VACCINATION STRATEGIES TO CONTROL COCCIDIOSIS IN POULTRY

by

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(Under the Direction of Brian James Jordan)

ABSTRACT

Coccidiosis is an economically significant enteric disease in poultry caused by parasitic protozoa in the genus *Eimeria.* Coccidiosis control is achieved with chemical anticoccidial agents, ionophore antibiotics, live vaccines, or a combination. Vaccines are mass applied at the hatchery, through a water spray diluent or a gel delivery system. The purpose of this research was to compare different coccidia vaccines, administration routes, and control combinations for preventing coccidiosis. The first project compared application parameters, the second characterized oocyst cycling, lesion scores, and protection from a pathogenic *E. maxima* challenge between different vaccines, and the final project was to compare vaccination and bioshuttle programs for the control of *E. tenella*. Regardless of the vaccine, administration method, or therapeutic combination, with sufficient time for cycling, chickens were protected from challenge in all trials.

INDEX WORDS: Coccidiosis, broiler, vaccination, bioshuttle, *Eimeria*

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CHAPTER 1

INTRODUCTION

Coccidiosis is an economically significant disease of poultry. With over 60 billion birds raised worldwide each year for meat and eggs, it is estimated that coccidiosis causes losses of over $3 billion a year (Blake & Tomley, 2014; Dalloul & Lillehoj, 2006). Approximately 80% of the estimated cost is due to production losses from subclinical and clinical disease. The remaining 20% is due to the money spent on prophylaxis drug and chemical use, and treatment (Williams, 1999). Coccidiosis in poultry is caused by parasitic protozoa in the genus *Eimeria*. There are several species that infect poultry and parasitize different regions of the gastrointestinal tract. Clinical signs range from decreased weight gain, increased feed conversion ratio, diarrhea, bloody droppings, and death (Johnson & Reid, 1970). Coccidiosis also predisposes chickens to secondary infection, most notably from *C. perfringens* causing necrotic enteritis, and can serve as carriers of foodborne pathogens (Collier et al., 2008; Qin, Arakawa, Baba, Fukata, & Sasai, 1996).

Control of coccidiosis is achieved through three programs: chemical drugs, ionophore antibiotics, and live vaccines, or a combination vaccine and drug program referred to as a bioshuttle (Jeffers, 1974; Vermeulen, Schaap, & Schetters, 2001). In order to prevent drug or ionophore resistance from building in the organisms between flocks, producers rotate the program in use, ranging from changing for every flock that is placed on a farm to changing seasonally. Most flocks are on a prophylaxis treatment program to prevent clinical disease.

Immunity is developed as the coccidial organism is replicating in the gastrointestinal tract. A one time infection with one hundred organisms or fewer will allow for the development of a

partial immune response to a homologous challenge, as will continuous infection with small number of oocysts (Chapman, Matsler, Muthavarapu, & Chapman, 2005) (Joyner & Norton, 1973, 1976). The immune response developed by a bird is species specific, and in some cases strain specific, meaning that protection gained against one organism will not confer immunity against another. In the chicken, diagnosis of coccidiosis is made using gross lesion scores, microscopic lesion scores, and oocyst count scores during necropsy (Goodwin, Brown, & Bounous, 1998). Depending on the species present, gross lesion scores are found at different locations in the gastrointestinal tract and can cause more severe pathogenic signs (Johnson & Reid, 1970). When measuring the ability of a program to provide protection against challenge, many parameters are examined including oocysts shed in feces, weight gain, gross lesion scores, microscopic lesion scores, and oocyst count scores (Barrios et al., 2017; Goodwin et al., 1998; Reid, Taylor, & Johnson, 1969).

Live vaccines have been available since 1952 (Edgar, King, & Flanagan, 1952). All vaccines contain a minimum of three species: *E. maxima, E. tenella,* and *E. acervulina.* There are attenuated and non-attenuated vaccines available, and all vaccines contain drug sensitive organisms. Vaccines also vary based on the number of oocysts present per dose (Price, Hafeez, Bulfon, & Barta, 2016). Over time, there has been a change in method of delivery of the vaccine which has included in the waterlines, being sprayed over the feed, eye drop vaccination, and spray or gel vaccination in the hatchery (Chapman et al., 2002). In the United States, the majority of the industry is utilizing a mass spray vaccine technique at the hatchery, though the concern over using a water vaccination system is that uneven application coverage occurs. This would result in missed chicks from vaccination, or uneven ingestion of a larger dose of oocysts (Chapman et al., 2002). There is increasing interest in use of a mass gel application at the hatchery, driven by the

recommendation of using a gel diluent with a certain low dose vaccine. The gel diluent works by increasing the length of time that chicks are exposed to the oocysts, allowing for elongated preening time (Danforth, Lee, Martin, & Dekich, 1997). One disadvantage of using live vaccines is that they cause damage to the bird gastrointestinal tract and cause decreased body weights when compared with unvaccinated controls (Conway, McKenzie, & Dayton, 1990; Crouch, Andrews, Ward, & Francis, 2003; Li, Kanu, Xiao, & Xiang, 2005).

Other control techniques include chemical drugs and ionophore antibiotics. The first drug found to have coccidiostatic activity was sulfanilamide in 1939, and since then a multitude of other drugs have been screened or developed to control coccidiosis (Levine, 1939). Producers will combine ionophore and chemical programs, adding in different drugs in the different diets throughout the birds' life. These programs are frequently rotated to prevent resistance building in the environmental organisms, with resistance first noted shortly after the widespread use of coccidiostats (Cuckler & Malanga, 1955). Another control program, a bioshuttle, combines the use of a live vaccine with a drug placed in the grower diet, allowing for the controlled development of immunity before adding a drug. Reasons for using a bioshuttle over other programs is to protect against organisms that were not present in the vaccine, and to lessen the economic impact of the live vaccine (Vermeulen et al., 2001).

The focus of this research is on the application parameters and protection of various live coccidiosis vaccines. The first trial was to determine the application parameters of gel and water diluents with high and low oocyst vaccines. The next trial determined the protection from an *E. maxima* challenge of the first groups, as well as vaccinal oocyst cycling. The final trial was to determine the protection from a pathogenic *E. tenella* utilizing vaccines and bioshuttle programs at different challenge timepoints.

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CHAPTER 2

LITERATURE REVIEW

Economic Significance

Coccidiosis is a disease of major economic importance in the poultry industry. More than 60 billion chickens are produced in the world each year, resulting in the production of more than 1.1 trillion eggs and 90 million tons of meat (Blake & Tomley, 2014). In the United States, 8.8 billion broilers and 244 million turkeys were raised, and 102 billion eggs were produced in 2016 (NASS, 2017). The combined value of production in the United States poultry industry was $38.7 billion in 2016, and $48.1 billion in 2015 (NASS, 2017). The total global economic impact of coccidiosis is estimated to be in excess of $3 billion per year, due to poor feed conversion, reduced egg production, failure to thrive, treatment, and prevention costs (Dalloul & Lillehoj, 2006). Approximately 80% of these costs are associated with the subclinical effects including the loss of performance parameters, and the final 20% of costs are associated with the cost of prophylaxis and treatment (Williams, 1999). Coccidiosis causes further loss and concerns of zoonotic foodborne disease as it is associated with increased intestinal colonization of *Clostridium perfringens* and *Salmonella enterica* serovars Typhimurium and Enteritidis (Baba, Fukata, & Arakawa, 1982; Collier et al., 2008; Qin, Arakawa, Baba, Fukata, & Sasai, 1996). In the UK more than 40% of all antimicrobials sold for the use of food animals are classified for the control of coccidial parasites (Blake & Tomley, 2014).

History, Taxonomy, and Lifecycle

Coccidiosis is caused by parasitic protozoa in the phylum Apicomplexa. Apicomplexa are a diverse phylum that including the genera *Eimeria, Isospora,* and *Toxoplasma.* While *Eimeria* species are characterized by having four sporocysts with two sporozoites each, *Isospora* species are characterized by having two sporocysts with four sporozoites each. In poultry, coccidiosis is caused by species in the genera *Eimeria*. The family *Eimeriidae* is characterized as being homoxenous, with merogony, gamogony, and formation of oocysts occurring in the same host (Perkins, Barta, Clopton, Peirce, & Upton, 2000). The number of described species is over 1,200 and hosts include birds, mammals, reptiles, and amphibia in a multitude of habitats (H. D. Chapman et al., 2013). In birds, *Eimeria* species primarily infect the intestinal tract, although *E. truncata* in the goose causes renal coccidiosis (Montgomery, Novilla, & Shillinger, 1978). Most *Eimeria* species infect only one bird species, although *E. dispersa* can infect many different species including the quail, turkey, partridge, and pheasant (Doran, 1978).

Using classic methodology, there have been nine *Eimeria* species described that infect the chicken. Classic methodology includes defined prepatent periods, oocyst morphology, location of infection, and cross protection from challenge (Tyzzer, 1929). *E. tenella* was originally described in 1891 (Railliet & Lucet, 1891), followed by *E. acervulina, E. maxima, E. mitis, E. necatrix, and E. praecox* (Tyzzer, 1929; Tyzzer, Theiler, & Jones, 1932). The next species to be described were *E. hagani* (Levine, 1938) and *E. brunetti* (Levine, 1942). The last species to be described and accepted was *E. mivati* (Edgar & Seibold, 1964). At this time, the validity of two species, *E. mivati* and *E. hagani* is currently being questioned using molecular techniques, though the original samples were not preserved (Vladimir Vrba, Poplstein, & Pakandl, 2011). The remaining species have been found in multiple countries across all six continents that raise poultry (Clark et al.,

2016). Additionally, with the increasing use of molecular data, there have been recent studies suggesting the presence of three additional species currently referred to as operational taxonomic units (OTU) X, Y, and Z, in the southern hemisphere (Cantacessi et al., 2008; Clark et al., 2016). The turkey is host to seven different distinct species of coccidia from the chicken *E. dispersa, E. meleagrimitis, E. gallopavonis, E. meleagridis, E. adenoides, E. innocua, E. subrotunda* (Ogedengbe, El-Sherry, Whale, & Barta, 2014). The major species to cause pathogenicity differ depending on the type of bird being raised. Broilers are most often impacted by *E. acervulina, E. maxima,* and *E. tenella*. The long-lived birds, including breeders and layers, face challenge from those three in addition to *E. necatrix* and *E. brunetti* (Mathis, Newman, Fitz-Coy, Lumpkins, & Charette, 2017). In turkeys, *E. meleagrimitis* and *E. adenoides* most frequently cause pathogenic signs.

As a significant enteric disease in domestic poultry, the different coccidia species parasitize different parts of the gastrointestinal tract. Clinical signs are related to the region of the lower gastrointestinal tract parasitized, and the pathogenicity of the species and strain. Site of infectivity for each species is predetermined, as the greatest number of sporozoites for each species are found at that site as compared to other places within the intestine following excystation (Shiotani, Baba, Fukata, Arakawa, & Nakanishi, 1992). A distinction between the presence of oocysts and clinical disease needs to be made, as not all infections lead to disease. The most pathogenic strains causing significant mortality in chickens include *E. tenella, E. necatrix,* and *E. brunetti*. Other species are an economical problem, causing significant impaired weight gain and increased feed conversion ratios. *E. maxima* causes diarrhea, decreased weight gain, increased feed conversion and is also involved in the pathogenicity of other significant enteric diseases such as necrotic enteritis (Allen & Fetterer, 2002; Conway & McKenzie, 2007). *E. mitis* and *E. praecox* are typically considered

the least pathogenic, but in high enough numbers can impact the growth and health of birds leading to decreased performance of the flock (Schwarz, Jenkins, Klopp, & Miska, 2009).

All *Eimeria* species that infect commercial poultry have the same general lifecycle, with primary differences between the species arising in host predilection and the target tissue. Infection occurs after ingestion of a sporulated oocyst by a susceptible bird. The oocyst wall and sporocysts are broken down by a combination of mechanical breakdown and enzymatic degradation in the ventriculus and proventriculus respectively, releasing sporozoites. The sporozoites travel along the intestine, invading epithelial cells in predetermined locations. Upon entering the cell, the sporozoite transforms into a trophozoite. As the trophozoite enlarges, it undergoes asexual replication (schizogony) resulting in the formation of a schizont, containing merozoites. The schizont ruptures the host cell and releases the merozoites, which infect other epithelial cells. Most species that infect domestic fowl undergo three cycles of asexual replication (McDonald & Rose, 1987). Upon completion of asexual replication, microgametocytes (male) and macrogametocytes (female) are formed and sexual replication begins. Sexual differentiation in *Eimeria* is not predetermined and male and female gametocytes can be formed from a clonal strain of merozoites (Cornelissen, 1988). Once mature, the microgametocyte ruptures and releases microgametes which fertilize the macrogametes to form a zygote. The zygote then matures into an immature oocyst, which ruptures the host cell and is shed in the feces. Once in the environment the oocyst must undergo sporulation before it is infective to other susceptible birds (Conway & McKenzie, 2007; Walker, Ferguson, Miller, & Smith, 2013). Precocious, or attenuated strains, undergo fewer cycles of asexual replication, produce fewer oocysts, and are less pathogenic than non-attenuated strains (Dalloul & Lillehoj, 2005).

For infection and repeated cycling to occur in poultry houses, the oocysts must undergo sporulation, forming mature sporozoites. There are many different factors that determine the optimal sporulation conditions, including temperature, access to oxygen, and moisture (Kheysin, 2013). Each species has a different optimal temperature for sporulation, ranging from 24° to 29°C (Edgar, 1955). *E. maxima* oocysts have the most efficient sporulation under drier litter conditions (16%) whereas sporulation percentage decreased with increasing moisture content (Waldenstedt, Lunden, Elwinger, Thebo, & Uggla, 1999). Heated floors and dry litter decreased fecal oocyst counts in birds with a primary or secondary infection, when compared to birds kept on wet litter (Abd El-Wahab et al., 2013). Time until the onset of sporulation increased with a lower temperature (21°C compared to 33°C) as well as a lower relative humidity (40% compared to 80%) (Graat, Henken, Ploeger, Noordhuizen, & Vertommen, 1994). Time until oocysts begin sporulating, in optimal conditions, also varies according to the species and can range from 15 to 30 hours, and maximum sporulation occurred eight to nine hours later (Edgar, 1955; Swayne et al., 2013). Additional factors to consider for oocyst cycling and reinfection is oocyst survival time in litter, where the number of surviving oocysts is in proportion to moisture levels, with longer survival of oocysts in higher moisture content. Oocyst survival also decreased with higher ambient temperatures (Reyna, McDougald, & Mathis, 1983). In commercial broiler production, survival time of oocysts is less important as oocysts are continuously shed from infected birds (Williams, 1995b).

Diagnosis

To diagnose coccidiosis, defined as the presence of clinical disease in birds, three procedures are traditionally used: gross lesions, oocyst count scores, and microscopic lesion

scores. Additionally, to detect the presence of oocysts on farms there are two methods of detection: fecal or litter oocyst counts and polymerase chain reaction (PCR).

The most commonly used method to diagnose clinical coccidiosis is to examine for the presence of gross lesions during a necropsy. Each species has a predilection for infection of specific sites of the intestinal tract, and most cause distinct lesions. Limitations to this method include the lack of gross lesion scores in light infections and with non-pathogenic strains. Birds are often infected with multiple species in field cases, confounding and skewing gross lesion scores for the species of interest (McDougald, Fuller, & Solis, 1986). Of the three major species found in broiler chickens, *E. acervulina* infects the duodenum, causing linear white streaks on the epithelial surface. *E. maxima* primarily infects the midgut and causes lesions ranging from petechia on the serosal surface, ballooning, and orange-tinged mucosal contents. *E. tenella* infects the ceca and causes lesions ranging from petechia on the serosa and mucosa, free blood in the lumen, and cecal cores (Johnson & Reid, 1970).

Oocyst count scores (OCS) have also been used to characterize infection and are more rewarding for species and strains that do not cause as severe gross lesions. To perform an OCS, a mucosal scraping of the area of interest is taken and the number of oocysts per high power field is counted, assigning a score ranging from 0 (no oocysts seen) to 4 (too numerous to count) (Goodwin, Brown, & Bounous, 1998; Swayne et al., 2013). Microscores can be affected by the virulence of the strain, with more oocysts present with more virulent strains (McDougald, Da Silva, Solis, & Braga, 1987).

The gold standard for detecting and diagnosing active coccidiosis infections is through histopathology and microscopic lesion scores. Samples are collected, fixed in formalin, sectioned, and stained for examination for the presence of *Eimeria* species, inflammatory response, and

disruption of the normal tissue (Goodwin et al., 1998; Idris, Bounous, Goodwin, Brown, & Krushinskie, 1997). The use of the three scoring systems simultaneously can reveal inconsistencies, as the lesion scores assigned based on the gross lesion scores underestimates the prevalence when compared with the damage and corresponding microscopic lesion scores (Idris et al., 1997). Microscopically, *E. tenella* in the ceca causes dilation and necrosis of the submucosal glands, multifocal areas of severe inflammation and foci of hemorrhage. Parasitic stages are found in the submucosal glands and can be located transmurally throughout the mucosa and submucosa in severe cases. *E. acervulina* in the duodenum causes villous atrophy, fusion of the villi, proliferation of epithelial cells, interstitial edema and mononuclear infiltrate at the submucosa membrane. Parasitic stages are intracellular and found clustered at the tips of the villi. *E. maxima* causes villous blunting and fusion, discrete hemorrhage, and mononuclear infiltrate in the lamina propria. Parasitic stages are found in submucosa and lamina propria (Swayne et al., 2013; Zulpo et al., 2007).

Other recognized methods to diagnose coccidial infections rely on litter or fresh feces instead of performing a necropsy on dead birds. Fecal and litter oocyst counts are used to quantify the number of oocysts that a bird is shedding, or that are found in the litter of infected flocks. Morphological distinction of the oocysts can be made to determine the number of species shed (Castañón, Fraga, Fernandez, Gruber, & Costa, 2007; Conway & McKenzie, 2007; Long, Millard, Joyner, & Norton, 1976). Care must be taken with this approach as many of the size measurements and morphologies overlap between the species. Additionally, strains within a species can have different morphologies present, making interpretation of the morphological differences inaccurate (Long & Joyner, 1984; Poplstein & Vrba, 2011).

For sample collection, it is important to collect the sample that will yield the information that is of interest, since fecal and oocyst litter counts represent different shedding patterns. Counting oocysts in feces indicates the number of oocysts shed at that particular moment, from that particular bird. It is highly sensitive for detecting short-term changes in oocyst production, especially when looking for a peak over a short time (Hodgson, 1970; Williams, 1995c). Fecal counts also can be utilized to detect vaccine uptake from the first cycle (Danforth, Lee, Martin, & Dekich, 1997). Litter counts reveal the total number of oocysts that have been shed and could potentially be ingested by foraging. Oocysts which have already been ingested and those that have been destroyed are missing from the total litter count. It can also give an approximation of the number of sporulated oocysts compared to unsporulated oocysts (Williams, 1995b). Many factors influence the litter oocyst counts including season, litter quality, and number of previous flocks raised on the litter, as well as the use of a synthetic drug in combination with an ionophore (Chapman & Johnson, 1992; Kling, Hanssens, & Grant, 1989; Reyna et al., 1983; Stayer, Pote, & Keirs, 1995). Litter oocyst concentration and species composition can provide valuable insight regarding developing immunity and protection from challenge under different coccidiosis control measures. Litter counts can be valuable in understanding the occurrence of *Eimeria* species in subclinical cases (Sun et al., 2009). In most flocks oocyst counts increase at three weeks, with a sharp peak at four weeks, and a sharp decline by weeks five and six (Chapman et al., 2016; Reyna et al., 1983). The coccidiosis control program being used also influences the abundance and type of *Eimeria* species found in the litter. (Jenkins, Parker, & Ritter, 2017; Lee et al., 2012). A delay in peak counts until week seven or eight can suggest incomplete vaccine coverage or drug resistance depending on the control program in place (Jenkins et al., 2017). In flocks raised without anticoccidials, oocysts in the litter peaked around four and a half weeks with higher numbers of

oocysts, whereas in flocks treated with anticoccidials litter oocyst counts peaked significantly later, around six weeks with significantly fewer oocysts (Williams et al., 1996). Litter counts in flocks raised with drug programs in place had significantly fewer oocysts present at the peak production (Chapman, 1999). Litter counts increase when consecutive flocks are raised on reused litter and can reach a million oocysts per gram of litter (Chapman & Johnson, 1992; Stanley, Gray, Daley, Krueger, & Sefton, 2004). In flocks receiving a live vaccine, the species composition in the litter is similar to that found in the vaccine (Jenkins, Klopp, Ritter, Miska, & Fetterer, 2010). When counting *E. maxima* specifically, the oocyst counts in the litter at two weeks in vaccinated flocks can be correlated to mortality occurring between three days and five weeks, presumably due to the development of necrotic enteritis (Alnassan et al., 2014; Collier et al., 2008; Jenkins et al., 2017).

Fecal oocyst counts are influenced by the prepatent period of each species and strain, with attenuated strains shedding earlier. Of the three species most commonly found in vaccines, *E. acervulina* has a shorter prepatent period, followed by *E. tenella*, and *E. maxima* has the longest prepatent period and latest peak shedding following infection (You, 2014). When examining the effect of crowding of ingested oocysts, the number of oocysts shed per oocyst administered starts to decrease. Another quantitative measure is described at the point where birds given increasing doses of oocysts yield progressively higher oocyst counts, until the maximally producing dose is reached, above which there is a decrease in oocyst yields per oocyst ingested. *E. acervulina* has the highest crowding threshold of approximately 900 oocysts, and a maximum reproductive potential of 3,049 oocysts produced per oocyst for each of the 1,370 oocysts given. *E. maxima* had a crowding effect starting at 39 oocysts, and a maximum reproductive potential at 8713 oocysts produced for each of the 63 oocysts inoculated. *E. tenella* had a crowding effect starting at 72 oocysts, and a maximum reproductive potential at 42,939 oocysts produced for each of the 241

oocysts inoculated (Williams, 2001). The numbers of oocysts that can be produced by a single oocyst of individual infection show the highly proliferative nature of *Eimeria* species and help to explain the rapid onset of outbreaks seen in clinical cases.

