Geny, A. Vandewalle, and M. R. Popoff. 2010. Clostridium septicum alpha-toxin forms pores and induces rapid cell necrosis. Toxicon 55:61–72.

Kotloff, K. L., S. S. Wasserman, G. A. Losonsky, W. Thomas, R. Nichols, R. Edelman, M. Bridwell, and T. P. Monath. 2001. Safety and Immunogenicity of Increasing Doses of aClostridium difficile Toxoid Vaccine Administered to Healthy Adults. Infection and immunity 69:988–995.

Lalitha, K., and K. Gopakumar. 2005. Influence of Temperature and pH on Growth and Toxin Production from Spores of Clostridium botulinum. Journal of aquatic food product technology 14:39–50.

Lancto, C. A., L. K. Foster, M. M. Kromm, B. McComb, J. Williams, J. Luke, A. Carnes, C. P. Hodgson, and D. N. Foster. 2014. A noncytolytic alpha toxin recombinant protein protects turkeys against Clostridium septicum challenge. Avian diseases 58:566–571.

Lee, K., H. S. Lillehoj, and G. R. Siragusa. 2010. Direct-fed microbials and their impact on the intestinal microflora and immune system of chickens. The journal of poultry science 47:106–114.

Lee, K., H. Lillehoj, M. Park, S. Jang, G. Ritter, Y. Hong, W. Jeong, H. Jeoung, D. An, and E. Lillehoj. 2012. Clostridium perfringens alpha-toxin and NetB toxin antibodies and their possible role in protection against necrotic enteritis and gangrenous dermatitis in broiler chickens. Avian diseases 56:230–233.

Li, G., H. S. Lillehoj, K. W. Lee, S. I. Jang, P. Marc, C. G. Gay, G. D. Ritter, D. A. Bautista, K. Phillips, A. P. Neumann, and others. 2010. An outbreak of gangrenous dermatitis in commercial broiler chickens. Avian pathology 39:247–253.

Lighty, M. E., F. Elvinger, R. D. Evans, N. Sriranganathan, T. LeRoith, and F. W. Pierson. 2016. Incidence of clostridial dermatitis (dermatitis) and factors for development of the disease in turkeys. Journal of Applied Poultry Research 25:104–112.

Neumann, A. P., and T. G. Rehberger. 2009. MLST analysis reveals a highly conserved core genome among poultry isolates of Clostridium septicum. Anaerobe 15:99–106.

Popoff, M. R. 2014. Clostridial pore-forming toxins: powerful virulence factors. Anaerobe 30:220–238.

Rahimi, S., S. Kathariou, J. L. Grimes, and R. M. Siletzky. 2011. Effect of direct-fed microbials on performance and Clostridium perfringens colonization of turkey poults. Poult. Sci. 90:2656–62.

Scaffidi, P., T. Misteli, and M. E. Bianchi. 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191.

Songer, J. G. 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9:216–34.

Tellez, G., N. R. Pumford, M. J. Morgan, A. D. Wolfenden, and B. M. Hargis. 2009. Evidence for Clostridium septicum as a primary cause of dermatitis in commercial turkeys. Journal of veterinary diagnostic investigation 21:374–377.

Thachil, A. J., B. McComb, M. M. Andersen, D. P. Shaw, D. A. Halvorson, and K. V. Nagaraja. 2010. Role of Clostridium perfringens and Clostridium septicum in causing turkey dermatitis. Avian Dis. 54:795–801.

Thachil, A., B. McComb, M. Early, C. Heeder, and K. Nagaraja. 2012. Clostridium perfringens and Clostridium septicum toxoid to control dermatitis in turkeys. Journal of Applied Poultry Research 21:358–366.

Thachil, A. J., B. McComb, M. Kromm, and K. V. Nagaraja. 2013. Vaccination of turkeys with Clostridium septicum bacterin-toxoid: evaluation of protection against clostridial dermatitis. Avian diseases 57:214–219.

Thachil, A. J., D. P. Shaw, and K. V. Nagaraja. 2014. Effects of dexamethasone immunosuppression on turkey clostridial dermatitis. Avian diseases 58:433–436.

Tweten, R. K. 2001. Clostridium perfringens beta toxin and Clostridium septicum alpha toxin: their mechanisms and possible role in pathogenesis. Veterinary microbiology 82:1–9.