In most cases, especially with the increasing use of live-vaccines, individual birds and flocks are most often infected by multiple *Eimeria* species (Long & Joyner, 1984). Coinfections can alter the pathogenicity of those species, as well as the severity of disease (Haug, Gjevre, Thebo, Mattsson, & Kaldhusdal, 2008; Jenkins, Allen, Wilkins, Klopp, & Miska, 2008). As further coccidiosis research is done it will become more important to recognize strain differences infecting flocks, in order to ensure that the vaccines in use continue to protect against the challenges in the field (Morgan & Godwin, 2017).

Many different methods of oocyst enumeration have been used for litter and fecal samples. The most common method of enumeration is to use a McMaster chamber (Conway & McKenzie, 2007; Eckert, 1995; Longet al., 1976). However, there are multiple methods to using this technique. The most traditional technique is very labor intensive to get to the stage where samples can be read. These steps include adding water, potentially diluting out small counts, having to filter out the large debris, and adding additional steps including centrifugation and the addition of a saturated sodium chloride solution before the oocysts are ready to count. Another method of counting removes the majority of the preparation steps and uses a mini-shaker to homogenize the fecal mixture before adding the saturated sodium chloride allowing for a much faster processing while still keeping the sensitivity of the test (Haug, Williams, & Larsen, 2006). Other techniques add a specific amount of fecal matter and use sugar floatation with success (Vadlejch et al., 2013). Another method is the use of a hemocytometer to count, although this method can be more variable with different oocyst numbers (Joyner & Norton, 1973; Peek & Landman, 2003).

Polymerase chain reaction can also be utilized to identify and speciate oocysts present. Depending on the region amplified, it is a highly sensitive and specific technique that minimizes the margin for error. It can also easily pick up the presence of multiple species, even in low numbers or in strains with low pathogenicity. Primarily it is used to identify species present in litter and fecal samples, but can also be utilized with tissue samples, both fresh and formalin fixed (Nolan, Tomley, Kaiser, & Blake, 2015). Polymerase chain reaction allows for the rapid and precise identification of *Eimeria* species, without bias. Two techniques, standard PCR and real-time (quantitative) PCR are most often used to identify the *Eimeria* species present in the litter on different farms in poultry production regions around the world.

For standard PCR, four regions of the genome are presently used to speciate: internal transcribed spacer 1 (ITS-1), internal transcribed spacer 2 (ITS-2), 18s rDNA, and cytochrome c oxidase subunit 1 gene (COI). Both species specific and universal primers have been developed for the ITS1 and COI genes (Barta et al., 1998; El-Sherry, Ogedengbe, Hafeez, & Barta, 2013; Lew, Anderson, Minchin, Jeston, & Jorgensen, 2003; Price, Hafeez, Bulfon, & Barta, 2016; Schnitzler & Shirley, 1999; Schnitzler, Thebo, Mattsson, Tomley, & Shirley, 1998). Recent attention has been focused on using the COI gene, as it is highly conserved within a species but is clearly identifiable between species (Price et al., 2016), as compared to the ITS-1 region that can be highly variable between different strains of the same species, potentially leading to the misidentification and misrepresentative of the species present (Poplstein & Vrba, 2011). Additionally, multiple genetic variants for both *E. maxima* and *E. mitis* are present in flocks, further complicating diagnosis using ITS-1 alone (Lew et al., 2003). Multiple divergent sequences have also been found in the 18s region in certain species of *Eimeria*, again limiting its usefulness as a diagnostic tool (El-Sherry et al., 2013; Schwarz et al., 2009). Using the ITS-2 region, three

additional populations, OUT-X, Y, and Z have been isolated in the southern hemisphere only. They were originally described in Australia but have since been found in high prevalence in Africa as well (Clark et al., 2016; Jatau et al., 2016). When compared to the classically described species, they most closely matched with *E. maxima*, *E. brunetti*, and *E. mitis* (Morgan & Godwin, 2017).

Real-time PCR has also been developed to quantify the number of oocysts of each species using different regions of the genome from standard PCR (Blake, Hesketh, Archer, Shirley, & Smith, 2006). Non-polymorphic sequence characterized amplification regions (SCARs) have been used, which are advantageous due to the lack of cross-reactivity between species (Vrba, Blake, & Poplstein, 2010). One drawback to qPCR is the method of DNA extraction and starting number of oocysts can affect the efficiency of the reaction potentially skewing the results (Cha, Talha, Lim, & Kim, 2014). Another drawback is the variability of the detection limit with qPCR, as for non-sporulated oocysts collected directly from the litter ranges from 40 to ~2,900 depending on the species specific primer and probe resulting in the potential for underrepresenting the presence of coccidiosis (Peek, Ter Veen, Dijkman, & Landman, 2017). Other real-time assays have been developed using the ITS-1 region and can detect as few as 100 oocysts from purified samples, but require 1000 oocysts when concentrating oocysts from fecal samples (Jenkins, Miska, & Klopp, 2006).

A different PCR based assay has also been developed to detect all seven, established, species, as well as the three operational taxonomic units. The assay, termed capillary electrophoresis, is designed with a set of generic primers that amplify a diagnostic fragment of the mitochondrial genome, and only requires one reaction to detect all species, as compared to a semi-nested PCR technique or qPCR, both of which rely on up to eight reactions to detect all species

(Godwin & Morgan, 2014, 2015). One disadvantage to this technique is that, like standard PCR, it does not allow for the quantification of oocysts.

Sensitivity of PCR detection of *Eimeria* species is at least 90% in cases that are detected using traditional floatation methods to count oocysts (Györke, Pop, & Cozma, 2013). In non-vaccinated flocks around the world, the most prevalent species varies according to PCR and includes 100% prevalence of *E. acervulina, E. maxima,* or *E. praecox*. Additionally, mixed infections on the same farm were common (Carvalho et al., 2011; Györke et al., 2013). Other studies have found different prevalence with all species being detected, but the most common being *E. maxima* and *E. praecox* by PCR. Gross lesion scores did not correlate with detection of the different species by PCR or floatation, suggesting immunity or non-pathogenic strains in these flocks (Carvalho et al., 2011).

The *Eimeria* species predominating in the flock also varies with respect to the type of chicken, and differs in respect to broilers, layers, and backyard flocks (Godwin & Morgan, 2015). Detection of *Eimeria* oocysts from litter from non-vaccinated birds in production facilities reaches 81-100%, depending on the type of flock and region of the world (Godwin & Morgan, 2015; Morris, Woods, Richards, & Gasser, 2007). The use of live oocyst vaccines increases the prevalence of positive farms, as oocysts are introduced to the facilities as soon as chicks are placed (Danforth, Lee, et al., 1997; Jenkins et al., 2017; Price et al., 2016).

Immunity

Each species of *Eimeria* induces a specific host response at their respective site of infection in the gastrointestinal tract. T-cells play a major role in the development of immunity. CD4+ T cells and intraepithelial lymphocytes are involved in primary infections, while CD8 T helper cells

are involved in secondary infections (Dalloul & Lillehoj, 2005; Hong, Lillehoj, Lillehoj, & Lee, 2006; H. Lillehoj, 1998). Cytokines also play a role in upregulating and controlling the T-cell response. (Hong, Lillehoj, Lee, Dalloul, & Lillehoj, 2006; Inagaki-Ohara et al., 2006; Rothwell et al., 2004). At low doses of infection with a single *E. acervulina, E. maxima,* or *E. tenella,* each species developed a different cell mediated reaction pattern in the host. Different inflammatory components were not restricted to the region of the gastrointestinal tract that was parasitized, especially in an *E. maxima* infection. There were no major changes to response after challenge with a mixed infection of all three species (Cornelissen, Swinkels, Boersma, & Rebel, 2009).

Birds are considered to have developed partial immunity when they have decreased oocyst shedding following challenge, decreased feed conversion ratios, decreased lesion scores, and most importantly increased body weight gains when compared to non-immunized birds (Williams & Catchpole, 2000). *E. maxima* is the most antigenic and requires a single oocyst or even sporocyst infection to confer a partial immune response, as measured by an increase in average weight gain following a challenge compared to previously unexposed birds. Oocysts were recovered from birds infected with single sporocysts, indicating an active replication of the organisms, as well as the likely sexual undifferentiation of sporozoites (Joyner & Norton, 1976; E.-H. Lee & Fernando, 1978). The oocysts produced after single sporozoite infection, once sporulated, were infectious as conferred by passing through previously unexposed birds.

E. acervulina and *E. tenella* are also capable of infecting birds and allowing for oocyst shedding with as few as a single sporocyst (Lee, Remmler, & Fernando, 1977; Lee & Winder, 1981). Fewer birds produced fewer oocysts after a single sporocyst or oocyst when inoculated with *E. maxima* than did with *E. tenella,* indicating that *E. maxima* is either less infective, or less prolific, making it more difficult to pick up infections. Having active replication with as few as

one sporocyst shows the utmost need for biosecurity, as an outbreak can be started, slowly, by introducing a few oocysts to a naïve flock. In an ideal situation, in a naïve bird, each infective sporozoite can give rise to up to 50 schizonts, each containing up to 600 merozoites, and in turn gametes and oocysts, allowing for the rapid proliferation of infective oocysts in the litter (Challey & Johnson, 1968; Long, 1959).

Birds infected with 100 *E. maxima* oocysts, and not allowed contact with oocysts shed in feces, did not develop complete immunity, measured by a decrease in oocyst production following challenge with a homologous strain, and no significant difference in weight gain from unchallenged birds. When birds were kept on litter, and in contact with their droppings, the birds had developed a complete immunologic response by four weeks of age (Chapman, Matsler, Muthavarapu, & Chapman, 2005). While increasing immunizing dose sizes in combination with subsequent immunizations was never able to incur complete protection against oocysts from a heterologous strain, it did increase protection against challenge. A benefit of partial protection is that fewer oocysts were shed compared to unvaccinated birds, decreasing environmental contamination and litter load and potential for future challenge (Blake et al., 2005). The incomplete protection can be due to having certain common antigens that can stimulate a partial immune protection, in addition to the lack of strain specific, more immunogenic antigens (Blake et al., 2004). Birds can develop immunity to drug resistant strains of *E. maxima*, and other species, by being immunized with the strain at a young age, even without vaccine cycling. With further vaccine cycling, the birds were better protected for challenge. Weight gain was decreased in the vaccinated groups at 21 days, prior to challenge, but there was no difference in weight gain at the end of the grow out period (Danforth, Watkins, Martin, & Dekich, 1997).

E. acervulina, *E. maxima*, and *E. tenella* oocysts that had been attenuated through irradiation and thereby rendered incapable of completing intracellular replication were found to elicit a host immune response, indicating that it is the early stages of infection, including sporozoites, trophozoites, and immature schizonts, and replication that create an immune response. The mechanism for generating immunity is also relatively similar across all species, as all three species, with different predilections for replication, generated an immune response (Jenkins, Augustine, Danforth, & Barta, 1991; Jenkins, Augustine, Barta, Castle, & Danforth, 1991; Jenkins, Seferian, Augustine, & Danforth, 1993).

Additionally, the minimum dose required to protect from homologous challenge is also dependent on the host genotype (Blake et al., 2005). When using the FP ($B^{15}B^{21}$) compared to the SC ($B^{2}B^{2}$) lines, chicks with the FP genome were protected from challenge and developed resistance to infection when a primary inoculation was within the first three weeks of life, whereas chicks with the SC genome were not able to develop protection from a challenge later in life. The FP genome is associated with high susceptibility and development of tumors associated with Marek's disease, whereas the SC genome is relatively resistant. The SC genome was also associated with a decreased reduction in the number of T cells present in the spleen at one day of age, further suggesting the importance of T cell mediated immunity in control of coccidiosis (Lillehoj, 1988). By manipulating the genetics of the pedigree flocks to a more resistant genotype, future flocks will be better protected from challenge from coccidiosis.

While cross protection is an important tool used to identify different species, it must also be noted that there are strains within a species that do not provide complete cross protection against one another. Of note, strains of *E. maxima* were isolated from farms across Canada and the United States, and it was found that although they were highly similar genotypically, certain strains

provided at least partial cross protection against challenge with other strains, resulting in maintaining weight gain, and reduced lesion scores. Other strains were only able to protect against a homologous challenge and did not have any ability to cross protect against the heterologous strains (Martin, Danforth, Barta, & Fernando, 1997). Studies have shown that the sporozoite proteins from these two strains are indistinguishable from one another using SDS-PAGE. Further studies show that there is some level of recognition of the different sporozoites from the host immune system, as shown by varied numbers of sporozoites reaching the crypt epithelium. Additionally, there were high numbers of sporozoites present in the lamina propria 72 hours after a homologous challenge, however the majority of the sporozoites failed to reach the crypt epithelium to further replicate. In the heterologous challenge, there was an increased number of sporozoites reaching the crypts in birds, following infection of the lamina propria. The degree of cross protection was not fully correlated to the sporozoite transportation success (Barta et al., 1998; Basak, Lee, Barta, & Fernando, 2006; Beattie, Fernando, & Barta, 2001; El-Ashram et al., 2015; Martin et al., 1997). This is important when developing vaccines as the strains included need to protect against heterologous challenges (Danforth, Lee, et al., 1997).

The widespread use of commercial vaccines, in places where the oocysts weren't initially isolated, results in variable protection due to geographic antigenic differences (Martin et al., 1997). DNA sequencing of the ITS-1 region is not able to predict which strains are cross-protective, although polymorphisms are associated with different pathogenicity of the isolates (Awad, El-Nahas, & Abu-Akkada, 2013).

Repeated exposure and infection with *Eimeria* species causes the production of *Eimeria* specific immunoglobulin IgG antibodies. These antibodies are transported to offspring via the egg yolk and can protect against challenge for the first two to three weeks of life (Rose, 1972; N. Smith,

Wallach, Petracca, Braun, & Eckert, 1994; Smith et al., 1994). Furthermore, *Eimeria* specific IgG levels in yolks and the sera of chicks correlates with the resistance to infection (Smith, Wallach, Miller, Braun, & Eckert, 1994). Studies have looked at the effect of using maternally-derived antibodies to protect offspring against an early challenge. Hens that were vaccinated with a mixed population of attenuated oocysts had progeny that had a significant decrease in gross lesion scores for certain species when compared to progeny of hens that were unvaccinated. By revaccinating hens, the progeny had a significant reduction of gross lesion scores of all species present. In addition to the reduction of gross lesion scores, progeny of vaccinated birds also shed significantly fewer oocysts than the progeny of non-vaccinated hens (Kitandu, Juranová, & Bedáňová, 2005)

Treatments that maintain weight gain and feed conversion ratio when compared to a control treatment, and only allow for minimal lesions to occur following a challenge, are generally considered protective against that specific strain (Allen, Danforth, & Vinyard, 2004). In general, virulence is described by a 30% reduction in body weight gain and a lesion score increase of 3. For species that cause mortality, 25% mortality of infected, non-medicated birds should be expected to mimic a naturally occurring infection. For a pathogenicity or sensitivity test, birds should be grown for at least ten to fourteen days before challenge. Medicated feed should be started at least 48 hours prior to the inoculation of oocysts in order to give time for the birds to ingest the feed (GFI, 2012). Oocyst output cannot reliably be used to evaluate protection, as the numbers of oocysts produced do not necessarily correlate with the magnitude of the inoculum (Williams, 2001).

Coccidiosis Control Measures

Coccidiosis control in poultry relies predominantly on the use of anticoccidial drugs and vaccination. Prior to 2000, anticoccidial drugs were utilized in ~95% of flocks where anticoccidial control was employed, including ~99% of commercial broiler flocks (Chapman & Jeffers, 2014). Recently, depending on the time of year, usage falls as low as 60%, driven by legislative and consumer pressure (Chapman & Jeffers, 2014; Godfray et al., 2010; Shirley, Smith, & Blake, 2007). Non-attenuated vaccines are included in the coccidiosis prevention program of at least 35-40% of US broiler companies, and is growing each year (Chapman & Jeffers, 2014).

Drugs

The first drug with demonstrated coccidiostatic activity was sulfanilamide in 1939, and it first began to have widespread commercial application in 1947 (Cuckler & Malanga, 1955; Levine, 1939). Since then a multitude of other drugs have been screened or developed to control coccidiosis, both for the use of prevention and treatment of disease (Shumard & Callender, 1970). In order to evaluate anticoccidial protection, oocyst counts and lesion scores were initially utilized (Reid, Taylor, & Johnson, 1969), however now weight gain and lesion scores are most often utilized.

The mechanism of action is different for many of the drugs in use and ranges from membrane function (ionophores), energy metabolism (quinolones), cofactor synthesis (amprolium, sulfonamides), and DNA synthesis (diclazuril) (Abbas, Iqbal, Blake, Khan, & Saleemi, 2011; Chapman, 1984). Each drug specifically targets a different life cycle stage, ranging from sporozoites, to second generation schizonts, to gametes. Each drug also functions best against one or multiple species of coccidia, *E. tenella* is the species most often studied due to the ability

to replicate in cell culture and embryos. In ionophore resistance, there is a considerable decrease in the uptake of ionophores in resistant strains when compared to sensitive strains, likely reflecting protein differences at the sporozoite stage resulting in an altered permeability of the cell membrane (Augustine, Smith, Danforth, & Ruff, 1987; C. K. Smith & Strout, 1979; Zhu, Johnson, & McDougald, 1994). Sulfa drugs work by inhibiting the folic acid pathway and resistance is conferred through mutations in two genes (Peterson, Walliker, & Wellems, 1988; Triglia & Cowman, 1994; Wang, 1975). Amprolium is a thiamine antagonist, and the resistant strain showed decreased sensitivity to the inhibitory effect due to changes at the molecular level of a target receptor (James, 1980). Quinolones work by blocking the electron transport chain in the parasite mitochondria, responsible for cellular respiration. Resistance is developed by lacking the ability to take up the drug, or developing an alternative biochemical pathway (Ryley & Betts, 1973; Wang, 1975). Limited studies have not shown increased virulence in the strains with drug resistance compared to sensitive strains when raised without the presence of anticoccidial agents (Williams, 2006a, 2006b).

Resistance is defined as a shift in susceptibility to a drug (Sangster, 2001). In association with coccidiosis, it means the ability of the strain to survive and replicate in the presence of a drug in doses higher than normally recommended. With the widespread use of drugs to control coccidiosis, currently all anticoccidials in use today have reports of resistance around the world, with a pattern or resistance noted as early as 1954 when resistance of field isolates to sulfanilamide was first detected (Abbas et al., 2011; Cuckler & Malanga, 1955). There are two types of resistance, acquired and cross resistance. Acquired resistance comes with decreases in sensitivity to drugs with the passage of time, such as resistance of *E. acervulina* and *E. tenella* to sulfaquinoxaline (Cuckler & Malanga, 1955). There is a direct relationship between the

concentration of a drug and the degree of resistance a particular strain shows, where using continuous low levels of a drug may allow for the initial selection of resistant organisms (Chapman, 1982). Cross resistance is when an organism is resistant to different compounds with a similar mode of action, despite lack of exposure to each drug (Chapman, 1997). Most commonly, if a strain is resistant to one ionophore it is likely to be resistant to the other ionophores, suggesting that a chemical with a different mechanism of action should be utilized (Abbas, Iqbal, Sindhu, Khan, & Arshad, 2008; Ryley & Betts, 1973).

The improper use of drugs can encourage the parasite to develop resistance. These include inadequate mixing of drugs, under dosing, use of the same anticoccidials for a long time, and frequency and timing of treatments (Chapman, 1997). From the parasite standpoint, rapid proliferation of resistant mutants and genetic diversity contribute to the development of resistance (Chapman, 1997). Multiple resistance occurs when a strain develops resistance to more than one drug with different mechanisms of action (Chapman, 1993). Different mechanisms of resistance exist for different drugs, though the exact mechanism of resistance for each drug may be unknown. Proposed mechanisms include metabolism of the drug to an inactive form, alteration in permeability, use of an alternative biochemical pathway, and modification to the target so sensitivity to inhibition is decreased (Chapman, 1984).

Anticoccidial sensitivity testing (AST) is performed to determine the efficacy of anticoccidial drugs against field strains of *Eimeria* species. AST is typically performed in battery cages in naïve birds (Chapman, 1998). The most commonly measured parameters are body weight gain, feed conversion ratio, gross lesion scores, and mortality (Barrios et al., 2017). One confounding factor of performing ASTs on field samples is there is no selection for *E. maxima* or

the species of interest, so there can be various strains with multiple resistance patterns and pathogenicity present in the sample (Chapman, Roberts, Shirley, & Williams, 2005).

Drug resistance, while typically involving the use of recording weight gains and gross lesion scores, can also be performed in cell culture for *E. tenella*. By using MDBK cells inoculated with *E. tenella* sporozoites, qPCR was able to detect a difference in replication with exposure to drugs, which were comparable to field isolates with known sensitivity and resistant patterns (Jenkins, O'Brien, Fuller, Mathis, & Fetterer, 2014; Thabet, Zhang, Alnassan, Daugschies, & Bangoura, 2017).

Drug sensitivity varies between flocks based on which anticoccidial drugs have been in use, but it can also vary between the *Eimeria* species found on a farm. Studies comparing the differences between *E. tenella* and *E. acervulina* suggest that *E. acervulina* gains resistance at a faster rate (Jeffers, 1974). In looking at the most recent flock, parasites recovered from vaccinated birds had an increased sensitivity to all drugs, while the parasites recovered from birds on an anticoccidial drug found that each species developed more resistance to one of the drugs in use. For example, *E. acervulina* was significantly more sensitive to monensin, and *E. maxima* and *E. tenella* were significantly more sensitive to diclazuril when a live vaccine was used (Peek & Landman, 2006). Resistance patterns in field isolates are consistent with the anticocidials that have been used and increase with the time that the drug has been used in the flocks. (McDougald et al., 1987; Rotibi, McDougald, & Solis, 1989).