United States Department of Agriculture, Animal and Plant Health Inspection Service. 2012. *Risk Factors Associated with Clostridial Dermatitis on U.S. Turkey-grower Farms.* Retrieved from https://www.aphis.usda.gov/animal_health/nahms/poultry/downloads/poultry10/Poultry10_is_RiskFactors.pdf

Useh, N. M., A. J. Nok, and K. A. N. Esievo. 2003. Pathogenesis and pathology of blackleg in ruminants: the role of toxins and neuraminidase. A short review. Vet Q 25:155–9.

Useh, N., A. Nok, and K. Esievo. 2006. Blackleg in ruminants. CAB Rev Perspect Agric Vet Sci Nutr Natur Resour 1:1–8.

Uzal, F. A. 2012. Evidence-based medicine concerning efficacy of vaccination against Clostridium chauvoei infection in cattle. Vet. Clin. North Am. Food Anim. Pract. 28:71–7, viii.

Vicuna, E., V. Kuttappan, R. Galarza-Seeber, J. Latorre, O. Faulkner, B. Hargis, G. Tellez, and L. Bielke. 2015. Effect of dexamethasone in feed on intestinal permeability, differential white blood cell counts, and immune organs in broiler chicks. Poultry science 94:2075–2080.

Wilder, T., J. Barbaree, K. Macklin, and R. Norton. 2001. Differences in the pathogenicity of various bacterial isolates used in an induction model for gangrenous dermatitis in broiler chickens. Avian diseases:659–662.

Chapter III. Evaluation of the Efficacy of a Candidate Turkey Cellulitis/Dermatitis Oil Emulsion Vaccine on Immune Response, Morbidity, and Mortality under Laboratory and Commercial Conditions

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ABSTRACT

Alpha-toxigenic *Clostridium septicum* (CS), the primary etiology of turkey cellulitis, results in devastating mortality. Various experimental vaccines have been evaluated to prevent this disease with variable but partial success. The ability of a CS bacterin-toxoid, in conjunction with adjuvants such as aluminum hydroxide, mannoslyated chitosan, or a water-in-oil Seppic Montanide 71 R VG adjuvant (OE) to induce immunity was evaluated in a 7-week study (Experiment 1). Poults (20/group) were vaccinated on day-of-hatch, boosted at 5 weeks-of-age and compared to unvaccinated controls. Antibody levels were determined by ELISA for all experiments. In experiment 1, initial vaccination with the OE resulted in significantly ($P<0.05$) higher antibody levels at 5 weeks-of-age, and at 7 weeks-of-age OE resulted in numerically increased antibody levels compared to all vaccinated groups. Efficacy of the OE vaccine was then evaluated in two field trials (Experiments 2 and 3) with treatments including a non-vaccinated control group and a vaccinated group (~50% each). Non-vaccinates were marked by dewclaw removal at the hatchery and were comingled during growout (Experiments 2 and 3). Experiment 2 consisted of 3 houses: House 1 (HS1), House 2 (HS2), and House 3 (HS3). Mortality associated with cellulitis was recorded once the first case was observed. Blood samples were obtained at 8, 12, and 16 weeks-of-age. Antibody levels (S/P ratio) in vaccinated groups for weeks 12 and 16 were significantly higher ($P<0.05$) than the control groups for all 3 houses. In HS1 and HS2, low CS-associated mortality was observed and there was no significant difference in mortality/total (%) between control and vaccinated group. In HS3, control mortality (%) was significantly ($P<0.001$) higher than mortality in vaccinated turkeys. Experiment 3 consisted of 6 farms with 1-4 houses/farm. Vaccination significantly ($P<0.05$) reduced CS-related mortalities as compared to controls in 5 of 6 farms in experiment 3 and antibody titers were significantly

(P<0.05) higher in vaccinated turkeys at 12 and 16 weeks for all 6 farms. Based on these results, W/O emulsion vaccines, such as this alpha-toxin bacterin-toxiod with Montanide 71 R VG adjuvant, can be used to increase antibody titers and may reduce related mortalities in the field.

Keywords: oil-emulsion vaccine; turkey cellulitis; *Clostridium septicum*; alpha-toxin; antibody titer

INTRODUCTION

Turkey cellulitis or clostridial dermatitis is primarily caused by *Clostridium septicum* (Tellez et al., 2009), a Gram positive, anaerobic rod primarily affecting commercial turkey flocks later in production (Clark et al., 2010). The incidence of cellulitis is costly due to the increase in production expenses at the onset of disease (Lighty et al., 2016). Cellulitis lesions occur in areas such as the breast region where discoloration, edema and gas accumulation are commonly observed (Clark et al., 2010). Pathogens, such as *C. perfringens*, have been affiliated with cellulitis, however *C. septicum* alpha toxin is the most virulent exotoxin responsible for necrosis associated with *C. septicum* infections (Kennedy et al., 2005). Subcutaneous challenge models (Tellez et al., 2009; Thachil et al., 2010; Thachil et al., 2014) have been more effective than oral challenge models (Thachil et al., 2014) possibly indicating that *C. septicum* primarily enters the animal through scratches or punctures of the skin rather than by oral inoculation, although the possibility of systemic seeding due to enteric translocation has not been eliminated. *C. septicum* is a ubiquitous pathogen which makes this disease difficult to prevent or control under commercial conditions (Clark et al., 2010). Antibiotic therapy can effectively treat this disease; however, a prevention method is needed to reduce the use of antibiotics. Experimental vaccines have been investigated for cellulitis, such as a *C. perfringens* and *C. septicum* bacterin-toxoid (Thachil et al., 2012), a recombinant *C. septicum* alpha toxin peptide vaccine (Lancto et al., 2014), and *C. septicum* bacterin-toxoids (Tellez et al., 2009; Thachil et al., 2013). Vaccination with a *C. septicum* bacterin-toxoid mineral oil vaccine reduced antibiotic usage and associated mortalities compared to non-vaccinated controls (Thachil et al., 2013). It has been hypothesized that water-in-oil emulsion vaccines create a depot or repository effect which stimulates and provides long term interaction with the immune system and antigen, thus elevating the overall

immune response (Aucouturier et al., 2001). Water-in-oil emulsion vaccines consist of a liquid/antigenic part and an oil part which are homogenized to achieve a target droplet size and uniformity. Aluminum hydroxide is commonly used as an adjuvant and may be an effective by creating a depot effect at the site of vaccination (He et al., 2015). Mannosylated chitosan has been combined with multiple antigens to enhance the immune response (Hargis et al., 2015). The purpose of these experiments was to evaluate and compare the efficacy of multiple adjuvants with a *C. septicum* bacterin-toxoid antigen to induce an immune response under laboratory conditions and then test the most efficacious vaccine under commercial conditions.

MATERIALS AND METHODS

Bacterial isolates

Isolation, identification, and culture of isolates used in these experiments has been previously described (Tellez et al., 2009). Briefly, two *Clostridium septicum* (CS) isolates from fluid emphysematous lesions of cellulitis in commercial turkeys that died acutely were purified and identified using commercial anaerobic identification panels (RapID ANA II anaerobic identification panels, Remel Inc., Lenexa, KS). From this, an experimental bacterin/toxoid was produced from two CS isolates that were capable of causing lesions consistent with turkey cellulitis and was recovered from induced lesions, as described. The bacterin was produced from an anaerobic 18h tryptic soy broth and sodium thioglycollate (0.5%) culture of CS, inactivated by the addition of formaldehyde (Fisher, Waltham, MA) to achieve a final concentration of 0.25%. Inactivation was timed to allow accumulation of toxin and 10^8 cells/mL (24- hr incubation) as verified by quantitative enumeration and hemolysin titration (Hang'ombe et al., 2005).

CS bacterin-toxoid Vaccine Preparation

Adjuvants were combined with the CS bacterin-toxoid as follows. Aluminum hydroxide (Rugby Labs, Duluth, GA.) was included as an adjuvant at 12% (v/v) (Experiment 1). Mannosylated chitosan (Hargis et al., 2015) was included at a 2:1 ratio with the bacterin-toxioid (Experiment 1 and 2). A commercial water-in-oil adjuvant (Seppic Montanide 71R VG) was included at 70%, per manufacturer's instructions (Experiment 1, 2 and 3). For the OE vaccine, the CS bacterin-toxoid was added to oil component and homogenized over a 45 second duration at low speed using a PRO 200 homogenizer, (PRO Scientific Inc., Oxford, CT). Following addition of antigen, oil emulsion was homogenized for 5 minutes. Emulsion stability decreased when homogenization time continued longer than 5 minutes. Droplets size for oil emulsion vaccine ranged from 1-2 μm. Stability of emulsion was evaluated and verified to be stable at 4C for at least 30 days. Sterility was verified by spread plating 100μl of prepared vaccine on sheep blood agar and incubating aerobically and anaerobically at 37C for 18h.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The indirect enzyme-linked immunosorbent assay (ELISA) used for measuring relative antibody levels against the potential CS etiology has been previously described (Tellez et al., 2009). This assay was used to show that vaccinated turkeys had increased levels of antibodies to CS or that turkeys with higher levels of antibodies were less likely to contract cellulitis. The assay was performed similarly to previously described methods (Roberts et al., 1987; Ameiss et al., 2004; Hang'ombe et al., 2005). Absorbance was read at 450 nm using a commercial microplate reader (BioTek MQX200, BioTek Instruments Inc., Winooski, VT.). The absorbance obtained for the

positive control, negative control, and experimental samples was used to calculate the sample to positive control ratios (S/P ratios) (Brown et al., 1991; Davies et al., 2003).