One study compared the drug sensitivity found in breeder pullets to broilers in the same study. In general, breeders and broilers were resistant to zoalene, but sensitive to nicarbazin. In addition, the breeders were also resistant to amprolium and sensitive to monensin. Broiler isolates in general have lost more sensitivity to ionophores due to long term use that is not present in the

breeder pullets. Resistance patterns also followed a history of use to that particular drug, with strains being more sensitive to drugs that had not been used in several years. One unanswered question with this study is if the oocysts are moving flocks between the breeders to the broilers, likely due to eggshell contamination, causing a seeding in the broiler houses with resistant species of parasites (Mathis, McDougald, & McMurray, 1984).

Despite the widespread drug resistance found in the field, there has been little evidence to suggest that in-feed anticoccidial drugs are used less frequently than before for this reason (H. D. Chapman et al., 2013). Most drugs, even when there is not resistance, allow for some parasites to complete their lifecycle allowing the birds to acquire natural immunity, even in the presence of very low oocyst numbers (Chapman, 1999). When performing litter counts, oocyst counts follow a similar bell curve in medicated and non-medicated flocks, though at lower counts in the medicated flocks, confirming the completion of the lifecycle (Chapman et al., 2016). In fact, a disadvantage to utilizing highly efficacious drugs is that birds never develop a protective immunity and are sensitive to a coccidiosis challenge during the drug withdrawal period just prior to marketing (Chapman, 2009; Witcombe & Smith, 2014).

With the increase in drug resistant parasite populations, companies have adopted various procedures in order to try to restore sensitivity in the field. One method is to include different compounds in the starter diet and the grower diet, known as a shuttle program. A rotation program is similar but utilizes different compounds in successive flocks. Both of these methods revolve around the theory that organisms may develop resistance to one compound, but can still be controlled through the use of a compound with a different mechanism of action (Rommel, 1982). An advantage to a shuttle program is the continued benefit in performance when looking at weight

gain compared to non-medicated birds, even when comparing across the same lesion scores (Conway, McKenzie, & Dayton, 1990).

Another method is by a massive introduction of drug sensitive coccidia to a house heavily contaminated with drug resistant coccidia (Jeffers, 1976) since oocyst composition in the litter from flocks vaccinated for coccidiosis tended to have a greater drug sensitivity than did flocks maintained on an anticoccidial drug (Jenkins et al., 2010). In experimental practice, an amprolium sensitive and resistant strain were mixed and passed through unmedicated chickens and, over the course of several passages, amprolium sensitivity was fully restored to the resulting strain (McLoughlin & Chute, 1979). In a field situation, this is most easily done with the use of live oocyst vaccines that contain oocysts of strains known to be sensitive to the different compounds (Chapman et al., 2002). Following programs that included placing the flocks on live vaccine, drug resistance has been restored in the field. (Chapman & Jeffers, 2014). In a controlled field experiment, farms that had diclazuril resistance were placed on two programs for successive flocks, either a non-diclazuril anticoccidial drug or a vaccination program. The flocks that were placed on a vaccination program showed an increased sensitivity to diclazuril, whereas the flocks on a different medication did not show any difference in resistance (Mathis & Broussard, 2006). Drug sensitivity to salinomycin has also been tested following successive flocks of broilers raised in floor pens on litter. At the end of the fifth flock, oocysts were isolated from the litter and anticoccidial sensitivity testing revealed that continual exposure over the course of five flocks resulted in oocysts that were partially resistant to the drug. In the absence of continual exposure, resistance went down, and at the end of the four unmedicated flocks there was a complete restoration of sensitivity to salinomycin (Chapman & Jeffers, 2015). One mechanism through which sensitivity is restored is the interbreeding and genetic recombination of a resistant strain

with a sensitive strain (Chapman, 1994). Precociousness, the trait found in the vaccines, can also recombine with field type strains, allowing for a decrease in pathogenicity in the wild type parasitic populations seeding the house (Jeffers, 1976; M. Shirley & Harvey, 1996). Bioshuttle programs, when birds are vaccinated with a drug sensitive live oocyst vaccine and then placed on an anticoccidial drug at two to four weeks post vaccination, allows the birds to continue developing an immune response while limiting the infection due to cycling and exposure to field strains, reducing the risk of coccidiosis (Vermeulen, Schaap, & Schetters, 2001, Li, Kanu, Xiao, & Xiang, 2005).

Vaccines

Use of live oocyst vaccines containing *Eimeria* species has been available in the United States since 1952 (Edgar, King, & Flanagan, 1952; Williams, 2002), when Coccivac® was first introduced to the market containing *E. tenella* oocysts. In 1985 Immucox® was first introduced to Canada (E.-H. Lee, 1987). The first attenuated vaccine, Paracox® was available in 1989 (M. Shirley, 1989; Williams, 1992) followed by Livacox® in 1992 (Bedrnik, Kučera, Firmanova, & Jurkovič, 1989).

Every commercial batch of vaccine must be tested for purity, potency, and safety. Birds used for propagation must be free from clinical signs of disease and serologically tested to ensure the absence of many viruses and bacteria (Chapman, Roberts, et al., 2005). When vaccines are developed, they must undergo rigorous testing, origin and history of the vaccine lines, virulence of the master seed, and purity, potency, and efficacy must all be known for licensure to be gained. A lesion-score based system is most frequently used to assess protection from challenge. Onset

and duration of immunity and immunological interference must all be determined as well (Chapman, Roberts, et al., 2005).

The live vaccines are given or applied to chicks shortly after or before hatch. Different methods of vaccination have evolved through the time and include the application of gel beads to the feed, introducing vaccine in the water line, vaccination in-ovo, ocular vaccination, and vaccination with a spray cabinet at the hatchery (Chapman et al., 2002). A dye is included in with the diluent to promote preening and ingestion of the vaccine by the chicks. Vaccines administered in the hatchery are given in a water based or gel based diluent (Awad et al., 2013; Danforth, Lee, et al., 1997; Dasgupta & Lee, 2000; Jenkins et al., 2012; Jenkins et al., 2013). Birds can also be vaccinated by eye-drop against *Eimeria* species and develop immunity, as the oocysts travel through the nasolacrimal duct to get to the gastrointestinal tract (Chapman, 1996; Chapman & Cherry, 1997).

Limited work has been done with vaccination of birds in ovo utilizing sporozoites or oocysts. With the proper buffer, all species were infective and were able to be recovered within a week following hatch. Embryos were vaccinated in the yolk sac on day 18 of incubation, however the prepatent period indicated that the chicks were not infected until the time of hatch. Chicks were protected from a homologous challenge at 14 days of age (Weber, Genteman, LeMay, Lewis Sr, & Evans, 2004). These results suggest that there is no benefit with vaccinating in ovo with oocysts compared to day of hatch as the chicks are not infected until hatch, and do not have an earlier development of immunity. However, when injecting with purified sporozoites, there was a slightly earlier shedding and peak of oocysts compared to vaccination at hatch (Weber & Evans, 2003; Weber et al., 2001).

Vaccines themselves differ on the number of oocysts present, species present, and the attenuation status of the oocysts. All major commercial vaccines for chickens contain strains of *E. acervulina, E. maxima*, and *E. tenella*, representing the most common species found on broiler farms. The presence of additional strains varies with manufacture, with some choosing to include *E. necatrix* and *E. brunetti* in vaccines designed for layers, as these strains tend to cause clinical signs in older birds. Other companies choose to include novel strains, including *E. mivati* and *E. mitis* (Chapman et al., 2002; Vermeulen et al., 2001). One vaccine contains all seven species (Dalloul & Lillehoj, 2005). Some vaccines include attenuated strains of different species, as they are less pathogenic and cause fewer economical losses than the fully pathogenic strains. Some vaccines also contain multiple strains of *E. maxima* to account for the immunovariability present (Dalloul & Lillehoj, 2005). A downside to using a live vaccine is that they may introduce new species or unexpected pathogens into a flock (Dalloul & Lillehoj, 2005). Vaccines can also fall into two different categories, high oocyst dose vaccines and low oocyst dose vaccines, that vary based on the number of oocysts present, ranging from less than 200 to roughly 3000 oocysts per dose (Price et al., 2016). All vaccines include drug-sensitive species of oocysts and can re-seed an environment with drug-sensitive species. Birds given a vaccine containing precocious oocysts performed as well as birds raised on anticoccidial drugs, a benefit over other vaccines (Crouch, Andrews, Ward, & Francis, 2003).

Other studies have examined the effectiveness of different application methods, comparing oocyst excretion and protection from challenge from gel-based, oral gavage, and spray vaccinated broilers (Dasgupta & Lee, 2000; Jenkins et al., 2012). It was found that when chicks are placed on litter and allowed contact with their feces to allow for oocyst recycling, the inconsistency of vaccine application method was negated and there was no statistically significant difference in

protection from challenge (Jenkins et al., 2013). One such study found that gel delivery elicited the greatest protection to challenge on floor raised broilers (Danforth, 1998). Use of gel also resulted in higher gross lesion scores and shed oocyst counts than do other vaccination methods, including immunization by gavage. The higher scores and counts indicate better vaccine cycling and should result in better protection from challenge. One reason could be due to the slightly slower ingestion of oocysts with gel than with the gavage when all oocysts are ingested at the same time (Danforth, Lee, et al., 1997).

Downfalls to using the live vaccines include the decreased weight gain and increased feed conversion ratio compared to non-vaccinated flocks, as the birds are being given a controlled challenge to develop immunity. Another downfall is the non-uniform application of the vaccine when using mass-application methods. Non-uniform application of vaccine results in uneven flock growth and protection as birds that are missed in the initial application and pick up an uncontrolled dose from the litter a week later (Chapman et al., 2002). Time until placement also influences the vaccine uptake. The antigenicity of different strains is also important, as there are field isolates that are not protected by the vaccine strain oocysts, so continuous studies need to be done when farms experience clinical signs despite being on vaccine programs (Danforth, Lee, et al., 1997). The decreased weight of immunized birds, as evident at four weeks, is reversed by seven to nine weeks when heavy broilers go to market, making it less important for heavy birds than light birds that are marketed sooner (Danforth, 1998). One reason behind decreased weight gains is light infections of *E. acervulina*, as described by low gross lesion scores, still negatively impact weight gain. Heavy infections, more consistent with field challenges produce marked reduction in growth rates, more apparent one week following inoculation, but can persist for three to four weeks (Reid & Johnson, 1970). Vaccination with non-attenuated strains also follows a pattern, with lesions due

to vaccine appearing on day 14 and persisting through day 28, with a peak in lesions found between 18 and 23 days (Chapman et al., 2002). Blood tinged droppings can also occur, with no noted adverse effects in health, behavior, or performance of the birds (Williams, 1994).

Despite a decrease in excystation efficiency in young birds, only 1.5% of sporulated oocysts were recovered in feces after inoculation in a two-day old chick within 24 hours of inoculation. These oocysts remained infective and could be ingested by another chick after placement (Shiotani et al., 1992; Williams, 1995a).

Another factor that affects the first cycle shedding of live-vaccine oocysts is the amount of time before chicks are first placed on feed. In the field, this time is influenced by processing and transportation time to a farm. When chicks are given access to feed immediately, they peak shedding at five days post vaccination, as the delay in access to feed increases by 12 hours, peak shedding is delayed by one day, meaning a 24 hour delay in access to feed results in peak shedding at seven days post vaccination, however there is no difference in number of oocysts shed from the three groups (Price, Freeman, Van-Heerden, & Barta, 2015). The delay in feed intake could result in slower development of immunity as reuptake of oocysts after ingestion is delayed.

Vaccine failure may be due to a large number of wild type oocysts present in a house when chicks are placed. If the litter and oocysts are not physically removed from the house, disinfectants have very little efficacy over killing the remaining oocysts. Clinical coccidiosis due to large numbers of *Eimeria tenella* have occurred, resulting in mortality between days 16 and 18. Oocyst cycling suggests the chicks ingest the wild type oocysts within the first three days of placement, and ingestion of the shed oocysts by day eleven to produce peak lesions five to seven days post challenge (Sainsbury, 1988).

Although gross lesions are relied upon for the diagnosis of coccidiosis in the field, they do not tell the full story of infection with the different species. Weight gains decreased slightly with increasing *E. acervulina* lesion scores, and there was a modest reduction with increasing *E. maxima* and *E. tenella* lesion scores. When salinomycin was included in the diet, there was a greater weight gain when compared to unmedicated birds with the same lesion scores (Conway et al., 1990). Mortality rates, and weight gain are poorly correlated with one another, in *E. tenella* challenges (Gardiner, 1955; Shumard & Callender, 1970). To get a full clinical evaluation, gross lesion scores must be evaluated in association with a second parameter to judge the severity of the infection.

Strains are considered attenuated when they have a shortened prepatent time due to decreased number of merogonic stages (Mathis et al., 2017; Williams, 1999). There are two methods to attenuate *Eimeria* strains. One method can be used with all species and is performed by collecting only the earliest oocysts while continuously passing the strain through naïve birds. The second method is used with *E. tenella* and allows for embryo adaptation of the strain, as *E. tenella* can replicate on the embryonic cell membranes (Bedrnik et al., 1989). There are presently three fully attenuated vaccines available on the market in Europe today. They contain different species ranging from five to seven. All of the vaccines had variable time to onset of immunity to the five challenge strains utilized, however none of them developed complete immunity to *E. maxima* by challenge 32 days post vaccination. The delay in increase and decrease in oocysts shed per gram of feces corresponded with the delay in onset of immunity, as seen in the lesion scores present. The use of attenuated vaccines did not cause the same decrease in weight gain that is found with the use of non-attenuated vaccines (Mathis et al., 2017).

Immunity to *Eimeria* species with the use of live oocyst vaccines is developed by applying a controlled number of non-pathogenic oocysts to day old chicks, and the immunity is enhanced by fecal-oral transmission of the shed oocysts (cycling). The importance of vaccine cycling is more notable in caged birds that should not typically have access to fecal material, breaking the fecal-oral transmission cycle. At one point, coccidiosis had been believed to be eliminated as a problem in caged birds (Bell & Weaver, 2002), though this has proven untrue as necrotic enteritis associated with *E. maxima* has been diagnosed as have developments of outbreaks of clinical coccidiosis in hens after being moved from the pullet house to the layer house (Gingerich, 2007; McDougald, Fuller, & McMurray, 1990; Soares, Cosstick, & Lee, 2004). Without the slow, controlled exposure to oocysts early in life, the pullets never develop immunity. In order to protect from these challenges, or chicks raised in cages, this means that a solid floor must be put down over the wire for the birds to uptake oocysts (Price, 2012; Price, Guerin, Newman, Hargis, & Barta, 2013).

When using a water diluent with a live-oocyst coccidiosis vaccine, there is the tendency of chicks to be missed by spray vaccination. In order to ensure all chicks receive a dose and to better mimic the gold standard of oral gavage, a gel diluent was introduced (Chapman et al., 2002). One problem associated with either vaccination method is to ensure even distribution of oocysts, though once the gel is properly mixed, oocysts do not settle out (Dasgupta & Lee, 2000). Gel vaccinated birds have a similar number of oocysts shed when compared to birds vaccinated by oral gavage, birds vaccinated by spray had fewer oocysts shed, suggesting fewer oocysts were ingested in each bird. Following challenge, there was no significant difference in weight gain and feed conversion between gel vaccinated and oral gavage birds, compared to a decrease in weight gain and an increase in feed conversion ratios of spray vaccinated birds, indicating that the birds vaccinated by spray were not as well protected from challenge as the other two groups (Jenkins et al., 2012). In

floor pen challenges, when birds have continuous contact with the litter, which allows for vaccinal oocyst recycling, the immunization efficacy increases as noted by a greater weight gain, decreased feed conversion ratio. Oocyst excretion following a pathogenic challenge was also greatly reduced when compared to non-immunized control birds, also indicating protection (Jenkins et al., 2013).

When there is non-uniform coverage of birds administered vaccine in mass applications, the vaccine cycling allows for those birds that did not initially ingest oocysts from the vaccine to still develop immunity (Price, Guerin, & Barta, 2014; Price et al., 2016). It also means that birds may not be protected from an early challenge, though they are protected from a challenge of the same dose and strain after cycling is completed (own data). Depending on the vaccine utilized, either two to three cycles are needed to develop protective immunity.

There has been development, although no commercially available vaccine, utilizing subunit and recombinant vaccines to vaccinate against coccidiosis. Trials using antigens of the three major species in broilers found that the birds had significantly higher body weights and significantly decreased feed conversion ratios three weeks after challenge. None of these immunization attempts have resulted in complete protection against oocyst challenge however (Jenkins, 2001; Vermeulen et al., 2001). Other advantages associated with recombinant vaccines is that only the antigens that stimulate the immune response are included, leaving out antigens that result in immunopathology and the stability of the proteins compared to the reliance on live, infective, oocysts. In natural infections, primary and secondary infections are stimulated by the CD4+ and CD8+ T cell responses. To stimulate these pathways, a subunit or recombinant vaccine needs to undergo intracellular processing as well to stimulate the major histocompatibility complex molecules (Jenkins, 2001).

Cleaning and disinfecting

The wall of coccidial organisms is impermeable to water-soluble substances but is permeable to lipid-soluble substances and small molecules (Belli, Smith, & Ferguson, 2006). This renders the organisms resistant to the most disinfectants, with the exception of ammonia, methyl bromide, phenol, and carbon disulphide (Williams et al., 1997). Disinfectants are tested for cidal effects both with the ability to prevent sporulation from occurring, and the ability of sporulated oocysts to infect after exposure. The ability of treated oocysts to infect is judged by the fecal output of oocyst, mucosal scrapings (OCS), and gross lesion scores and is compared to non-treated oocysts. For a product to gain certification as a coccidial disinfectant, a minimum inhibitory activity of 95% must be reached (Daugschies, Böse, Marx, Teich, & Friedhoff, 2002). The most effective disinfectants used are ammonia and phenol. Phenol is the least affected by the presence of organic matter, as the inhibitory activity of the others is greatly reduced. Increasing the temperature also increases the efficacy for disinfectants and decreases the contact time required to kill the oocysts. Increasing this temperature from 25°C to 40°C, reduced the contact time necessary to kill the oocysts from 24 hours to 15 minutes for both 10% phenol and 10% ammonia (Samaha, Haggag, Nossair, & Habib, 2013). Other studies have found that using a 10% solution of liquid ammonia will kill 100% of oocysts in 45 minutes, at a standard room temperature. When volatized, 25 mg/liter will destroy 100% of oocysts within one hour though the concentration decreases considerably when the ammonia comes into contact with fecal material and other organic materials. Even at the reduced concentrations, given enough time all oocysts that come into contact with the ammonia gas will be destroyed (Horton-Smith, Taylor, & Turtle, 1940). For all oocysts in the house to be exposed to ammonia, a thorough cleanout must be performed, otherwise those in the litter will not necessarily be exposed. These contact times, and chemical side effects do not

allow for widespread application on farms. Gluteraldehyde and Karnovsky's fixatives resulted in complete crenation of oocysts after this time, however none of these chemicals can be used for mass disinfection due to health hazards and contact time required (Duszynski & Gardner, 1991).

Other methods of disinfecting include high temperatures, desiccation, freezing, and ultraviolet light. Oocysts from coccidial species are rapidly destroyed as the heat increases, however this effect does not start until approximately 60°C, where it took several minutes to kill oocysts, to 70°C and above when it took a few seconds to destroy oocysts (Fayer, 1994). At low temperatures, the colder the temperature the less time it took until the oocyst was destroyed and no longer capable of infection. At 10°C, a few oocysts were still infective after seven days (Fayer & Nerad, 1996). Oocysts are also sensitive to desiccation, with 100% dead after only 4 hours (Robertson, Campbell, & Smith, 1992). The use of ultraviolet light alone does not seem to kill 100% of the organisms on a surface, however it can reduce sporulation rates to <5% in clear water. One of the drawbacks to using UV light is that it is impractical for all surfaces in a field to be exposed to a light source (Kniel, Shearer, Cascarino, Wilkins, & Jenkins, 2007; M. Lee & Lee, 2005).

Additional common laboratory chemicals that are unable to penetrate the oocyst wall include potassium dichromate, formalin, ethanol, bleach, and sulphuric acid. Potassium dichromate, a chemical that had been used as a preservative as it is biocidal, is used for oocyst sporulation and long term (weeks-months) storage of oocysts (Upadhyay, Goyal, Kumar, Ghai, & Singh, 2014). Sodium hypochlorite, household bleach, is used to clean oocysts and remove bacterial and other contamination. Additional chemicals, such as 10% buffered formalin and 70% ethanol, allow for some degradation of oocysts over the course of four months at 23°C (Duszynski & Gardner, 1991).

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CHAPTER 3

COMPARISON OF THE APPLICATION PARAMETERS OF COCCIDIA VACCINES BY GEL AND SPRAY[1]

[1]Tensa, L.R. and B.J. Jordan. To be submitted to *Journal of Applied Poultry Research*.