EXPERIMENTAL DESIGN

Laboratory Trial

Experiment 1 was conducted comparing the effects of adjuvants including aluminum hydroxide, mannosylated chitosan, or commercial water-in-oil adjuvant, Seppic Montanide 71R VG with the CS bacterin-toxoid. 100 day-of-hatch commercial cross poults were obtained from a local hatchery and transferred to the University of Arkansas JKS Poultry Health Laboratory (Fayetteville, AR). Poults (n=20 per treatment) were randomly assigned to one of five treatment groups and then individually neck tagged according to treatment. Treatment groups were subcutaneously (neck) vaccinated with respective candidate vaccines on day-of-hatch (prime) with 0.25mL and boosted at 5 weeks-of-age with 0.5mL. Treatment groups included 1) non-vaccinated control, 2) alum prime + oil emulsion (OE) boost, 3) mannosylated chitosan (MCA) prime + MCA boost, 4) MCA prime + alum boost, 5) and an oil emulsion prime + oil emulsion group boost. Turkeys were comingled and provided feed and water *ad libitum* for the 7-week duration of the experiment. Serum samples were obtained at 2, 5 and 7 weeks-of-age. No cellulitis-associated lesions or mortalities were observed in this experiment. Vaccine injection site lesions were scored post-mortem by a licensed veterinarian as a 0 (not present), 1 (detectable but insignificant), 2 (moderate), and 3 (clinically meaningful lesions).

Field Trial 1

Experiment 2 included 3 houses (HS) with each having a non-vaccinated control and vaccinated group. Control turkeys were distinguished by the removal of the dewclaw and were comingled

with vaccinated turkeys. Vaccinated turkeys were primed at day-of-hatch with a CS bacterin/toxoid MCA vaccine and subcutaneously boosted at 8 weeks-of-age with the CS bacterin/toxoid oil emulsion vaccine (0.5mL). Allocation treatments was as follows: HS 1 consisted of 5800 control turkeys and 4800 vaccinated turkeys, HS 2 consisted of 4800 control turkeys and 5000 vaccinated turkeys, and HS 3 with 4100 control turkeys and 5100 vaccinated turkeys. Mortality was calculated based on percent of each group. Blood samples were obtained from 20 per treatment per house at 8, 12, and 16 weeks-of-age. Mortality estimates were reported from 13-16 weeks-of-age. Antibiotic treatment was administered when cellulitis mortalities were observed, however, the duration of antibiotic therapy was not provided in this trial.

Field Trial 2

Experiment 3 consisted of 6 farms with 1-4 houses per farm as described in **Table 4**. Non-vaccinated control and vaccinated group were allocated evenly. Control turkeys were distinguished by the removal of the dewclaw and were comingled with vaccinated turkeys. Turkeys were subcutaneously vaccinated (0.5mL) at 8 weeks-of-age with CS toxoid oil emulsion vaccine. Blood samples were obtained from 10 per treatment per house at 8, 12, and 16-20 weeks-of-age based on collection date determined by producer. Mortality estimates were reported from 13-21 weeks-of-age. Similar to experiment 2, antibiotic therapy was administered at onset of disease and duration of treatment was recorded (**Table 5**).

Statistical Analysis

All data were subjected to Analysis of Variance as a completely randomized design using the General Linear Models procedure of SAS (SAS Institute, 2002). Antibody response data is expressed as mean ± standard error, in all experiments. Significant differences among means

were determined by using Tukey's multiple-range test (Experiment 1) at $P < 0.05$. Chi-squared test of independence (Zar, 1984) was used to determine significant ($P < 0.05$) differences for mortality.

RESULTS AND DISCUSSION

Toxoid vaccines have successfully prevented Clostridial diseases, such as blackleg in ruminants (Useh et al., 2006; Uzal et al., 2012). Vaccines for turkey cellulitis including a *C. septicum* bacterin-toxiod combined with an aluminum hydroxide adjuvant (Tellez et al., 2009), a recombinant *C. septicum* alpha toxin peptide (Lancto et al., 2014) and both a *C. septicum* bacterin-toxiod and *C. septicum* and *C. perfringens* bacterin-toxoid mixed with an oil emulsion adjuvant (Thachil et al., 2012; Thachil et al., 2013) have been evaluated and proven to have some protective effects.

In experiment 1, at 2 weeks post-prime, a group vaccinated day-of-hatch with MCA CS bacterin-toxoid had significantly ($P<0.05$) higher antibody levels than the non-vaccinated control, but was not statistically different than vaccinated groups. At 5 weeks post-prime, turkeys vaccinated on day-of-hatch with the CS oil emulsion vaccine had significantly ($P<0.05$) higher antibody levels than all groups. By 7 weeks-of-age, all vaccinated groups had markedly ($P<0.05$) higher antibody levels than the control however there was no statistical difference between vaccinated groups. CS oil emulsion prime + CS oil emulsion boost group resulted in the most elevated immune response compared to all treatment groups followed by the Alum prime + CS oil emulsion boost group (**Table 1**). We identified that prime and boost vaccination with the *C. septicum* bacterin-toxoid oil emulsion vaccine elicits a more robust immune response than the alternatives evaluated (**Table 1**). This, in part, may be due to the fact that oil emulsion vaccines provide long term protection against the antigenic portion included in a vaccine due to the

accumulation of a depot at the injection site (Aucouturier et al., 2001). Antigen should be released slowly from this repository over an extended period thus increasing encounter of antigen with antigen presenting cells. Continuous interaction between antigen and antigen presenting cells provides constant stimulation of the immune system and subsequent antibody production (Awate et al., 2014). Vaccine injection sites were evaluated post mortem to determine if there were any lesions associated with the adjuvants tested in experiment 1. MCA prime + MCA boost and MCA prime + alum boost groups had no observed lesions at vaccine injection site. However, in the both oil emulsion vaccine groups there were lesions present at the injection site (**Table 2**). Injection site lesions associated with oil emulsion vaccination were consistent with lesions induced by commercial oil emulsion vaccines (data not shown). There were no cellulitis related morbidities or mortalities in experiment 1 (**Table 2**).

In experiment 2, there was no difference in titer between the control and vaccinated group at 8 weeks post-prime with the CS MCA vaccine. However, at 12 and 16 weeks-of-age (4 and 8 weeks post-boost with the CS oil emulsion vaccine), the vaccinated group had significantly ($P<0.05$) higher antibody levels than the control group in all three houses (**Table 3)**. Vaccinated turkeys in experiment 2 were primed with the *C. septicum* MCA vaccine which may have stimulated a primary immune response. The prime vaccination could explain the higher antibody levels that were observed in the vaccinated group for this experiment. Mortality was reported from 13-16 weeks-of-age although the results are complicated due to the intermittent use of antibiotics. In HS 1 and 2, CS-related mortalities were low in both groups and there was no mortality differences observed between the control and vaccinated group. However, in HS 3, there was a significant ($P<0.001$) reduction in cellulitis related mortalities in the vaccinated group (**Table 3**). The significantly lower mortality observed in the vaccinated treatment group

may be a result a higher bacterial challenge being present in this house, thus the vaccinated group had a sufficient immune response to the *C. septicum* bacterin-toxoid oil emulsion vaccine and were protected whereas the non-vaccinated controls were naïve to the antigen.

Experiment 3 consisted of 6 farms with 1-4 houses per farm. At vaccination (8 weeks-of-age), there was no antibody level differences between control and vaccinated groups. At 12 weeks-of-age (4 weeks post vaccination), vaccinated groups had a significantly ($P<0.05$) more robust immune response than the control groups. Vaccinated group antibody titers were significantly ($P<0.05$) higher than control groups at 16-20 weeks-of-age (**Table 4**). Mortality was reported between 13-21 weeks-of-age. Cellulitis-associated mortalities were significantly ($P<0.05$) lower in vaccinated groups than control groups in 5 of the 6 houses. There were 24% less cellulitis-related mortalities in the vaccinated groups in experiment 3 (**Table 5**). Similar to experiment 2, if antibiotic treatment was required, both the control and the vaccinated groups were subjected to the treatment. Previously, Thachil et al., (2013) evaluated a CS bacterin-toxoid oil emulsion vaccine and observed a significant reduction in antibiotic usage and cellulitis mortalities in the vaccinated group. Vaccinate antibody titers were elevated and had complete protection against *C. septicum* challenge while non-vaccinates had low antibody levels with 100% mortality 24 hours post challenge (Thachil et al., 2013). This indicates that an oil emulsion vaccine for cellulitis can protect against experimental subcutaneous challenge (Thachil et al., 2013).