Summary

Coccidiosis is an economically significant enteric disease caused by *Eimeria spp.* Control of the disease is achieved through various means, including chemical anticoccidial drugs, ionophore antibiotics, and vaccination. Differences between the vaccines include the number of oocysts per dose (varying by as much as tenfold between vaccines), attenuation status of the oocysts, and the species present within the vaccine. Coccidia vaccines are typically administered via spray cabinet to day old chicks, however a new gel-based delivery system that claims to elongate preening time and increase oocyst ingestion has been introduced and is specifically recommended for certain low dose vaccines. The purpose of this trial was to compare the application properties between high and low oocyst dose vaccines administered via gel and spray delivery systems to determine if application systems could potentially affect application success. The vaccines were mixed into gel and spray diluents per manufacturer's instructions, and samples were taken to assess how well the oocysts remained in suspension. Gel and spray application patterns were assessed by measuring the size and number of droplets applied onto a plexiglass sheet in a chick basket. Different size droplets were collected and oocyst enumeration and speciation were performed. Results show that no settling occurred after mixing in either diluent. As expected, the number of oocysts per droplet increased as droplet size of the spray administration increased but stayed constant in the uniform droplet size of gel administration. There is a consistent number of oocysts found in each of the sections across the plexiglass sheet. Taken together, this data will aid poultry producers in deciding which delivery system will provide the best protection in their production system.

Description of Problem

Coccidiosis is an important intestinal parasite in chickens caused by apicomplexan protozoa in the genus *Eimeria*. Multiple *Eimeria* species infect the chicken, the most significant in the broiler industry being *E. maxima, E. tenella,* and *E. acervulina.* Clinical infection with these species causes decreased weight gain, increased feed conversion ratio, mortality, and predisposition to secondary infections (Allen & Fetterer, 2002; Conway & McKenzie, 2007; Johnson & Reid, 1970; Reid & Johnson, 1970). Combined, these factors make coccidiosis a disease with significant economic impact. More than 60 billion chickens are produced worldwide each year, and the total global impact of coccidiosis is estimated to be in excess of $3 billion per year (Blake & Tomley, 2014; Dalloul & Lillehoj, 2006). Approximately 80% of these losses are associated with the subclinical loss in performance parameters, including decreased weight gain and increased feed conversion, and the remaining 20% of costs include the cost of prophylaxis and treatment measures (Williams, 1999). In addition, coccidiosis is associated with increased intestinal colonization of *Clostridium perfringens* and *Salmonella enterica* serovars Typhimurium and Enteriditis, leading to further economic losses (Baba, Fukata, & Arakawa, 1982; Collier et al., 2008; Qin, Arakawa, Baba, Fukata, & Sasai, 1996).

Historically, coccidiosis has been controlled using chemicals and ionophore antibiotics, but there is increasing use of live coccidiosis vaccines due to consumer preferences, anticoccidial drug resistance difficulties, and governmental regulations (Jeffers, 1976; Vermeulen, Schaap, & Schetters, 2001; *Veterinary feed directive*, 2015). These vaccines contain drug-sensitive sporulated oocysts of different species, with *E. maxima, E. tenella,* and *E. acervulina* present in all vaccines. The first commercially available coccidiosis vaccine in the United States was introduced in 1952 (Edgar, King, & Flanagan, 1952), with many other vaccines introduced since then (Lee, 1987;

Shirley, 1989; Williams, 1992). These vaccines vary based on the number of total oocysts present in each vaccine, the attenuation status of the organisms present, and the number of species present (Chapman et al., 2002; Dalloul & Lillehoj, 2005; Vermeulen et al., 2001). The total number of oocysts in a vaccine varies tremendously and ranges from less than 200 to approximately 3000 oocysts per dose (Price, Hafeez, Bulfon, & Barta, 2016).

Many different application methods have been used to apply the live oocyst vaccines, including administration on the farm through the drinking water, spraying on feed, use of gel droplets applied to the feed, and administration at the hatchery using ocular vaccination, gel bead delivery, and spray cabinets (Awad, El-Nahas, & Abu-Akkada, 2013; Chapman, 1996; Chapman et al., 2002; H. D. Chapman & Cherry, 1997; Danforth, Lee, Martin, & Dekich, 1997; Dasgupta & Lee, 2000; Jenkins et al., 2012; Jenkins et al., 2013). In the United States, coccidiosis vaccines are most commonly administered through a spray cabinet to day old chicks at the hatchery, though there is increasing interest for use of a gel diluent as studies have implicated that vaccination utilizing a water spray delivery system can result in uneven vaccine application resulting in chicks that do not receive any vaccine or ingest fewer or more oocysts than others (Chapman et al., 2002; Price, Guerin, & Barta, 2014). The chicks that do not receive vaccine in the hatchery will then be exposed to uncontrolled amounts of oocysts in the environment as other chicks shed oocysts, which can result in worsened clinical signs in those birds.

Previous studies examining the effectiveness of different application methods of coccidia vaccines compared oocyst excretion and protection from challenge from gel-based, oral gavage, and spray vaccinated broilers (Albanese, Tensa, Aston, Hilt, & Jordan, 2018; Dasgupta & Lee, 2000; Jenkins et al., 2012) and have reached different conclusions about level of protection. Some studies found that gel administration elicited the greatest protection against an *E. maxima*

challenge, while other studies found no difference in protection but differences in number of oocysts shed between different delivery methods (Albanese et al., 2018; Danforth, 1998; Jenkins et al., 2013). This study was performed to determine if the diluent used with the vaccine and method of vaccine delivery greatly influenced the number of oocysts applied in either a high or low dose vaccine.

Materials and Methods

Vaccines

Two vaccines were used for these studies: a high oocyst dose vaccine, Coccivac®-B52 from Merck Animal Health, and a low oocyst dose vaccine, Immucox® III from CEVA Animal Health.

Oocyst Enumeration

Oocysts were enumerated for all parts of the trial utilizing a McMaster's chamber. Vaccine was mixed with an appropriate dilution of saturated salt water based on the concentration of oocysts. The resulting sample was then mixed and pipetted into a McMaster's chamber. The chamber was allowed to sit for three minutes so oocysts could rise to the top of the chamber, then were counted using the method of Conway and McKenzie. Oocysts were speciated according to the morphological characteristics of the different species present in the vaccine, including size and shape (Conway & McKenzie, 2007; Eckert, 1995; Long, Millard, Joyner, & Norton, 1976).

Sporulation rate

To assess for sporulation, a sample was taken directly from each vaccine vial and diluted to an appropriate dilution for counting using a McMaster's chamber (Coccivac®-B52 was diluted 1:1000; Immucox® III was diluted 1:10). Based on the presence or absence of sporocysts and sporozoites within the oocyst, each oocyst was categorized as sporulated or unsporulated (Conway & McKenzie, 2007). All samples were read in triplicate and counted in accordance with species and sporulation status of the oocysts.

Vaccine Mixing

Each vaccine was mixed with either the CEVA gel diluent or water, at a dosage of 250 mls reconstituted gel per 1000 doses or 240 mls of water per 1000 doses. The CEVA gel powder was reconstituted according to manufacturer's protocol prior to mixing in the vaccine. To ensure even mixing of vaccine throughout the gel diluent, a handheld electric whisk was used to mix for three minutes. Vaccine in water diluent was mixed by stirring and inversion of the sealed vaccine container.

Settling

To determine settling in water, one bottle of each vaccine was mixed in the appropriate amount of water and was continually aerated as is recommended to maintain a uniform distribution of oocysts. Samples were drawn from three levels, the top, middle, and bottom, every 15 minutes for two hours. To determine settling in gel, one bottle of each vaccine was mixed in the appropriate amount of reconstituted gel. Samples were drawn from the same three levels every 15 minutes for two hours, and at 24 hours. All samples were read using a McMaster's chamber in triplicate.

Application Pattern

A commercial spray cabinet utilizing two angled spray nozzles or a gel drop bar cabinet was used to apply the vaccines in different diluents onto a sheet of plexiglass placed on top of a chick basket to determine the application pattern of each delivery method.

Oocysts per Droplet

Each sheet of Plexiglass was divided into six even sections, the left, middle, and right section in the front and back of the plate. Droplets were collected from each section. For the vaccines applied via spray administration, droplets were categorized into 5 sizes based on recoverable volume: extra-small (<1 ul), small (1 ul), medium (5 ul), large (10 ul), and extra-large (15-30 ul). Five droplets of each obtainable size (small to extra-large) were acquired from each section and every oocyst present in the droplet was counted using salt floatation in a McMaster's chamber as described. For the gel vaccine application only one size droplet was formed (~30 ul). Five droplets were randomly collected from each section of the plexiglass and every oocyst present in the droplet was counted using salt floatation in a McMaster's chamber. Three replicates were obtained by collecting droplets from three sheets of plexiglass representing three separate passes through the application system for each vaccine and diluent combination.

Oocysts per Dose

A dose of 24 mls per 100 chicks was used to calculate oocyst per dose present in the water diluent. A dose of 25 mls per 100 chicks was used as the dosage for oocysts present in the gel diluent. The total number of oocysts per bottle was divided by the number of doses present in the bottle to determine the number of oocysts per dose present in the bottle. For the dosage out of the

working stock and nozzle, the number of oocysts per milliliter was divided by the number of doses present per milliliter for each respective diluent. In order to determine the oocysts present per dose for the gel diluent it was assumed that chicks ingested gel droplets for a dose of 250 microliters per chick. To determine the number of oocysts present per dose for the gel diluent it was assumed that the chick would ingest an equal number of each size droplet to ingest a 240 microliter dose per chick.

Results and Discussion

Vaccine

Both vaccines showed a sporulation rate of >95%, indicating the majority of oocysts present could be capable of infection. The three species common to both vaccines were present in the same proportions with *E. acervulina* the highest, followed by *E. maxima,* and *E. tenella.* The high oocyst dose vaccine, Coccivac®-B52, contained ~ 1630 oocysts per dose, approximately six times the number of oocysts in the low oocyst dose vaccine, Immucox® III, which contained approximately 270 oocysts per dose (Figure 3.1).

Mixing

Although oocysts are extremely small, less than 30 micrometers, it is widely known that they settle when diluted in water unless a method to continuously agitate the solution is used (Landers, 1960; Long et al., 1976). As seen in this trial, when properly agitated, no settling occurred for either vaccine when mixed in a water diluent (Figure 3.2). Conversely, when using a more viscous gel product, the oocysts did not settle and did not require any continual agitation (Danforth et al., 1997). The gel diluent also claims it can be stored for one day after reconstitution

and prior to use, so a sample was collected at 24 hours post mixing to ensure that oocysts remained in suspension. Neither vaccine exhibited settling at this timepoint, indicating that a uniform application of vaccine in gel can still occur at 24 hours. It should be noted however, that diluting vaccine in gel diluent requires more rigorous mixing protocols and could be unevenly mixed if not done correctly. This would result in uneven vaccine distribution during application.

Spray pattern

When vaccines in water-based diluent were sprayed on the plexiglass, the extra-large droplets were located primarily at the left and right edges (Figure 3.3A). There was an even distribution of the remaining sizes of droplets from side to side and front to back. When vaccines were applied in gel via gel drop bar, similar sized droplets were present across the entire sheet of plexiglass, with the 32 rows of droplets corresponding to the 32 "tips" present on the drop bar (Figure 3.3B).

Oocysts per droplet

Coccivac®-B52 applied in water diluent had oocysts present at each droplet size, and as the droplets increased in size, the number of oocysts increased (Figure 4A). Oocyst counts per species remained in the same proportion as the vaccine bottle until the final time point, when *E. mivati* became the most common oocyst found. Immucox® III applied in a water diluent, when averaged across the three replicates, did not contain *E. tenella* in all droplets of any size (Figure 3.4B). Coccivac®-B52 applied in gel diluent had all four species present in the single droplet size, and in similar proportions as the vaccine bottle (Figure 3.4C). The droplets also did not contain an average of at least one oocyst of the two other species until the large droplet size. Immucox® III

applied in gel diluent contained multiple oocysts of each species in each droplet (Figure 3.4D). As seen with Coccivac®-B52, Immucox® III applied by either method maintained species proportionality with what was observed in the vaccine bottle. A common concern about mass application in hatcheries is uneven distribution across the chick basket (Chapman et al., 2002), though, in this trial, none of the vaccine and diluent combinations showed any difference in total number of oocysts present in the different sized droplets between the six sections on the plexiglass (Figure 3.5).

Dosages

Oocysts per dose were calculated at each collection point; vaccine directly from the bottle, vaccine mixed in the respective diluent, vaccine collected directly out of the nozzle or gel bar of the vaccination cabinet, and from the droplets applied to the plexiglass sheet in the chick basket (Figure 3.6). Comparing dosage numbers for spray application, there is a general decline in oocyst numbers for each collection point. This decrease is especially present between the oocyst counts in the working stock compared to oocyst counts from the spray nozzle, potentially due to the shearing effects of aerosolization from the nozzle. There is an additional decline in oocyst counts from the nozzle to what was collected on the plexiglass, which can be attributed to the smaller droplets that never made it to the plexiglass sheet due to external factors (air movement, natural fall rate of liquid droplets). This decline in vaccine reaching the chicks has also been noted in spray applied respiratory vaccines as well (Jordan, 2017). The gel diluent and gel drop bar did not show this decline in oocysts reaching chick level, most likely due to the larger size and weight of the gel droplets, which are not influenced by external factors.

For coccidiosis vaccination to be successful, oocysts must reach the chicks in a uniform manner and this study shows that, regardless of vaccine or diluent, oocysts did reach the level of chicks in the chick basket. Oocysts from each vaccine were evenly distributed and remained in the proper proportion for each delivery method, indicating that each delivery system can effectively deliver vaccine without differentially affecting any particular coccidia species in the vaccines. Differences were seen in the effective dose of oocysts reaching chick level, with the main effect coming from application method. It remains to be seen how the difference in effective oocyst dose reaching chick level will influence vaccine oocyst ingestion by the chicks and, thereby, influence vaccine coverage and protection from challenge. In conclusion, this data demonstrates the similarities and differences between application characteristics of high and low oocyst dose coccidia vaccines when applied in water and gel diluents.

Conclusions and Applications

1. Oocysts from both vaccines in both diluents remained in suspension when properly handled, leading to consistent application.

2. The spray pattern was consistent for both vaccines from front to back of the plexiglass, with the largest droplets concentrated on the left and right edges. The gel application pattern was consistent from left to right and front to back.

3. Oocyst counts were proportionally consistent through all droplet sizes in the vaccine application.

4. Both vaccines in the water diluent showed a loss of oocysts per dose from the bottle to the time that the vaccine reached the point it could be ingested by the chick, while the vaccines in gel diluent did not demonstrate a loss in oocysts.

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Figures

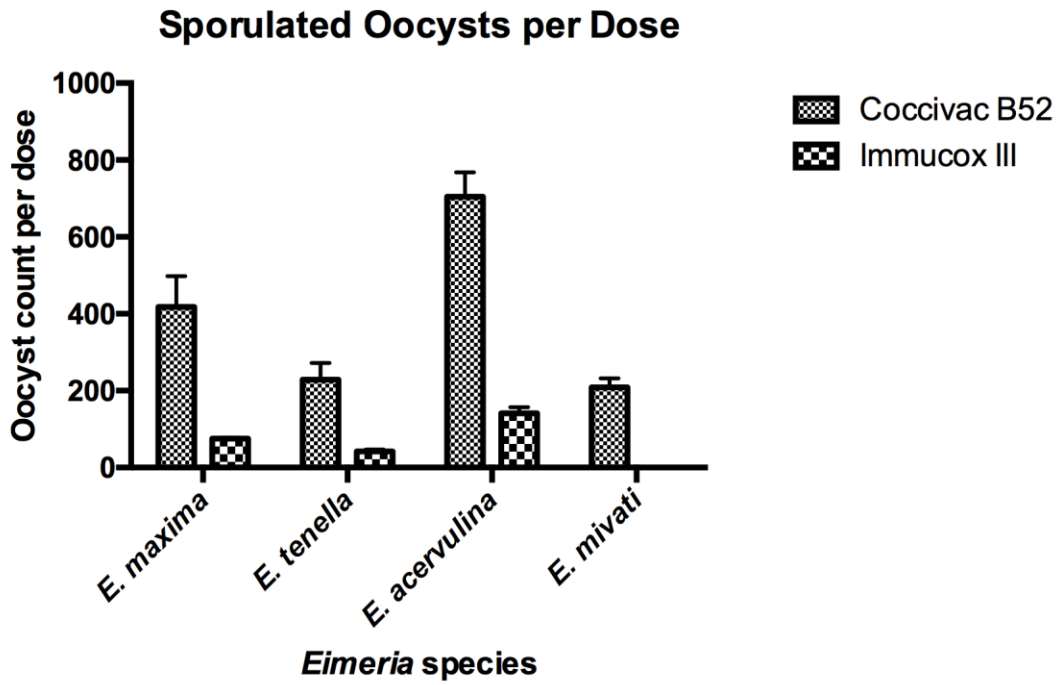

Figure 3.1: Number of oocysts present per 1,000 doses. (A) Coccivac®-B52 and (B) Immucox® III.

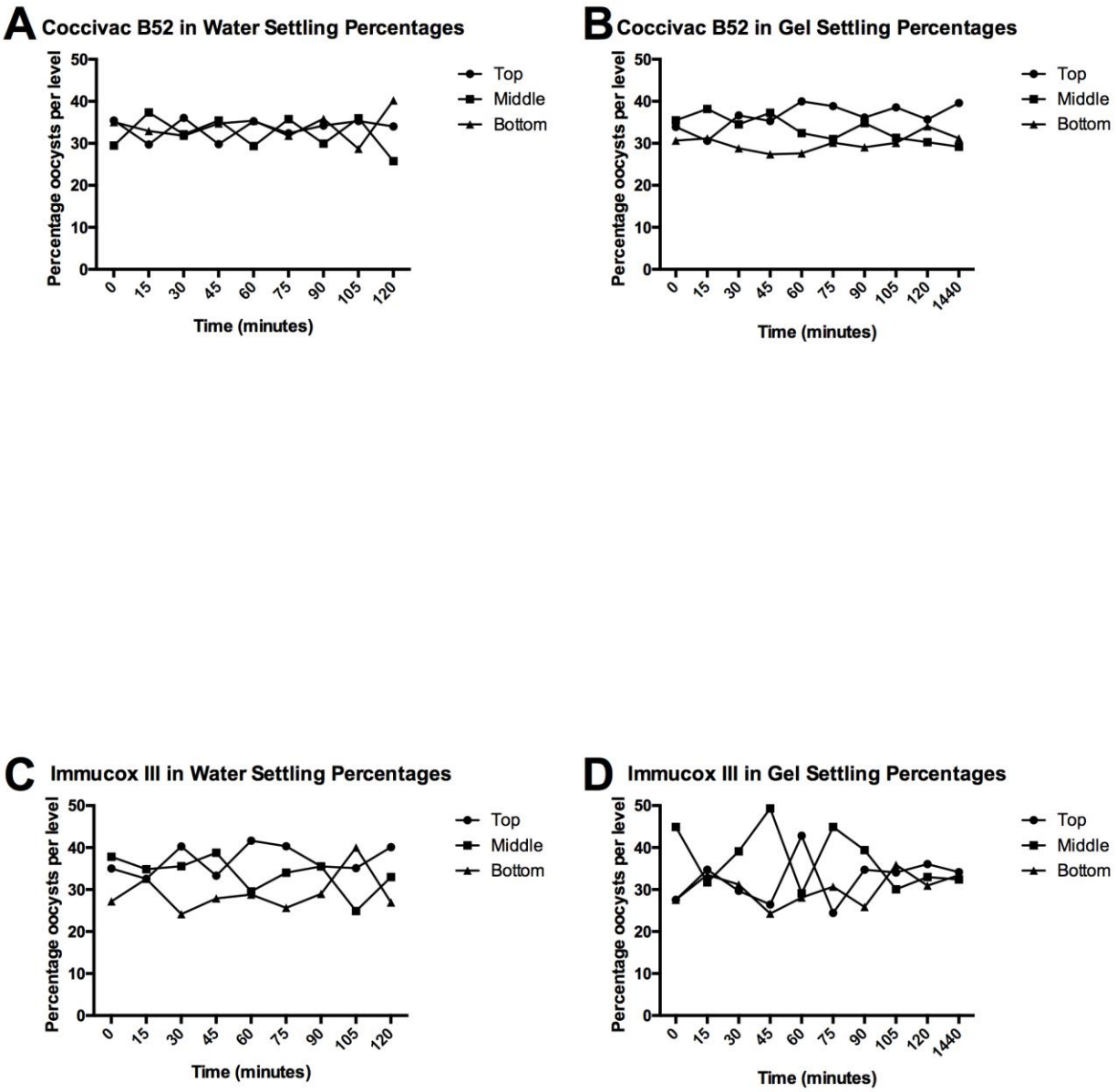

Figure 3.2: Determining oocyst settling over time, by a percent of oocysts present per level of the working stock of the vaccine. (A) Coccivac®-B52 in water. (B) Immucox® III in water. (C) Coccivac®-B52 in gel. (D) Immucox® III in gel.