These data show that the *C. septicum* bacterin-toxoid oil emulsion vaccine used in these experiments elicits robust immune response and may reduce the incidence of cellulitis related mortalities. Vaccine efficacy without antibiotic treatment under commercial conditions is currently unknown. Further studies need to be conducted to determine effectiveness of a

subsequent boost vaccination in a field study, protective ability of this *C. septicum* bacterin-toxoid oil emulsion vaccine against experimental *C. septicum* challenge, and evaluation of vaccine efficacy without antibiotic treatment.

References

Ameiss KA, Danforth HD, McElroy AP, et al.: 2004, Immunogenicity of ad libitum drinking water administration of bovine serum albumin in Leghorn chickens. Poult Sci 83:1535–1538.

Aucouturier, J., L. Dupuis, and V. Ganne. 2001. Adjuvants designed for veterinary and human vaccines. Vaccine 19:2666–72.

Awate, S., L. A. B. Babiuk, and G. Mutwiri. 2013. Mechanisms of action of adjuvants. Frontiers in immunology 4:114.

Brown MB, Stoll ML, Scasserra AE, et al.: 1991, Detection of antibodies to *Mycoplasma gallisepticum* in egg yolk versus serum samples. J Clin Microbiol 29:2901–2903.

Clark, S., R. Porter, B. McComb, R. Lipper, S. Olson, S. Nohner, and H. L. Shivaprasad. 2010. Clostridial cellulitis and cellulitis: an emerging disease of turkeys. Avian Dis. 54:788–94.

Davies RH, Heath PJ, Coxon SM, et al.: 2003, Evaluation of the use of pooled serum, pooled muscle tissue fluid (meat juice) and pooled faeces for monitoring pig herds for *Salmonella*. J Appl Microbiol 95:1016–1025.

Hang'ombe MB, Kohda T, Mukamoto M, et al.: 2005, Relationship between *Clostridium septicum* alpha-toxin activity and binding to erythrocyte membranes. J Vet Med Sci 67:69–74.

Hargis, Billy M., Neil R. Pumford, Marion Morgan, Srichaitanya Shivaramaiah, Guillermo Tellez, and Amanda Wolfenden. "Novel mucosal adjuvants and delivery systems." U.S. Patent Application 14/439,536, filed October 22, 2015.

He, P., Y. Zou, and Z. Hu. 2015. Advances in aluminum hydroxide-based adjuvant research and its mechanism. Human vaccines & immunotherapeutics 11:477–488.

Kennedy, C. L., E. O. Krejany, L. F. Young, J. R. O'Connor, M. M. Awad, R. L. Boyd, J. J. Emmins, D. Lyras, and J. I. Rood. 2005. The alpha-toxin of Clostridium septicum is essential for virulence. Mol. Microbiol. 57:1357–66.

Lancto, C. A., L. K. Foster, M. M. Kromm, B. McComb, J. Williams, J. Luke, A. Carnes, C. P. Hodgson, and D. N. Foster. 2014. A noncytolytic alpha toxin recombinant protein protects turkeys against Clostridium septicum challenge. Avian diseases 58:566–571.

Lighty, M. E., F. Elvinger, R. D. Evans, N. Sriranganathan, T. LeRoith, and F. W. Pierson. 2016. Incidence of clostridial cellulitis (cellulitis) and factors for development of the disease in turkeys. Journal of Applied Poultry Research 25:104–112.

Roberts DW, Pumford NR, Potter DW, et al.: 1987, A sensitive immunochemical assay for acetaminophen-protein adducts. J Pharmacol Exp Ther 241:527–533

SAS Institute. 2002. SAS User Guide. Version 9.1. SAS Institute., Cary, NC.

Tellez, G., N. R. Pumford, M. J. Morgan, A. D. Wolfenden, and B. M. Hargis. 2009. Evidence for Clostridium septicum as a primary cause of cellulitis in commercial turkeys. Journal of veterinary diagnostic investigation 21:374–377.

Thachil, A. J., B. McComb, M. M. Andersen, D. P. Shaw, D. A. Halvorson, and K. V. Nagaraja. 2010. Role of Clostridium perfringens and Clostridium septicum in causing turkey cellulitis. Avian Dis. 54:795–801.

Thachil, A., B. McComb, M. Early, C. Heeder, and K. Nagaraja. 2012. Clostridium perfringens and Clostridium septicum toxoid to control cellulitis in turkeys. Journal of Applied Poultry Research 21:358–366.

Thachil, A. J., B. McComb, M. Kromm, and K. V. Nagaraja. 2013. Vaccination of turkeys with Clostridium septicum bacterin-toxoid: evaluation of protection against clostridial cellulitis. Avian diseases 57:214–219.

Thachil, A. J., D. P. Shaw, and K. V. Nagaraja. 2014. Effects of dexamethasone immunosuppression on turkey clostridial dermatitis. Avian diseases 58:433–436.

Useh, N., A. Nok, and K. Esievo. 2006. Blackleg in ruminants. CAB Rev Perspect Agric Vet Sci Nutr Natur Resour 1:1–8.

Uzal, F. A. 2012. Evidence-based medicine concerning efficacy of vaccination against Clostridium chauvoei infection in cattle. Vet. Clin. North Am. Food Anim. Pract. 28:71–7, viii.

Zar , J. 1984. Pages 348–351 in Biostatistical Analysis. 2nd ed. Prentice-Hall, Englewood Cliffs, NJ.

List of Tables

Table 1. Antibody response to *Clostridium septicum* following vaccination with various CS bacterin-toxoid/adjuvant combinations (Experiment 1)

	2 weeks post-prime	5 weeks post-prime, pre-boost	7 weeks post-prime, 2 weeks post-boost	Mortality*,[1]
Control	0.14 ± 0.02[b]	0.44 ± 0.04[b]	0.34 ± 0.06 [b]	1/12 (8.33%)
Alum + OE	0.16 ± 0.02 [ab]	0.51 ± 0.06 [b]	1.48 ± 0.13[a]	0/15 (0%)
MCA + MCA	0.16 ± 0.03 [ab]	0.55 ± 0.06 [b]	1.30 ± 0.14 [a]	1/17 (5.89%)
MCA + Alum	0.31 ± 0.05 [a]	0.35 ± 0.06 [b]	1.25 ± 0.13 [a]	1/17 (5.89%)
OE + OE	0.26 ± 0.06 [ab]	1.06 ± 0.14 [a]	1.63 ± 0.17 [a]	1/18 (5.56%)

[a, b] Indicates significant differences ($P < 0.05$) between treatment groups by age
Turkeys were primed on day-of-hatch with 0.25mL and boosted at 5 weeks-of-age with respective vaccine
1 Several neck tags were lost in each group. Mortality was calculated on the basis of the number of birds with retained neck tags at the end of the experiment
*No cellulitis lesions observed and no challenge was administered in this experiment

Table 2. Localized reactions at vaccine injection site evaluated post-mortem at 7 weeks-of-age (Experiment 1)

	Score			
Treatment	0	1	2	3
Non-vaccinated Control	12/12 (100%)	0/12 (0%)	0/12	0/12 (0%)
Alum + OE	5/15 (33%)	9/15 (60%)	1/15 (6.7%)	0/15 (0%)
MCA + MCA	17/17 (100%)	0/17 (0%)	0/17 (0%)	0/17 (0%)
MCA + Alum	17/17 (100%)	0/17(0%)	0/17 (0%)	0/17 (0%)
OE + OE	6/18 (33.3%)	9/18 (50%)	3/18 (16.7%)	0/18 (0%)

Turkeys were comingled for the duration of the trial. Injection site lesions were not evaluated for turkeys which lost neck tags
Scoring: 0 (not present), 1 (detectable but insignificant), 2 (moderate), and 3 (clinically meaningful lesions)

Table 3. Cellulitis-related mortalities and antibody response *Clostridium septicum* at 8, 12, and 16 weeks post-vaccination (Experiment 2)

	8 weeks post-prime, pre-boost	12 weeks post-prime, 4 weeks post-boost	16 weeks post-prime, 8 weeks post-boost	Late mortality associated with cellulitis
HS 1 Control	0.25 ± 0.01 [a]	0.39 ± 0.05 [b]	0.37 ± 0.02 [b]	81
HS 1 Vaccinated	0.30 ± 0.02 [a]	2.16 ± 0.16 [a]	1.57 ± 0.08 [a]	80
HS 2 Control	0.25 ± 0.02 [a]	0.46 ± 0.09 [b]	0.41 ± 0.07 [b]	70
HS 2 Vaccinated	0.29 ± 0.02 [a]	1.71 ± 0.13 [a]	2.40 ± 0.15 [a]	80
HS 3 Control	0.24 ± 0.02 [a]	0.32 ± 0.03 [b]	0.56 ± 0.18 [b]	148
HS 3 Vaccinated	0.28 ± 0.06[a]	1.60 ± 0.11[a]	2.12 ± 0.15[a]	78*