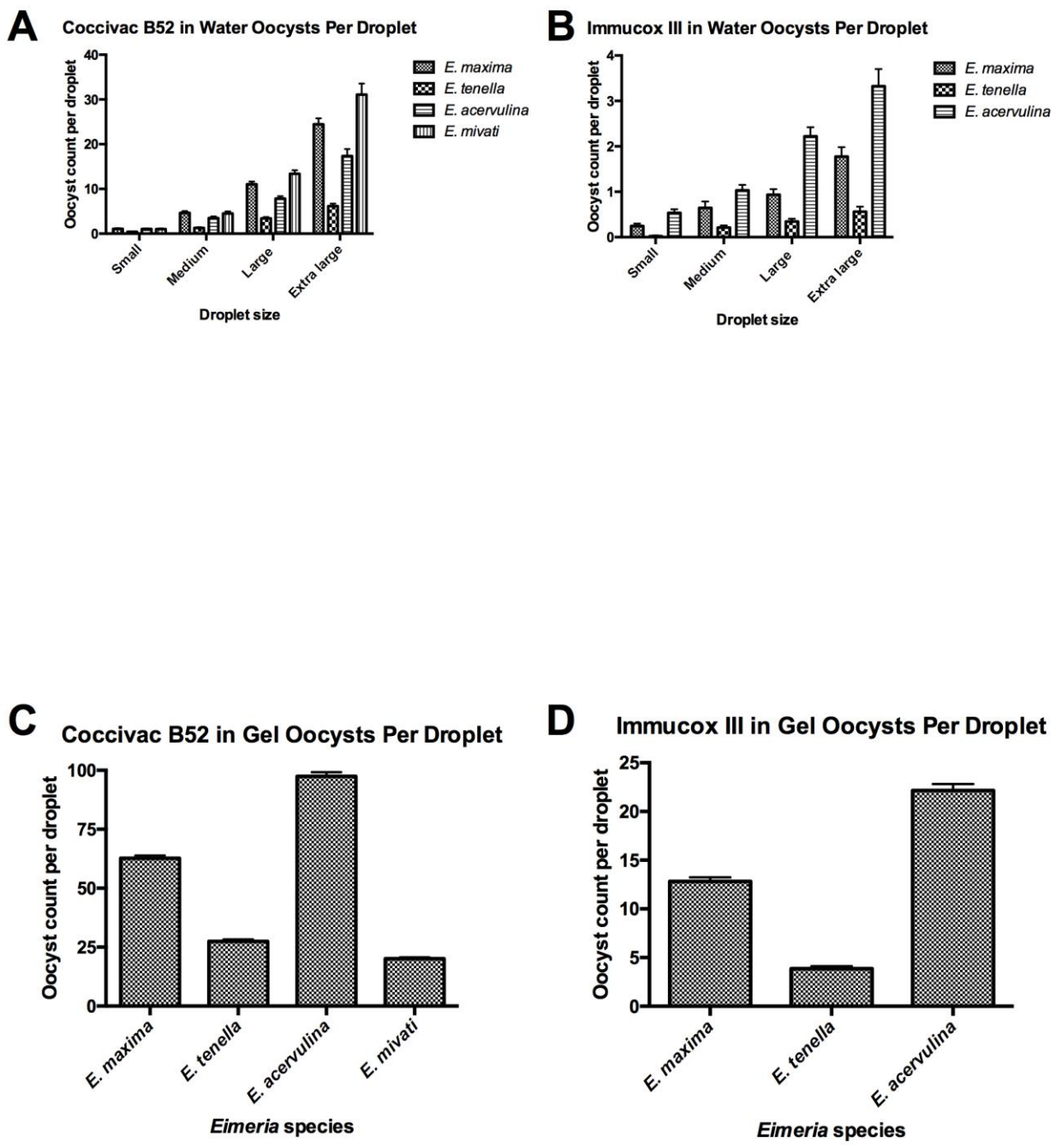

Figure 3.3: Number of oocysts present per droplet according to droplet size. Water diluent droplet size: small (1 ul), medium (5 ul), large (10 ul), and extra-large (15-30 ul). Gel diluent droplet size ~30 ul. (A) Coccivac®-B52 in water. (B) Immucox® III in water. (C) Coccivac®-B52 in gel. (D) Immucox® III in gel.

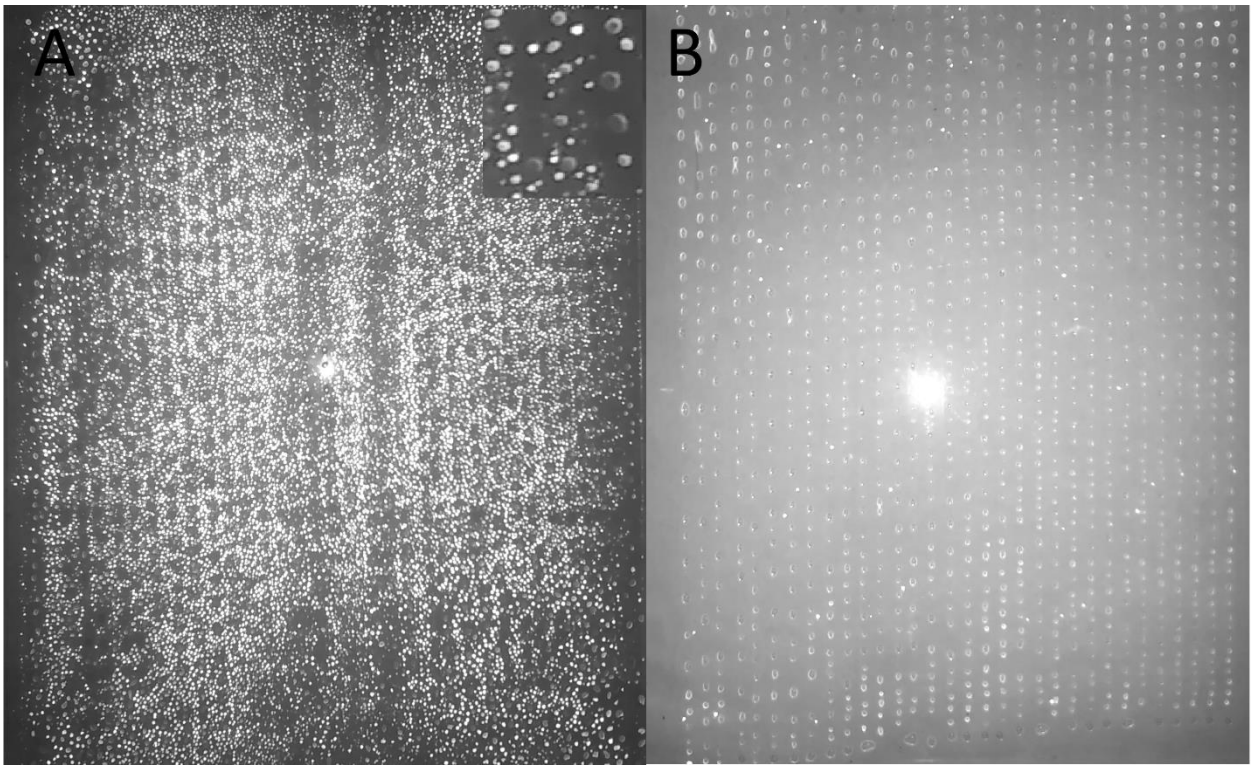

Figure 3.4: Image of plexiglass after being sprayed with each. (A) Spray applied with a two nozzle spray system. Insert magnification to show the variety of droplet size present. (B) Gel diluent applied with a gel drop bar

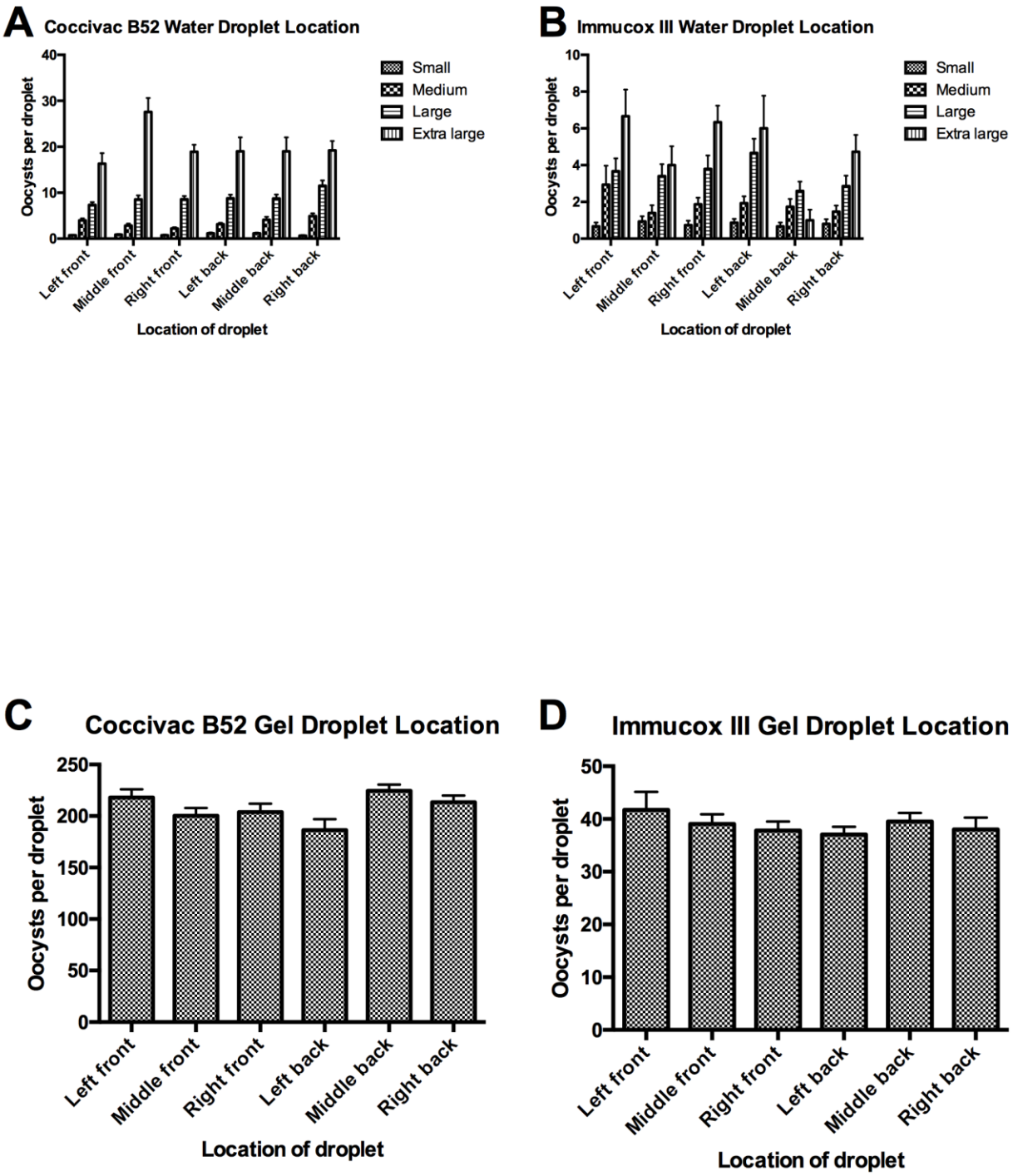

Figure 3.5: Number of oocysts present per droplet size according to the section of the droplet. (A) Coccivac®-B52 in water. (B) Immucox® III in water. (C) Coccivac®-B52 in gel. (D) Immucox® III in gel.

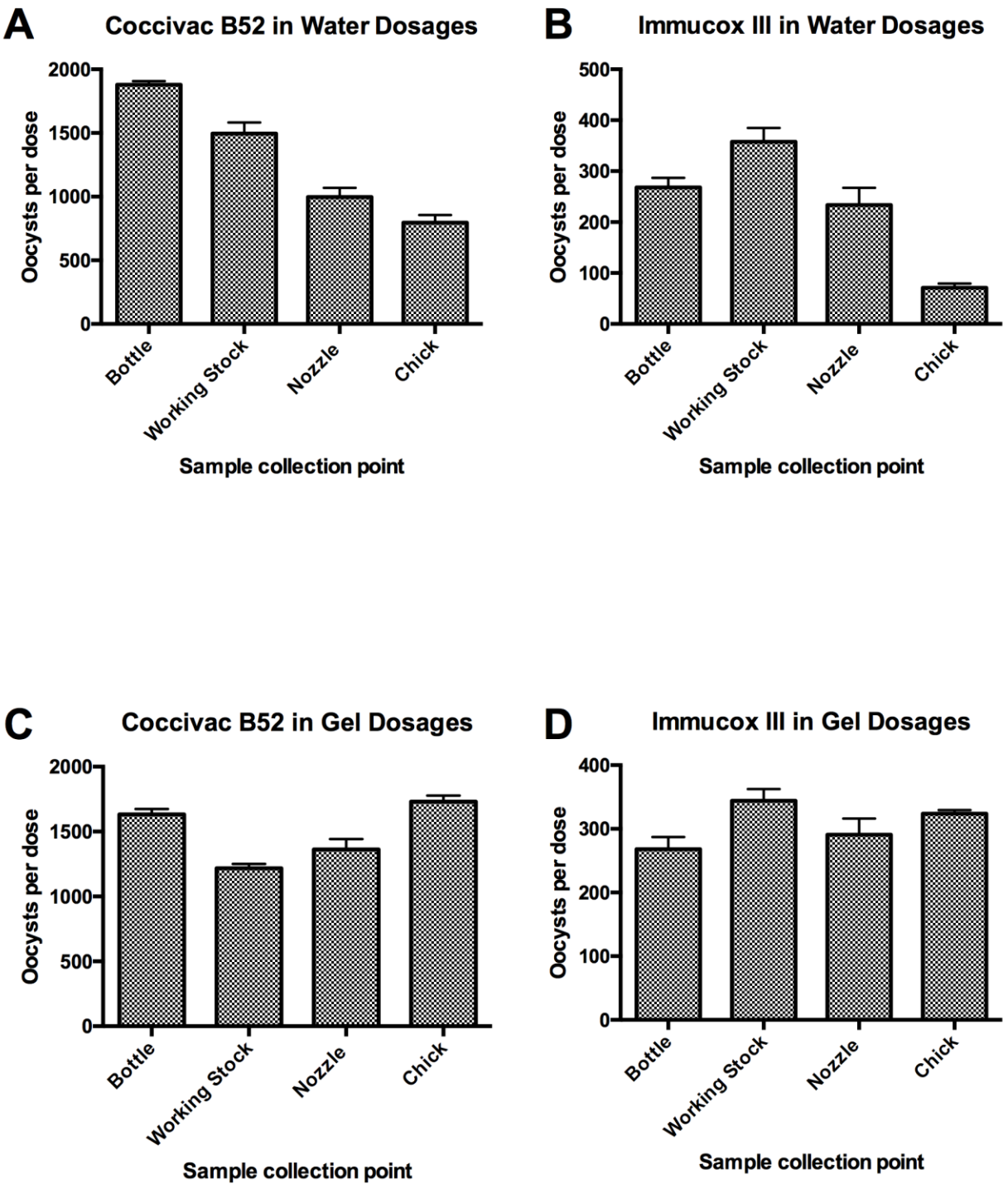

Figure 3.6: Number of oocysts present per dose, according to the sample collection point during vaccine application. (A) Coccivac®-B52 in water. (B) Immucox® III in water. (C) Coccivac®-B52 in gel. (D) Immucox® III in gel.

CHAPTER 4

CHARACTERIZATION OF GEL VERSUS SPRAY APPLICATION AND PROTECTION FROM CHALLENGE FOR HIGH OOCYST AND LOW OOCYST DOSE COCCIDIA VACCINATIONS[1]

[1]Tensa, L.R., G.A. Albanese, and B.J. Jordan. To be submitted to *Poultry Science.*

Abstract

Coccidiosis is an economically significant enteric disease in poultry caused by parasitic protozoa in the genus *Eimeria.* Coccidiosis control is performed through the use of chemical anticoccidial agents, ionophore antibiotics, live vaccines, or a combination program. Live coccidia vaccines have been available for several decades and have been administered a variety of ways, including at the hatchery, in the water lines, or sprayed on the feed. Now, vaccines are typically mass applied at the hatchery, through a water spray diluent, but certain low dose vaccines recommend the use of a gel delivery system. The gel diluent is designed to elongate the chicks exposure to the oocysts by lengthening the time the chicks can preen the droplets, thereby allowing for increased oocyst ingestion. The purpose of this trial was to compare vaccinal oocyst cycling, lesion scores, and protection from a pathogenic *E. maxima* challenge between high and low oocyst dose vaccines administered in either a gel and spray delivery systems to determine if the vaccine or administration route truly makes a difference in protection. Our results demonstrate that all chickens in all vaccinated groups were shedding all *Eimeria* species present in each vaccine by the second cycle, but differences in lesion scores from vaccine cycling were seen. All vaccinated groups were protected from an *E. maxima* challenge, though there were differences in post challenge lesion scores across the vaccinated groups. Since all vaccine and diluent groups were protective against an *E. maxima* challenge, producers can choose which combination best serves their production system.

Introduction

Coccidiosis is an important enteric protozoal parasite disease in commercial poultry operations and is caused by numerous *Eimeria* species. Clinical and subclinical losses from infection with coccidia include decreased weight gain, increased feed conversion ratio, mortality, and predisposition to secondary infections depending on the *Eimeria* species infecting the flock (Allen & Fetterer, 2002; Collier et al., 2008; Johnson & Reid, 1970). Worldwide, approximately 60 billion chickens are produced for meat and egg production, and the estimated global impact of coccidiosis is in excess of $3 billion per year due to subclinical losses in performance, prophylaxis control, and treatment measures (Blake & Tomley, 2014; Dalloul & Lillehoj, 2006; Williams, 1999).

Historically, coccidiosis has been controlled using chemical drugs and ionophore antibiotics, however with the growing demand for antibiotic free poultry and increasing drug resistance to available anticoccidials, there is a reemergence in the use of live coccidiosis vaccines. Coccidiosis vaccines are the only control strategy that induce full development of immunity, which is species specific (Rose & Long, 1962). Several coccidia vaccines are available to the industry, with major differences arising from variations in number of oocysts per dose, inclusion of novel or precocious strains, and attenuation status of the oocysts (Vermeulen, Schaap, & Schetters, 2001) (Chapman et al., 2002; Dalloul & Lillehoj, 2005; Price, Hafeez, Bulfon, & Barta, 2016). The range in oocyst counts in vaccines is due in part to immunity developing from few oocysts being protective against challenge from a homologous strain (Blake et al., 2005). In addition to the differences between vaccines, there has been increased interest and use of gel based diluents, compared to the more widely used water diluent, especially with the use of a low oocyst dose vaccine (Jenkins et al., 2012; Jenkins et al., 2013).

Various vaccination challenge studies have yielded different results using different vaccines and application methods. When looking at various parameters to determine protection from challenge, including weight gain, feed conversion ratio, oocysts shedding, and lesion scores, results have been mixed. When comparing a high dose vaccine given in multiple application routes, one study found all groups showed similar cycling and protection from challenge (Albanese, Tensa, Aston, Hilt, & Jordan, 2018). A similar trial, using a low dose vaccine, found gel application to be the superior method of vaccine delivery for providing an immunizing dose (Danforth, Lee, Martin, & Dekich, 1997). Previous research has compared application parameters of different coccidiosis vaccines in gel and spray diluents and has shown that there is uniform application of oocysts in droplets across a chick basket. In addition, if the gel suspension is properly mixed initially, the oocysts remain in suspension for the duration of the application period. Oocysts will also remain in suspension in a water diluent, provided there is constant agitation (Tensa & Jordan, 2018).

The aim of this study is to perform an in-depth comparison of two vaccines: high and low oocyst dose, given via two different diluents: water and gel. The first portion of the trial was to determine the cycling characteristics of the vaccinal oocysts, including gross and microscopic lesion scores caused by the vaccine along with oocyst shedding. The second portion of the trial is to determine protection from an *E. maxima* challenge as determined by gross and microscopic lesion scores and oocyst count scores. This study will help determine if there is an advantage to either vaccine or delivery system, in a direct comparison.

Materials and Methods

Vaccines

Two vaccines and two diluents were used. Coccivac®-B52 is a high oocyst dose vaccine that contains *Eimeria acervulina, E. maxima, E. mivati, and E. tenella*. Immucox® III is a low oocyst dose vaccine that contains *E. acervulina, E. maxima,* and *E. tenella.*

Vaccine Groups

For the trial, group A was vaccinated with Coccivac®-B52 in a gel diluent, group B was vaccinated with Coccivac®-B52 in a water diluent, group C was vaccinated with Immucox® III in a gel diluent, and group D was vaccinated with Immucox® III in a water diluent (Table 4.1).

For the water diluent, each vaccine was mixed at a ratio of 240 mls water to 1000 doses of vaccine. The vaccine was applied using a commercially available spray vaccine cabinet. For the gel application, 2.5 L of water and one 70 g packet of Ceva gel were mixed with an immersion blender, until the gel powder was fully dissolved. Each vaccine was mixed at a ratio of 250 mls reconstituted gel to 1000 doses of vaccine and mixed with an immersion blender for three minutes, until the vaccine was evenly distributed. The vaccines in gel diluent were applied using a commercially available gel drop bar vaccine cabinet.

Oocyst Enumeration

Oocysts were enumerated to determine oocysts shed per gram feces utilizing a McMaster's chamber. All feces were collected individually from birds housed in isolators. Water was added in a 10:1 ratio and left to soak overnight. The following day, the fecal water slurry was filtered with cheesecloth. A 15 mL sample was centrifuged at 1500 rpm for 5 minutes. The supernatant was

discarded and the pellet was resuspended in saturated salt water. The resulting sample was then mixed and pipetted into a McMaster's chamber. The chamber was allowed to sit for three minutes so oocysts could rise to the top of the chamber, then were counted using the method of Conway and McKenzie (Conway & McKenzie, 2007).

Animal and Oocyst Model

The challenge strain of *E. maxima* was isolated from a bird with clinical *E. maxima* at the University of Georgia Poultry Diagnostic and Research Center Diagnostic Laboratory. A single oocyst propagation and pathogenicity test was performed. It was determined that a 100,000 oocyst challenge gave an average gross lesion score of 3 and was utilized for this study.

Non-vaccinated Ross broiler chicken embryos were purchased from a commercial source and hatched at the Poultry Diagnostic and Research Center (Athens, GA). Once hatched, chicks were randomly assorted into one of the experimental groups. All birds were exposed to 20 hours of light daily and offered a non-medicated feed and water ad libitum throughout the duration of this experiment. Animal care and use protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Experimental Design

Five hundred one-day-old broiler chicks were divided into 5 different groups, four vaccinated and one non-vaccinated. The four vaccinated groups (A-D) were vaccinated with the diluted vaccines with commercial vaccination equipment and placed on litter in separate colony housing units, while the non-vaccinated group was placed directly into a separate colony housing unit without being exposed to vaccine (Table 1). All birds were fed a typical nonmedicated broiler

starter/grower diet. At day three post-vaccination, twenty birds from each vaccinated group were moved into individual isolators to collect feces and count oocyst shedding corresponding to cycle 1. All feces were collected individually from each bird on days four through eleven post-vaccination. On day eleven, all birds in isolators were euthanized and twenty new birds from each group were moved from the colony houses and placed into individual isolators. Feces were collected from these birds from days 12-22 post-vaccination to represent oocysts shed during cycle 2. All oocysts were speciated according to oocyst size and morphology and enumerated utilizing a McMaster chamber. At day sixteen, each colony house was culled to thirty birds. The colony house was divided into two pens, with one pen housing twenty birds that were challenged with 100,000 sporulated *E. maxima* oocysts, and the second pen containing ten unchallenged birds. Following challenge, birds remained in the colony houses on litter for 7 days post challenge, a necropsy was performed on all challenged and non-challenged birds, and gross lesion scores, microscopic lesion scores, and oocyst count scores were obtained.

Histological Sampling and Microscopic Lesion Scores

Sections of the gastrointestinal tract were collected from five nonchallenged birds from each treatment group at seven, fourteen, and twenty-three days post vaccination and fixed in 10% neutral buffered formalin. Sections from the duodenum were collected from the distal duodenal loop, sections from the jejunum were collected 2 cm proximal to Meckel's diverticulum, sections from the ileum were collected from proximal to the ileo-cecal ligament, and samples from the ceca were collected from the distal end of the left ceca. In addition, samples were collected from the challenged birds seven days post challenge. Two-centimeter sections of each portion of the

intestine were routinely paraffin embedded, sectioned, and stained with hematoxylin and eosin stain.

Microscopic lesion scores (MLS) were assigned to histological sections according to the *Eimeria spp.* present in the duodenum, jejunum, ileum, and ceca. The duodenum was examined for *E. maxima* and *E. acervulina*, the jejunum was examined for *E. maxima*, the ileum was examined for *E. maxima*, and the ceca were examined for the presence of *E. tenella.* Four fields per section of intestine were examined for the developmental stages of *Eimeria* species with the 10x objective. The MLS is the sum of A plus B, where A represents the distribution of stages along the examined segment and B represents the severity of infection in the examined section. The total scores were calculated per treatment group, and divided by 2, to allow for direct comparison between the oocyst count score and gross lesion score (Goodwin, Brown, & Bounous, 1998).

Gross Lesion Scores

Gross lesion scores (GLS) for *Eimeria maxima, E. tenella, and E. acervulina* (Reid & Johnson, 1970) were taken at seven days post vaccination, fourteen days post vaccination, twenty three days post vaccination, and seven days post challenge. GLS for each species ranged from 0 (no lesions) to 4 (severe lesions).

Oocyst Count Scores

Oocyst count scores (OCS) were collected for *E. maxima* seven days post challenge. A 10x objective lens, with 10x oculars, was used for oocyst count scores. A direct smear was taken from the midgut, just distal to Meckel's diverticulum, and numbers of *E. maxima* oocysts per field were counted. Scores were assigned as follows: 0 = no oocysts seen, 1 = 1-20 oocysts per field, 2 = 21-

50 oocysts per field, 3 = 51-100 oocysts per field, and 4 = too numerous to count (Goodwin et al., 1998).

Biometrics

All GLS, MLS, and OCS were analyzed utilizing one-way analysis of variance with post-hoc Tukey's multiple comparisons (GraphPad Prism, GraphPad Software, La Jolla California USA). All comparisons were considered significant at a level of $P < 0.05$.

Results

Vaccination

Based on the vaccine group, the total oocysts per dose and total *E. maxima* oocysts per dose varied. *E. maxima* is represented separately as an *E. maxima* challenge model was utilized. Group A contained the most oocysts, followed by groups B and C, while group D had the fewest oocysts present (Table 4.2).

Oocyst Cycling

In cycle one, small oocyst (*E. acervulina, E. tenella,* and *E. mivati)* shedding peaked at day seven for groups A, B, and D, with group D shedding the most oocysts, followed by group B then group A. Oocyst shedding in Group C peaked at day six. More than 95% of vaccinated birds in all groups shed small oocysts on days six or seven post vaccination (Figure 4.1C, D, Table 4.3).

For the first cycle of *E. maxima,* oocyst shedding from chickens in groups C and D peaked at day seven with 75 and 85% of birds shedding respectively. Chickens in groups A and B shed

oocysts at a much lower rate, with both groups peaking at 30% of birds shedding *E. maxima* on days eight and seven, respectively (Figure 4.1A, B, Table 4.4).

To determine if *E. maxima* shed in the first cycle sporulated in the environment and were re-ingested by the chickens, cycle two of oocyst shedding was counted. All four groups reached 100% of birds shedding *E. maxima* during the second cycle. Groups C and D reached peak shedding sooner than groups A and B (Figure 4.1E, F, Table 4.4).

Gross Lesion Scores

Gross lesions for the three major *Eimeria spp* (*E. maxima, E. acervulina,* and *E. tenella)* present in the vaccines were evaluated during peak shedding from the first, second, and third vaccinal oocyst cycles. During cycle one, no gross lesions were seen for any species in any of the vaccinated groups. During cycle two, there were no significant differences seen between any of the groups when comparing the same species. All four groups had *E. acervulina* scores averaging between one and two. Only the groups vaccinated by gel (groups A and C) had any *E. maxima* gross lesions present, though the average scores were less than one. By cycle three, there were no longer *E. acervulina* gross lesion scores present. All groups had *E. maxima* scores averaging between 0.5 and 1.5. Group A had a significantly higher *E. tenella* score than the other groups (Figure 4.2).

Microscopic Lesion Scores

For cycle one in the duodenum for both *E. acervulina* and *E. maxima,* there were no significant differences seen between the vaccinated or nonvaccinated groups, and all scores present were below one. For *E. maxima* in the jejunum and ileum, only group C averaged a score above

one and was significantly higher than the non-vaccinated group. In the ceca, groups A, B, and C had lesion scores that averaged less than one (Figure 4.3A).

In cycle two, all four groups had approximately the same average lesion score for *E. acervulina* in the duodenum, which was significantly higher than the non-vaccinated group. Only groups C and D had *E. maxima* present in the duodenum. In the jejunum, groups C and D had significantly higher lesion scores than the other three groups, and group B was not significantly different from the non-vaccinated group. When examining *E. maxima* in the ileum, group D was the only group with a significantly higher lesion score than the non-vaccinated group. Examining *E. tenella* scores in the ceca, groups A, B, and C had significantly higher lesion scores than the non-vaccinated group (Figure 4.3B).

In cycle three, no significant lesions were seen in the duodenum for *E. acervulina* in any of the groups. Only group A was significantly higher than the non-vaccinated group for *E. maxima* in the duodenum. In the ileum, groups A, C, and D had significantly higher scores than the non-vaccinated group for *E. maxima*. No groups had lesions in the ileum associated with *E. maxima.* In the ceca, groups B and C had significantly higher lesion scores for *E. tenella* than the non-vaccinated group (Figure 4.3C).

Post-challenge Gross Lesion Scores

All challenged groups had gross lesion scores that were significantly higher than the non-vaccinated/non-challenged group. All vaccinated groups had significantly lower gross lesion scores than the non-vaccinated/challenged group indicating protection from challenge (Figure 4.4A).

Post-challenge Microscopic Lesion Scores

Microscopic lesion scores were taken in the duodenum, jejunum, ileum, and cecum to determine which tissues were impacted by a challenge from *E. maxima.* No lesions were seen in the duodenum due to *E. acervulina*. The non-vaccinated/challenged group had the highest scores attributed to *E. maxima* in the duodenum, jejunum, and ileum, with scores greater than three. In the duodenum, all groups scored significantly higher than the non-vaccinated/non-challenged group. In the ileum, all vaccinated groups had significantly lower lesion scores than the non-vaccinated/non-challenged group (Figure 4.4B).

Post-challenge Oocyst Count Scores

All challenged groups had significantly higher oocyst count scores than the non-vaccinated/non-challenged group. Only the vaccines administered in water scored significantly lower than the non-vaccinated/challenged group (Figure 4.4C).

Discussion

Although all vaccines and diluent combinations were protective against an *E. maxima* challenge, there were differences between the different vaccinated groups in vaccinal oocyst cycling, vaccinal oocyst lesion scores, and lesion scores post challenge. As seen in other studies, oocyst shedding is not always correlated with the oocysts that are ingested. One reason is oocyst production is limited due to a crowding threshold, as there is only so many cells for replicating organisms to infect and replicate, especially in young birds with developing gastrointestinal tracts (Williams, 2001). Additionally, coinfections can alter the number of oocysts shed for each species present, especially in cases with overlapping areas of infection such as *E. maxima* and *E.*

acervulina, and the severity of disease (Haug, Gjevre, Thebo, Mattsson, & Kaldhusdal, 2008; M. Jenkins, Allen, Wilkins, Klopp, & Miska, 2008).

When counting vaccinal oocyst shedding, shedding in cycle one indicates vaccine uptake by the chicks, and oocyst shedding in cycle two indicates sporulation of oocysts in the litter and reuptake by the chicks. Small oocyst shedding by more than 90% of birds in all vaccinated groups in cycle one indicates that the vaccine was ingested by the majority of the chicks at the time of vaccination. In this trial, the vaccine that contained more total oocysts, more strains, and more species, Coccivac®-B52, had poor *E. maxima* oocyst shedding in the first cycle in both number of oocysts shed per gram of feces and percent of birds shedding *E. maxima*. The decrease in *E. maxima* shedding in the first cycle in the high oocyst vaccine could be due to the competition of *E. maxima* and *E. acervulina* for the same cells as they can both replicate in the duodenum and jejunum. Since *E. acervulina* has a shorter preparent period, it is able to infect more epithelial cells prior to *E. maxima* replication (Tyzzer, 1929; Tyzzer, Theiler, & Jones, 1932). In cycle two, all birds shed *E. maxima.* In cycle two, groups C and D peaked shedding *E. maxima* earlier than the groups A and B, due to the earlier shedding and sporulation of *E. maxima* oocysts that were shed during cycle one (Price et al., 2016).

The highest lesion scores due to vaccine cycling were seen in cycle two in all four groups, coinciding with increased oocyst replication and shedding while birds were still developing immunity. When evaluating protection from challenge, gross lesion scores were significantly decreased in all vaccinated groups when compared to a non-vaccinated/challenged control group, however all vaccinated groups also had significantly higher gross lesion scores than the non-vaccinated/non-challenged group indicating that vaccine and diluent combinations may have only afforded partial protection from challenge after two vaccine cycles. The antigenic diversity of *E.*

maxima is well known, and the partial protection could be due to heterologous challenge from the field isolate (Barta et al., 1998).

In addition to the significant findings, there was a numerical trend of higher scores in each group vaccinated with the gel diluent compared to the group that received the same vaccine in a water diluent during vaccinal oocyst cycling and following challenge with *E. maxima*. This trend held true for gross lesion scores, microscopic lesion scores, and oocyst count scores. Further work needs to be done to determine if this was an effect of the gel diluent. One proposed benefit of using a gel diluent is higher vaccine oocyst intake and it has been postulated that this, in combination with non-attenuated vaccines like the ones used in this study, could lead to higher lesion scores from the vaccine. Further studies evaluating precocious and non-precocious vaccines would need to be performed to investigate this hypothesis. Alternatively, one study has attributed higher lesion scores to better protection, however all groups had significant decreases in gross lesion scores following challenge, so the increase in lesion scores is likely not related to protection level in this trial (Danforth et al., 1997). Further work needs to be done to determine if the higher lesion scores are biologically significant, either in predisposing to secondary disease or contributing to lower levels of protection during other critical time points.

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Tables and Figures

Table 4.1: Vaccine and diluent groups.

Group	Vaccine	Diluent
A	High dose (Coccivac®-B52)	Gel
B	High dose (Coccivac®-B52)	Water
C	Low dose (Immucox® III)	Gel
D	Low dose (Immucox® III)	Water
E	Nonvaccinated/challenged	
F	Nonvaccinated/non-challenged	

Table 4.2: Sporulated oocysts per dose for each vaccinated group.

Experimental group	Total oocyst/dose	*E. maxima* oocysts/dose
Group A	1361	352
Group B	997	352
Group C	290	96
Group D	233	38

Table 4.3: Mean oocysts shed per gram feces and percent coefficient of variation (%CV) for total oocyst shedding of each group for cycle one.

	Cycle 1							
Experimental group	d 4	d 5	d 6	d 7	d 8	d 9	d 10	d 11
Group A	0, 0	4232.1, 116.7	3415.0, 138.2	5936.3, 173.0	1874.3, 233.6	1140.6, 213.6	360.2, 233.4	186.8, 340.5
Group B	0, 0	6596.6, 126.3	5599.5, 153.3	10068.4, 143.9	5442.7, 311.6	3868.6, 224.0	1073.9, 168.7	2017.7, 305.5
Group C	3.3, 447.2	2204.6, 227.4	8524.3, 244.8	2938.1, 167.0	306.8, 172.4	110.0, 129.4	306.8, 242.6	92.4, 397.6
Group D	0, 0	533.6, 266.1	1287.3, 127.4	12649.7, 350.6	200.1, 234.7	216.7, 266.7	290.1, 152.7	33.4, 189.2

Table 4.4: Mean oocysts shed per gram feces and percent coefficient of variation (%CV) for *E. maxima* shedding of each group at each timepoint.

	Cycle 1										
Experimental group	d 4	d 5	d 6	d 7	d 8	d 9	d 10	d 11			
Group A	0, 0	0, 0	0, 0	0, 0	130.1, 327.5	36.7, 253.6	333.5, 362.2	56.7, 447.2			
Group B	0, 0	0.3, 447.2	0, 0	170.1, 258.4	196.8, 367.3	40.0, 447.2	3.3, 447.2	283.5, 302.4			
Group C	0, 0	30.0, 309.9	2227.8, 193.4	3071.7, 183.7	423.5, 175.8	83.4, 163.9	303.5, 304.1	113.4, 227.5			
Group D	0, 0	0, 0	1497.4, 179.4	1790.9, 179.8	156.7, 172.6	270.1, 281.1	196.8, 206.8	110.0, 290.5			
	Cycle 2										
	d 12	d 13	d 14	d 15	d 16	d 17	d 18	d 19	d 20	d 21	d 22
Group A	33.4, 447.2	0, 0	565.2, 269.3	2265.5, 175.3	4720.9, 145.6	25514.5, 109.1	5416.7, 160.8	16232.7, 262.8	3247.2, 181.2	979.4, 122.5	691.6, 285.0
Group B	1000.5, 447.2	70.0, 292.3	36.7, 330.9	246.8, 308.2	1941.0, 241.5	12989.8, 143.2	2141.1, 117.8	2467.9, 152.1	1274.0, 120.8	323.5, 93.0	253.5, 188.0
Group C	4335.5, 346.8	133.4, 261.6	4044.1, 197.1	16074.7, 211.2	1660.5, 251.7	1119.9, 178.9	35.1, 392.3	0, 0	2489.0, 145.0	2731.2, 134.4	0, 0
Group D	433.6, 447.2	600.3, 271.9	14844.1, 200.0	8250.8, 122.5	5926.3, 108.0	2254.5, 108.0	1040.5, 143.1	610.3, 154.3	1607.5, 135.0	516.9, 170.2	520.3, 338.9

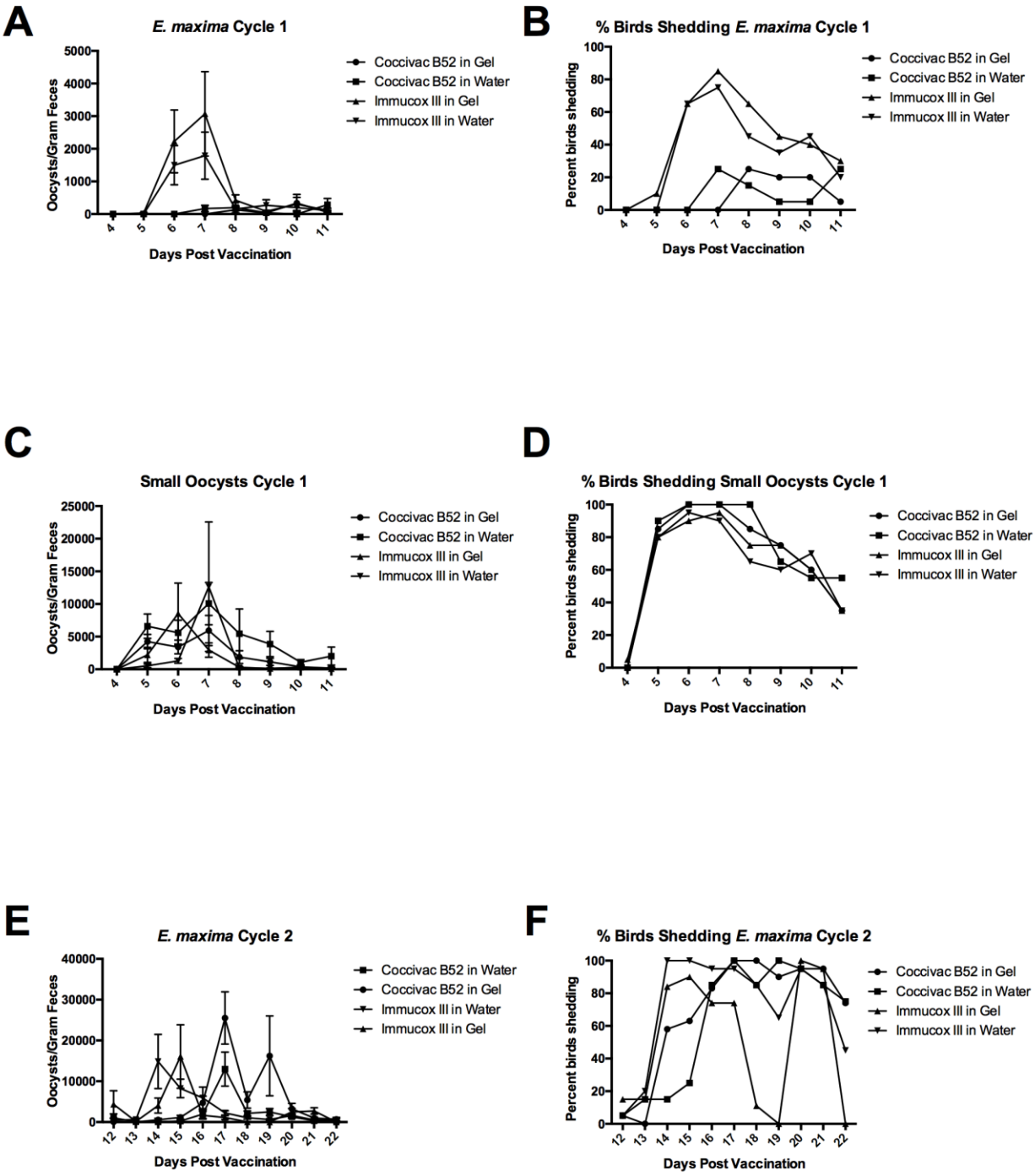
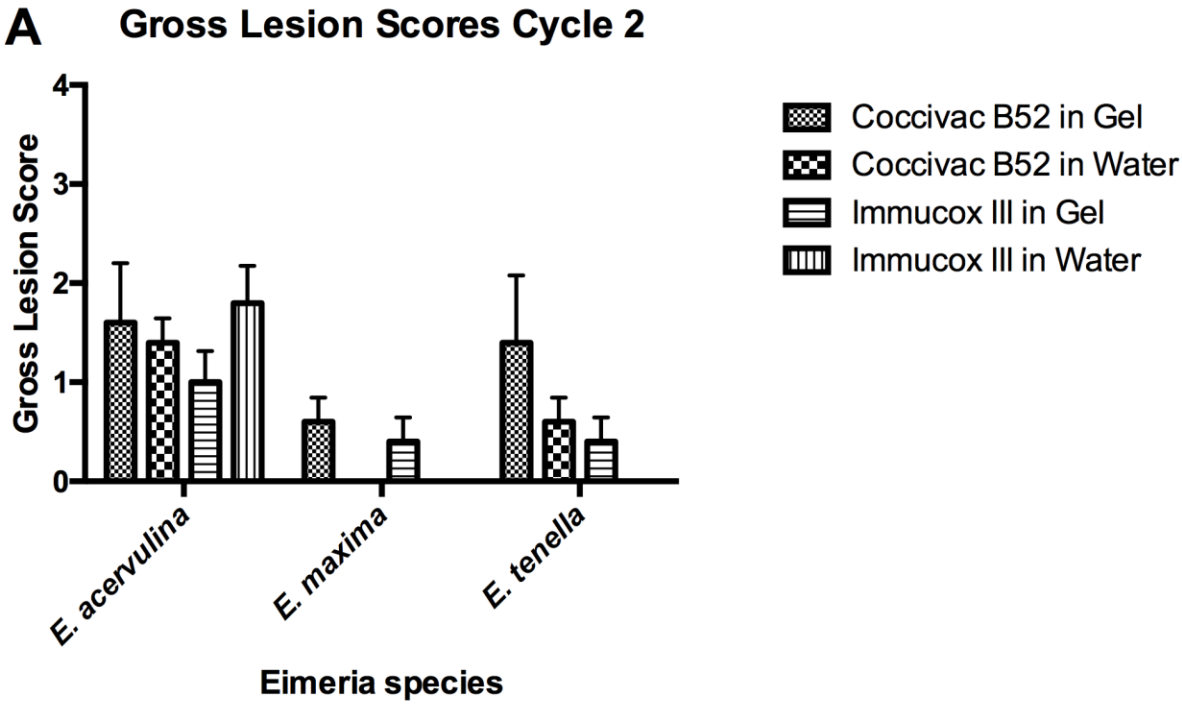

A
E. maxima Cycle 1
Oocysts/Gram Feces
Days Post Vaccination
Coccivac B52 in Gel
Coccivac B52 in Water
Immucox III in Gel
Immucox III in Water
B
% Birds Shedding E. maxima Cycle 1
Percent birds shedding
Days Post Vaccination
C
Small Oocysts Cycle 1
D
% Birds Shedding Small Oocysts Cycle 1
E
E. maxima Cycle 2
F
% Birds Shedding E. maxima Cycle 2

Figure 4.1: Vaccinal oocyst shedding. (A) Oocyst per gram counts for *E. maxima* in cycle one, (B) percentage of birds shedding *E. maxima* in cycle one. (C) Oocyst per gram counts for small oocysts in cycle one, (D) percentage of birds shedding small oocysts in cycle one. (E) Oocyst per gram counts for *E. maxima* in cycle two, (F) percentage of birds shedding *E. maxima* in cycle two.