[a, b] Indicates significant differences ($P < 0.05$) between control and treatment group by house
Vaccinated turkeys were primed with CS bacterin-toxoid MCA vaccine day-of-hatch and boosted (0.5mL) with CS bacterin-toxoid oil emulsion vaccine at 8 weeks-of-age
n=20/group
*Indicates significant difference ($P < 0.001$) between control and vaccinated group by house
Mortality reported between 13 and 16 weeks-of-age

Table 4. Antibody response at 8, 12, and 16-20 weeks-of-age (Experiment 3)

			Antibody Response (S/P) ratio		
	n/farm (# of houses) †	Treatment	8 weeks	12 weeks	16-20 weeks*
Farm 1	13608 (2)	Control	0.43 ± 0.02 [a]	0.56 ± 0.03 [b]	1.28 ± 0.02 [b]
		Vaccinated	0.40 ± 0.02 [a]	0.88 ± 0.07 [a]	2.01 ± 0.16 [a]
Farm 2	21600 (4)	Control	0.31 ± 0.01 [a]	0.81 ± 0.03 [b]	1.01 ± 0.04 [b]
		Vaccinated	0.30 ± 0.01 [a]	1.29 ± 0.10 [a]	1.91 ± 0.12 [a]
Farm 3	21600 (4)	Control	0.37 ± 0.02 [a]	0.64 ± 0.02 [b]	1.08 ± 0.04 [b]
		Vaccinated	0.38 ± 0.01 [a]	1.02 ± 0.06 [a]	2.16 ± 0.09 [a]
Farm 4	6792 (1)	Control	0.61 ± 0.04 [a]	0.77 ± 0.06 [b]	1.12 ± 0.11 [b]
		Vaccinated	0.63 ± 0.04 [a]	1.15 ± 0.15 [a]	1.57 ± 0.15 [a]
Farm 5	36288 (3)	Control	0.51 ± 0.03 [a]	0.66 ± 0.03 [b]	0.98 ± 0.06 [b]
		Vaccinated	0.51 ± 0.01 [a]	0.99 ± 0.06 [a]	1.39 ± 0.13 [a]
Farm 6	18774 (3)	Control	0.53 ± 0.02 [a]	0.92 ± 0.05 [a]	1.38 ± 0.08 [a]
		Vaccinated	0.52 ± 0.02 [a]	1.19 ± 0.05 [b]	1.90 ± 0.12 [b]

[a, b] Indicates significant differences ($P < 0.05$) between control and treatment group by farm

*Collection date determined by producer

Vaccinated turkeys were subcutaneously vaccinated (0.5mL) with CS bacterin-toxoid oil emulsion vaccine at 8 weeks-of-age n=10/group

†n/treatment was allocated evenly between control and vaccinated group for all houses

Table 5. Late cellulitis-associated mortalities (Experiment 3)

	Control Mortality	Vaccinated Mortality	Total Late Mortality	Total Late Mortality as % of Farm	Antibiotic Treatment (# of days)
Farm 1	131 (76%)	42 (24%)*	173	1.27%	42
Farm 2	10 (24%)*	31(76%)	41	0.19%	14
Farm 3	19 (86%)	3 (14%)*	22	0.10%	0
Farm 4	160 (71%)	64 (29%)*	224	3.30%	35
Farm 5	749 (59%)	514 (41%)*	1263	3.48%	42
Farm 6	266 (63%)	158 (37%)*	424	2.26%	35
Exp 2 total†	1335 (62%)	812 (38%)	2147	1.81%	-

*Indicates significant differences ($P < 0.05$) between control and treatment group by farm
(%) indicative of a percent of total late mortalities
†Statistical analysis not conducted due to variability between farms

Chapter IV. Conclusion

C. septicum alpha-toxin is the primary virulent toxin responsible for inducing cellulitis. Preventative measures, such as improved flock management and direct-fed microbials, can reduce the risk of disease, however antibiotic therapy is still required. An effective vaccine could provide immune protection for flocks if vaccinated prior to infection. Oil emulsion vaccines induce long term robust immunity to the antigenic portion of the vaccine, such as the *C. septicum* bacterin-toxoid evaluated in these experiments. Although booster vaccination under field conditions are not ideal, it may be necessary to prevent turkey cellulitis related mortalities without antibiotic treatment.

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To: Billy Hargis
Fr: Craig Coon
Date: February 5th, 2018
Subject: IACUC Approval
Expiration Date: December 31st, 1969

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18073:** *Evaluation of the effect of an autogenous Clostridium septicum bacterin-toxoid vaccine under laboratory conditions*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond December 31st, 1969 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Brittany Danielle-Mahaf, and Amanda Wolfenden. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

18073