A
Gross Lesion Scores Cycle 2
Gross Lesion Score
0
1
2
3
4
E. acervulina
E. maxima
E. tenella
Eimeria species
Coccivac B52 in Gel
Coccivac B52 in Water
Immucox III in Gel
Immucox III in Water

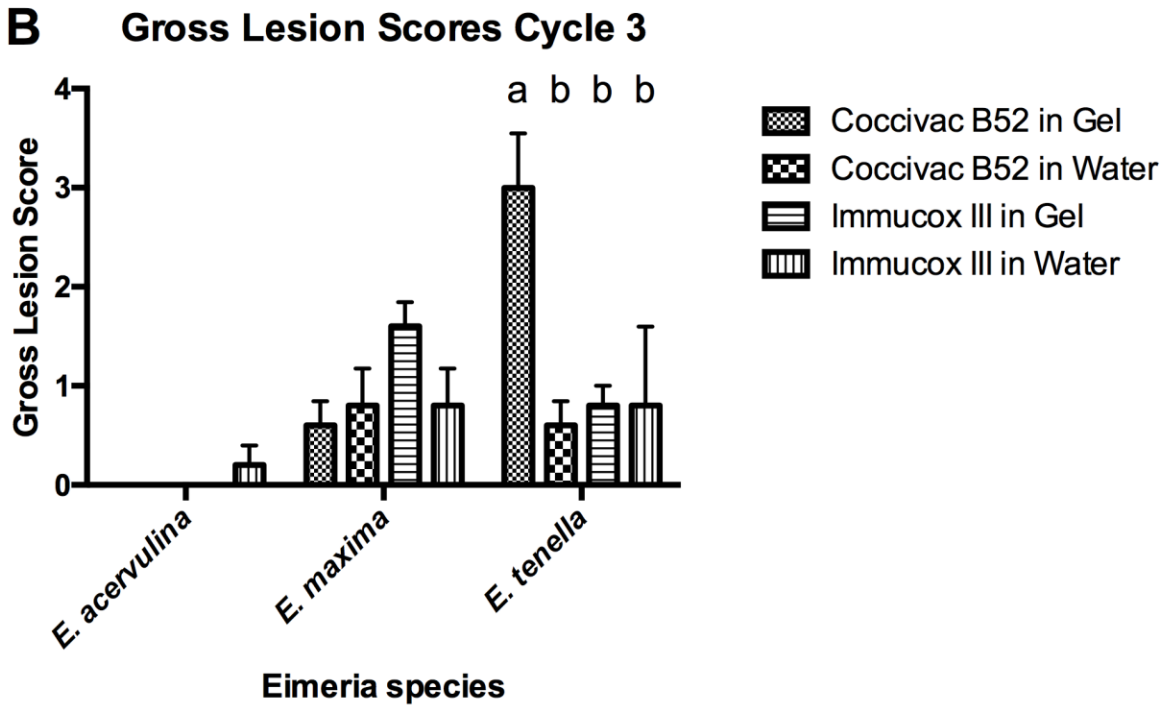
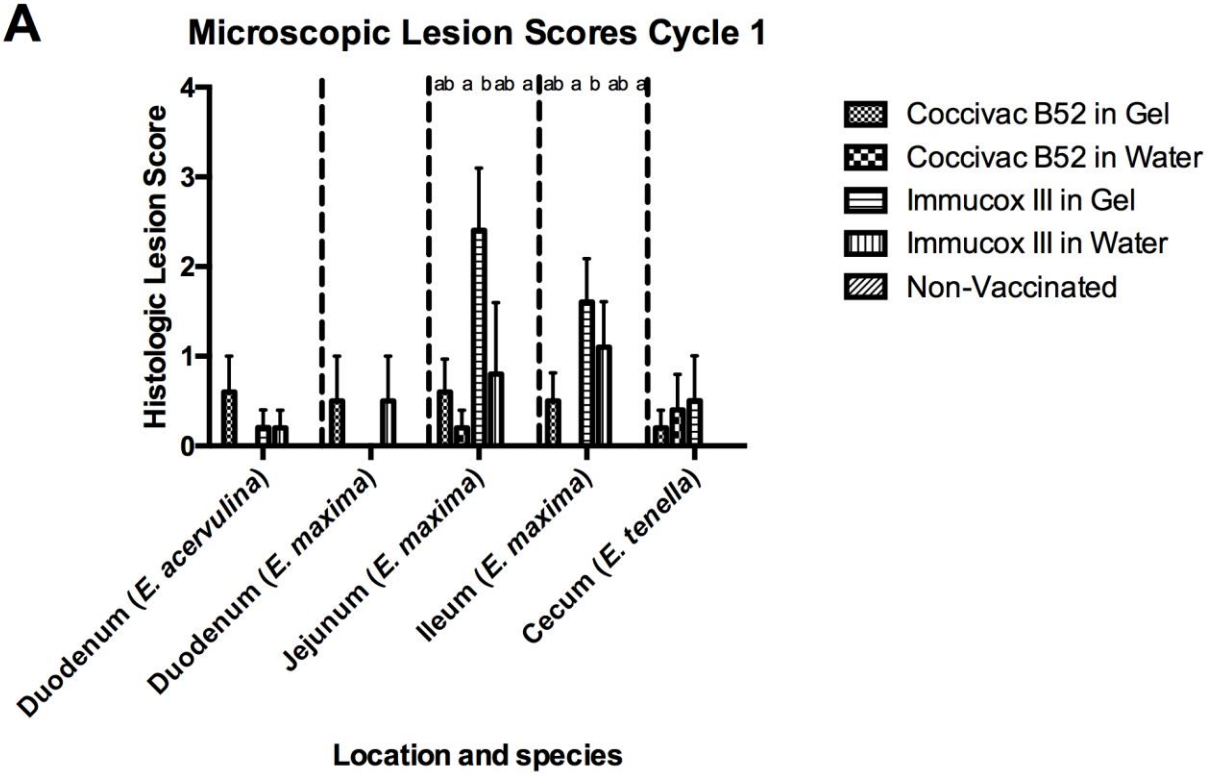
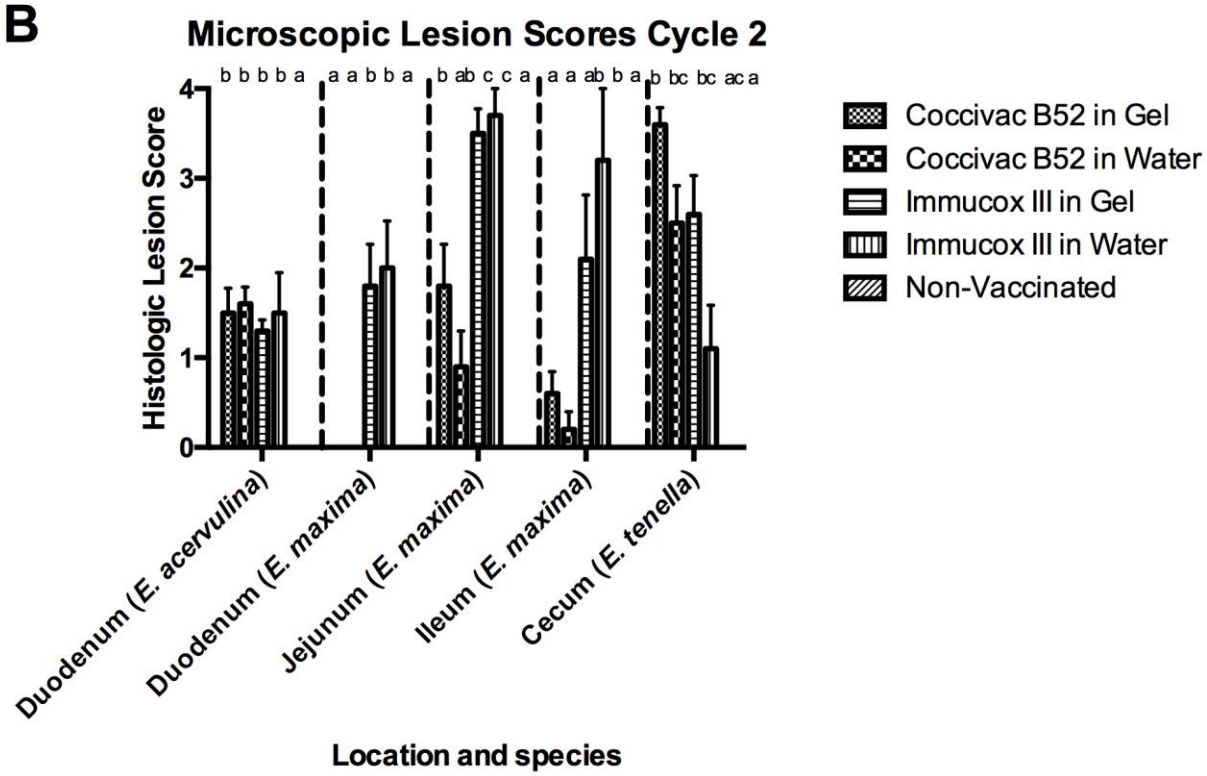

B
Gross Lesion Scores Cycle 3
a b b b
Gross Lesion Score
0
1
2
3
4
E. acervulina
E. maxima
E. tenella
Eimeria species
Coccivac B52 in Gel
Coccivac B52 in Water
Immucox III in Gel
Immucox III in Water

Figure 4.2: Gross lesion scores present during vaccinal cycling. (A) GLS for *E. maxima*, *E. tenella*, and *E. acervulina* at 14 days post-vaccination. (B) GLS for *E. maxima*, *E. tenella*, and *E. acervulina* at 23 days post-vaccination.

A


Microscopic Lesion Scores Cycle 1
Histologic Lesion Score
0
1
2
3
4
ab a b ab a
ab a b ab a
Duodenum (E. acervulina)
Duodenum (E. maxima)
Jejunum (E. maxima)
Ileum (E. maxima)
Cecum (E. tenella)
Location and species
Coccivac B52 in Gel
Coccivac B52 in Water
Immucox III in Gel
Immucox III in Water
Non-Vaccinated

B


Microscopic Lesion Scores Cycle 2
Histologic Lesion Score
0
1
2
3
4
b b b b a
a a b b a
b ab c c a
a a ab b a
b bc bc ac a
Duodenum (E. acervulina)
Duodenum (E. maxima)
Jejunum (E. maxima)
Ileum (E. maxima)
Cecum (E. tenella)
Location and species
Coccivac B52 in Gel
Coccivac B52 in Water
Immucox III in Gel
Immucox III in Water
Non-Vaccinated

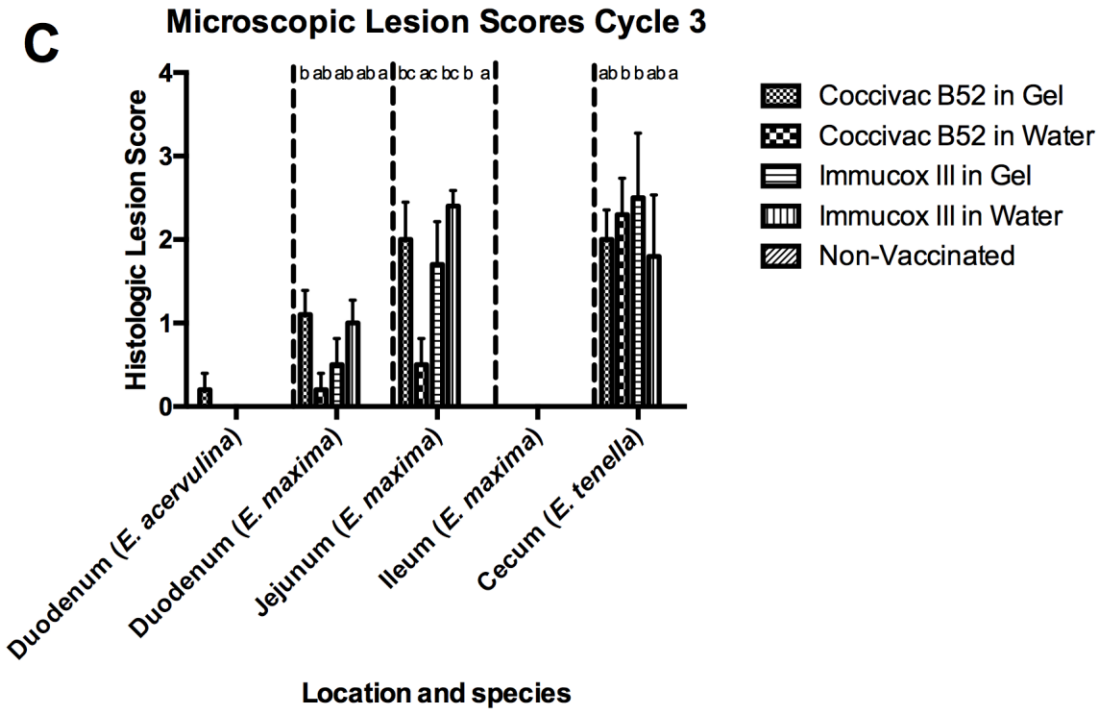
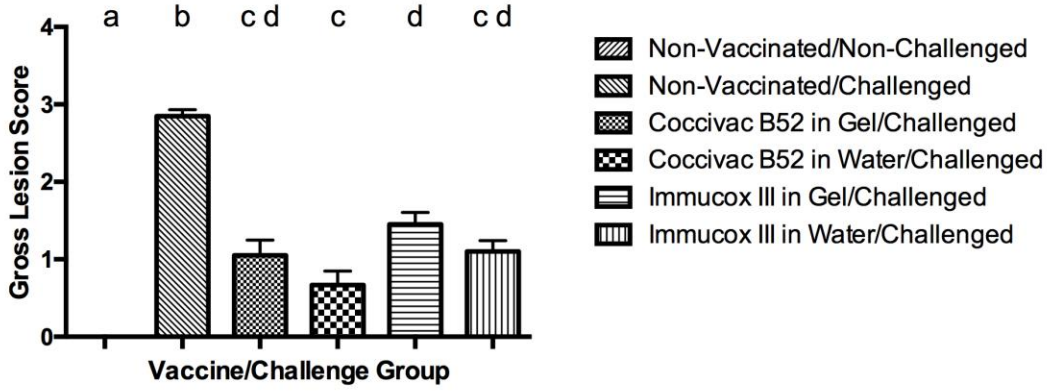


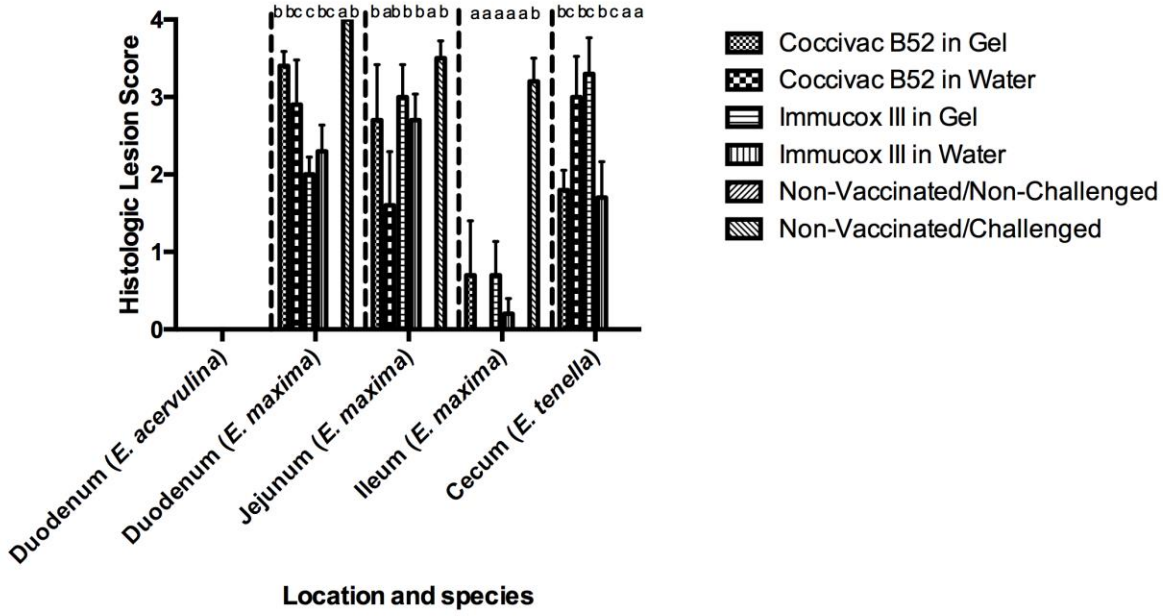
Figure 4.3: Microscopic lesion scores present during vaccinal cycling. (A) MLS for *E. maxima*, *E. tenella*, and *E. acervulina* at 7 days post-vaccination. (B) MLS for *E. maxima*, *E. tenella*, and *E. acervulina* at 14 days post-vaccination. (C) MLS for *E. maxima*, *E. tenella*, and *E. acervulina* at 23 days post-vaccination.

A ***E. maxima*** **Gross Lesion Scores Post Challenge**



B

Microscopic Lesion Scores Post Challenge



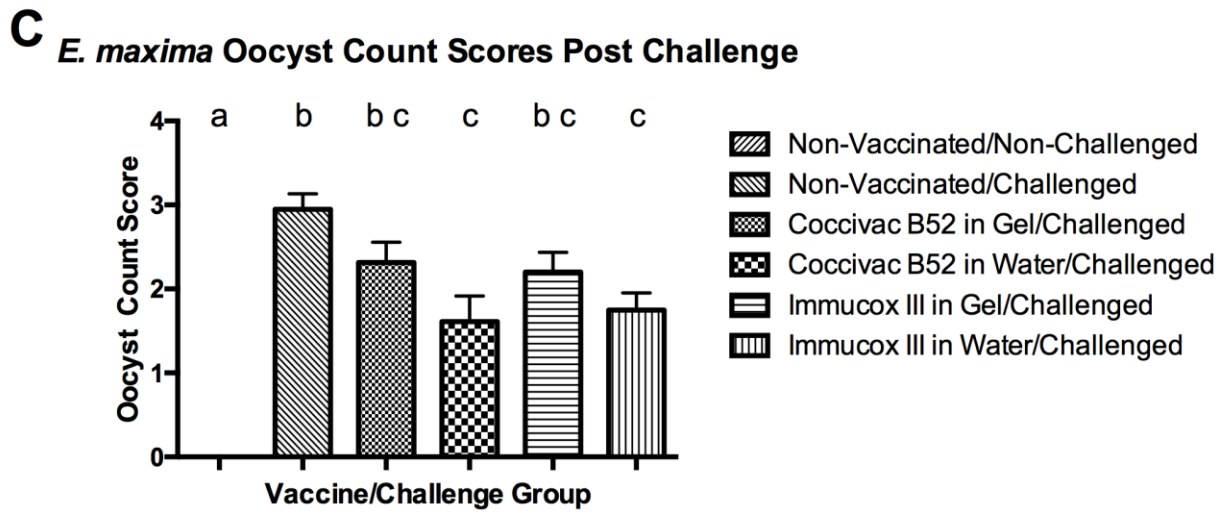


Figure 4.4: Post-challenge lesion scores. (A) *E. maxima* gross lesion scores in the jejunum. (B) Microscopic lesion scores for all species. (C) *E. maxima* oocyst count scores in the jejunum.

CHAPTER 5

EVALUATION OF DIFFERENT COCCIDIA VACCINES, WITH OR WITHOUT A BIOSHUTTLE, FOR CONTROL OF A PATHOGENIC *E. TENELLA*[1]

[1]Tensa, L.R., G.A. Albanese, B.J. Jordan. To be submitted to *Avian Diseases*.

Summary

Coccidiosis is an economically significant enteric disease caused by *Eimeria spp.* One species, *E. tenella,* infects the ceca of chickens causing bloody feces, thickening and sloughing of the cecal wall, and mortality. For the past several years, a commercial poultry integrator in the south has had a pathogenic *E. tenella* challenge on multiple broiler complexes. These challenges have been unresponsive to multiple interventions, including the use of multiple coccidiosis vaccines and anticoccidial treatments. The purpose of this trial was to evaluate the protection properties of two coccidiosis vaccines used alone or in combination with salinomycin against the pathogenic isolate in a controlled setting, to help determine if other management factors could be exacerbating the situation in the field.

For this trial two challenge times were used; an early challenge, at 11 days, to coincide with when lesions and mortality were seen in the complexes, and a late challenge, at 21 days, to determine if further cycling of vaccinal oocysts would induce immunity. Litter samples were collected from all groups to determine vaccinal oocyst cycling. Body weights, gross lesion scores, and oocyst count scores were collected to determine if birds were protected compared to non-vaccinated challenged and non-challenged controls. Additional birds were grown to 35 days to determine the lasting effects from the challenge and vaccination on processing weights.

Oocysts per gram of litter counts showed that both vaccines were infective and all species of *Eimeria* were shed in the first cycle, though at different levels from each group of vaccinated birds. In the early challenge, the vaccinated birds were not protected when compared to the non-vaccinated challenged control. In the late challenge, all vaccinated groups had significantly lower lesion scores than the non-vaccinated challenged control. After the early challenge, the groups vaccinated with vaccine 1 had significantly decreased weight gain compared to the other groups.

Before the late challenge, all vaccinated groups had significantly lower body weights than the non-vaccinated groups. Results from this trial indicate that for the early challenge neither vaccine, with or without the ionophore, was protective against this pathogenic field isolate of *E. tenella*. For the late challenge, once the vaccines completed cycling to induce immunity, both commercial vaccines were protective against the isolate.

Introduction

Coccidiosis is a major economical disease in commercial poultry, leading to poor performance from decreased weight gain and increased feed conversion ratios. Worldwide, coccidiosis results in economic losses of approximately $3 billion per year. Eighty percent of the cost of coccidiosis is associated with subclinical to clinical signs, while the remaining 20% is associated with prophylaxis control and treatment (Blake & Tomley, 2014; Williams, 1999). One of the major species described in chickens, *E. tenella,* causes thickening and sloughing of the cecal wall leading to bloody feces, cecal cores, and mortality in infected birds (Reid & Johnson, 1970). *E. tenella* can cause variable mortality rates in different strains of chickens infected with the same dose ranging from no mortality to 27%, and a reduction in growth due to anorexia in surviving birds (Pinard-Van Der Laan, Monvoisin, Pery, Hamet, & Thomas, 1998; Witlock, Ruff, & Chute, 1981).

Coccidiosis control in broilers has historically been achieved through the use of medicated poultry feed with either ionophore antibiotics or chemical anticoccidial drugs, though recently there has been a reemergence of the use of coccidiosis vaccines. The vaccines marketed for use in broiler type chickens contain oocysts from drug-sensitive *E. acervulina, E. maxima,* and *E. tenella* (Chapman et al., 2002; M. W. Shirley & Long, 1990), as these are the most common species that cause pathology. Immunity is developed in chicks through vaccinal oocyst ingestion, infection, shedding into the litter, sporulation in the litter, and re-ingestion to repeat the process. In the first cycle of infection, the number of shed oocysts is relatively low for each species, and birds are reinfected through oocyst ingestion and shed higher numbers of oocysts in the second cycle (Long et al., 1986; M. Shirley, 1989).

Vaccinal oocyst cycling studies have demonstrated that the peak of shedding varies by species, and is highest for *E. tenella* at 7 days post inoculation (You, 2014). After the first cycle, chickens immunized with a live coccidia vaccine generally have rising oocyst numbers in the litter by 2 weeks, peaking at 4-6 weeks before declining (Jenkins, Parker, & Ritter, 2017). A significantly slower increase in oocysts per gram of feces following vaccination has been correlated with a slower onset of immunity, as determined by lesion scores following challenge (Mathis, Newman, Fitz-Coy, Lumpkins, & Charette, 2017). It has also been shown that full immunity to all species of oocysts in a vaccine may not occur at the same time within a vaccine, or between vaccines (Mathis et al., 2017).

Coccidia vaccines function very differently from ionophores or chemicals in that they induce full immunity in the bird after multiple rounds of oocyst cycling. A side effect of vaccination however, is decreased weight gain and increased feed conversion ratios of vaccinated birds compared to unvaccinated birds (Sokale et al., 2018; Waldenstedt, Lunden, Elwinger, Thebo, & Uggla, 1999); though compensatory weight gain at the end of the grow period can negate the effects (G. Mathis & Lang, 1999). One attempt at reducing performance losses from vaccination is to vaccinate at one day of age and then place the birds on a bioshuttle program, where an anticoccidial is added to the grower diet. Bioshuttle programs allow birds to develop immunity against coccidia by cycling vaccinal oocysts, but the anticoccidial drug should reduce the infection rate in the second cycle, thereby reducing gut damage (Chapman et al., 2002). A field evaluation of a bioshuttle program comparing over 15 million birds per treatment group found that a bioshuttle improved adjusted feed conversion, increased body weight, and reduced average days to process compared to the use of a coccidiosis vaccine alone (Montoya & Quiroz, 2013).

The purpose of this trial is to determine the protection of three different control programs against a pathogenic *E. tenella* challenge. The three control measures utilized were vaccination alone, ionophore (salinomycin) alone, and vaccination in combination with salinomycin. Two different live non-attenuated vaccines were utilized to determine if one provided better protection against this strain of *E. tenella*. Additionally, two challenge timepoints—one corresponding to field challenge and a later challenge—were utilized to determine if further vaccine cycling would be protective.

Materials and Methods

Experimental Design

Six hundred non-vaccinated day old chicks were obtained from the commercial poultry company experiencing the *E. tenella* challenge and were brought to the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia (UGA) for the experiment. At the same time, starter and medicated and non-medicated grower feeds were obtained from the commercial company's feed mill to use for the trial. Water and feed were provided ad-lib for the duration of the trial. Animal care and use protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

The chicks were randomly divided into six groups of 100 chicks per group and weighed prior to placement. Chicks were then vaccinated with Groups 1 and 3 receiving vaccine 1, and groups 2 and 4 receiving with vaccine 2. Groups 5 and 6 were kept non-vaccinated for controls (Table 5.1). All groups received the same starter diet with bacitracin methylene disalicylate (BMD) from days 1-14. On day 14, Groups 1 and 2 were switched to a grower diet containing BMD, while

Groups 3-6 received the grower diet containing BMD and salinomycin at a concentration of 60 g/ton. All groups were kept on litter in pens in a curtain sided house.

On days 6-8 post-vaccination, litter samples were collected from groups 1-4 to determine vaccinal oocyst cycling. Ten samples of approximately 10 grams each were randomly collected from each pen for counting each day. Eleven days post vaccination, half the birds from groups 1-5 were challenged with 15,000 sporulated *E. tenella* oocysts. Once birds had been challenged, they were placed into new pens to prevent exposure of non-challenged birds to oocysts. Twenty-eight days post vaccination, the remaining birds were challenged. Challenged birds were weighed and necropsies were performed on days 18, 28, and 35 to determine effects from challenge on gross lesion scores and oocyst count scores (Table 5.2).

Monoculture and Pathogenicity Testing of E. tenella Challenge

Two field isolates from separate cases of clinical coccidiosis caused by pathogenic *E. tenella* were used to prepare single oocyst monocultures for testing. For pathogenicity testing, doses of 1,000, 5,000, 10,000, 15,000 and 20,000 sporulated oocysts were tested. Five birds per dosage received challenge via gavage, and seven days later a necropsy was performed, and gross lesions in the ceca caused by *E. tenella* were scored 0-4 (Reid & Johnson, 1970). A challenge of 15,000 sporulated oocysts per bird was utilized as the challenge for this trial, as this consistently elicited a gross lesion score of 3 in naïve chickens.

Oocyst Cycling

Oocysts were enumerated from the litter during cycle one for all vaccinated birds based on species present in the vaccine. Litter samples were soaked in 10x water overnight before being

filtered, centrifuged, and resuspended in salt water. The resulting sample was then mixed and pipetted into a McMaster's chamber. The chamber was allowed to sit for three minutes so oocysts could rise to the top of the chamber, then were counted using the method of Conway and McKenzie (Conway & McKenzie, 2007). Oocyst counts are reported in oocysts/gram of litter.

Gross Lesion Scores

Gross lesions in the ceca were scored on a 0-4 scale, with 0 representing no lesions and 4 designating cecal cores. Additionally, any bird that died due to *E. tenella* was assigned a score of four (Reid & Johnson, 1970).

Oocyst Count Scores

Mucosal scrapings were taken from the right ceca and placed on a slide with a coverslip to obtain oocyst count scores. Using a 10x objective, a score of 0-4 was assigned, with a score of 0 representing no oocysts present, a score of 1 representing 1-20 oocysts, a score of 2 representing 21-50 oocysts, a score of 3 representing 51-100 oocysts and 4 representing too many oocysts to count (Goodwin, Brown, & Bounous, 1998).

Biometrics

All body weight, weight gain, GLS, and OCS data were analyzed utilizing one-way analysis of variance with post-hoc Tukey's multiple comparisons (GraphPad Prism, GraphPad Software, La Jolla California USA). All comparisons were considered significant at a level of $P < 0.05$.

Results

Pathogenicity testing

For the pathogenicity test, following challenge of naïve birds 1,000 oocysts produced lesion scores averaging one, 15,000 oocysts produced lesion scores averaging three, and 20,000 oocysts resulted in 20% mortality. For this trial, a challenge of 15,000 oocysts was utilized.

Vaccinal Oocyst Shedding

E. maxima vaccinal oocyst shedding from chickens in all groups peaked on day 7 post vaccination, with oocyst counts per gram of litter ranging between 2,000 and 7,000 oocysts. Shedding of *E. tenella* from all groups peaked on day 7 as well, however both groups vaccinated with vaccine 1 failed to shed any significant quantity of oocysts. *E. acervulina* shedding peaked on day six or seven in all groups, with groups vaccinated with vaccine 2 shedding higher numbers of *E. acervulina* than groups vaccinated with vaccine 1 (Figure 5.1).

Early Challenge Body Weight

Body weights on day 1, just prior to vaccination, were uniform regardless of the challenge group (Figure 5.2). On day 11 post vaccination, at the time of challenge, there were no significant differences in body weights between any of the groups. By day 18, seven days post challenge and at the time of necropsy, body weights in the vaccinated and non-vaccinated groups that received salinomycin in the grower diet were not significantly different compared to the non-vaccinated/challenged group, while weights in groups that did not receive salinomycin were significantly decreased.

Late Challenge Body Weight

As with the early challenge group, there were no significant differences in body weights at day one. At challenge, day 21, three of the vaccinated groups had significantly decreased body weights compared to the non-vaccinated groups (Figure 5.3). At day 28, all vaccinated groups weighed significantly less than the non-vaccinated/non-challenged group. At day 35, again all vaccinated groups weighed significantly less than the non-vaccinated/non-challenged group. At both day 28 and day 35 of the trial the body weight of the non-vaccinated/challenged group was not significantly different from any other group.

Lesion and Oocyst Count Scores

All challenged groups from the early challenge had significantly higher gross lesion and oocyst count scores than the non-vaccinated/non-challenged group and were not different from the non-vaccinated/challenged group (Figure 5.4). For the late challenge groups, all vaccinated groups had significantly higher lesion scores than the non-vaccinated/non-challenged group but had significantly lower gross lesion scores than the non-vaccinated/challenged group. All groups followed the same pattern for oocyst count scores as well. By day 35, all gross lesions caused by *E. tenella* resolved, with the exception of the late non-vaccinated/challenged group where three of the five birds examined had gross lesion scores of one.

Discussion

The trial was designed due to a commercial integrator facing a pathogenic *E. tenella* challenge on multiple farms. The farms reported bloody feces and mortality occurring between 16 and 18 days. Based on the field situation, the trial took into account two different challenge

timepoints, an earlier challenge to coincide with the conditions seen in the field, and a later challenge to see if further vaccine cycling would provide protective immunity. In the early challenge, none of the challenged groups were protected based on gross lesion scores. In the last challenge, the vaccinated groups and groups on a bioshuttle program showed decreased gross lesion scores compared to the expected lesion score of three based on the pathogenicity test, while the non-vaccinated group did not have a reduction in gross lesion scores.

When comparing body weights in this study, there was a significant decrease in body weight at 35 days post challenge in all groups that received the vaccine or bioshuttle program. The decrease in body weights is similar to results seen in previous studies also looking at small birds placed on a coccidiosis vaccination program (Chapman et al., 2002; Danforth, 1998). The reason behind the decrease in weight gain, is that a live oocyst vaccine is given to the birds and the replication of the oocysts, particularly *E. maxima* and *E. acervulina*, results in controlled exposure, and subclinical signs of coccidiosis. When a longer lived, larger bird is grown, there is compensatory weight gain following the development of immunity in vaccinated birds so the decrease weight of birds is not recognized at the time of processing (Chapman et al., 2002; G. Mathis & Lang, 1999; Montoya & Quiroz, 2013). If a different challenge model had been utilized that causes a decrease in body weights like *E. maxima*, there would have been a more significant decrease in body weight gain in the non-vaccinated challenged groups at the early and late challenge time points, and the effects of utilizing the vaccine on body weight gain may not have been as noticeable.

E. maxima is known to be the most immunogenic of the coccidia species that infect chickens, while *E. tenella* is not very immunogenic and takes longer to develop immunity in the bird (Long & Millard, 1979; Rose & Long, 1962). Other studies have noted development of cecal

cores in birds after challenge due to an incomplete development of an immune response against *E. tenella* as many as 20 weeks after vaccination (Long, Johnson, & Wyatt, 1980; M. Shirley & Millard, 1986). Birds given repeated immunizing doses still developed severe gross lesions, however other clinical effects of the challenge were not seen (Long et al., 1980; Long, Johnson, & Wyatt, 1981). Based on the body of research, and findings from this study, it is unlikely that vaccination alone will prevent mortality occurring from an early *E. tenella* challenge.

The results from this study show the potential for salinomycin resistance in this field isolate of *E. tenella.* Resistance has been documented since shortly after the widespread use of anticoccidial drugs (Cuckler & Malanga, 1955). As high as 71% of *E. tenella* strains have been resistant to monensin, another ionophore, and as high as 86% of *E. tenella* isolates have been resistant to salinomycin (McDougald, Fuller, & Solis, 1986). Additionally, as the live oocyst vaccines contain drug sensitive oocysts, drug resistance of the coccidian organisms is higher in flocks in which an anticoccidial drug is utilized (M. Jenkins, Klopp, Ritter, Miska, & Fetterer, 2010).

To combat the ongoing *E. tenella* challenge, different control strategies could be undertaken. These control strategies can include a total barn cleanout to reduce the number of oocysts present in the house, use of an anticoccidial agent with greater activity against *E. tenella,* and use of an anticoccidial drug in the starter diet (Peek & Landman, 2011).

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Tables and Figures

Table 5.1: Experimental design.

Day	Procedure	Early Challenge	Late Challenge
1	Weigh, vaccinate 100 birds		
11		Weigh, challenge 50 birds	
14	Switch to grower diet, containing salinomycin		
18		Weigh, necropsy 20 birds	
21			Weigh, challenge 50 birds
28			Weigh, necropsy 20 birds
35		Weigh 30 birds	Weigh 30 birds

Table 5.2: Group and treatment assignments.

Group	Treatment
1	Vaccine 1
2	Vaccine 2
3	Vaccine 1 with salinomycin
4	Vaccine 2 with salinomycin
5	Nonvaccinated with salinomycin/challenged
6	Nonvaccinated with salinomycin/nonchallenged

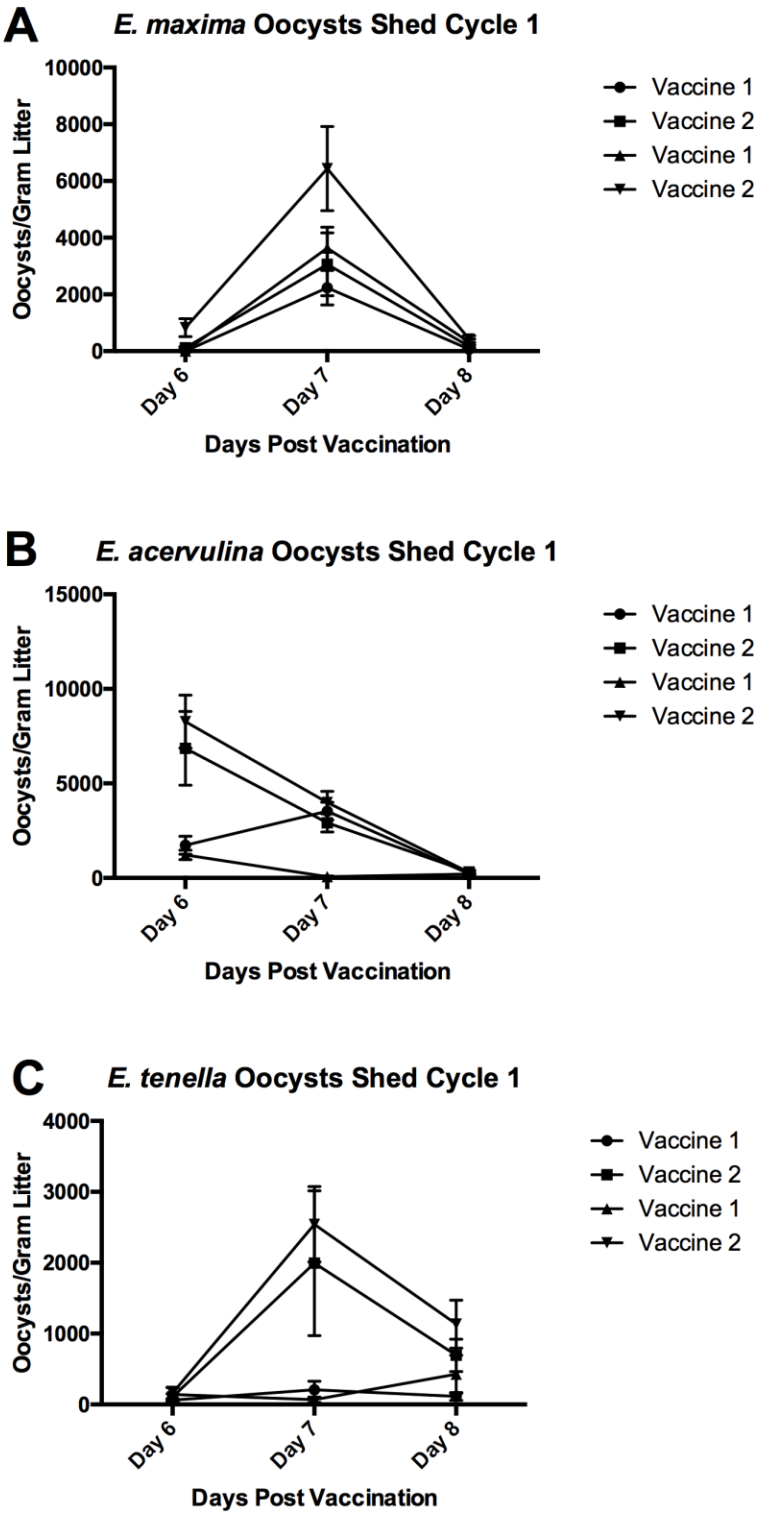
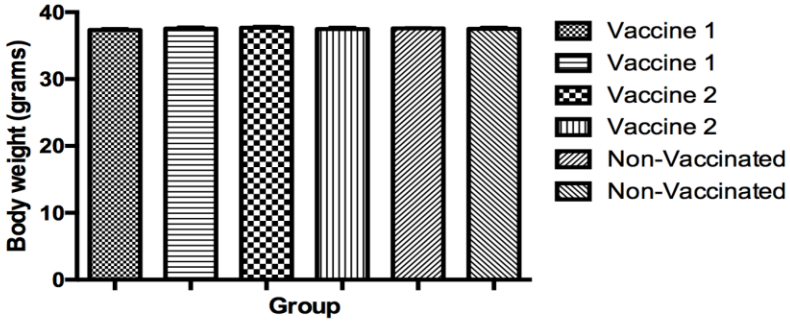
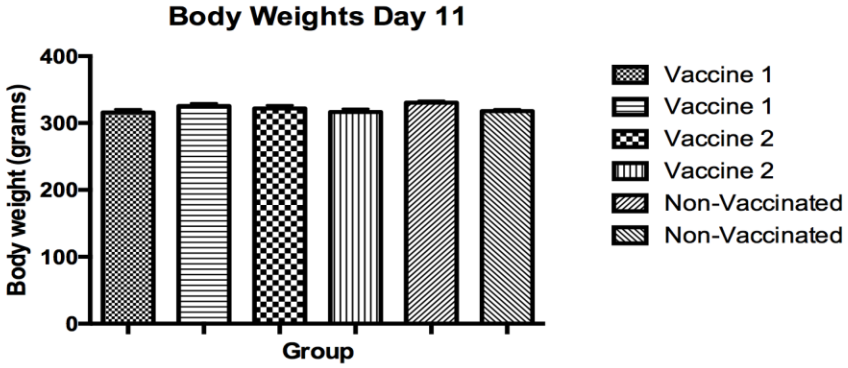


Figure 5.1: Oocysts per gram litter days 6 through 8 post vaccination. (A) *E. maxima.* (B) *E. acervulina*. (C) *E. tenella.*

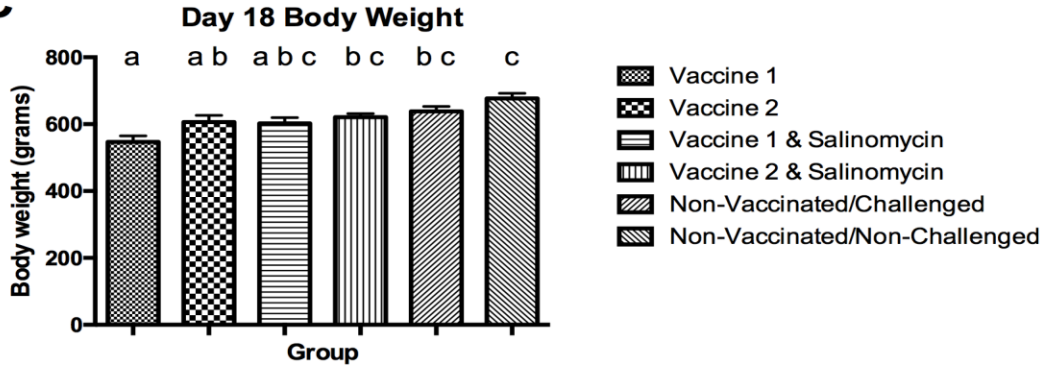
A


Body Weights Day 1
Body weight (grams)
40
30
20
10
0
Group
Vaccine 1
Vaccine 1
Vaccine 2
Vaccine 2
Non-Vaccinated
Non-Vaccinated

B


Body Weights Day 11
Body weight (grams)
400
300
200
100
0
Group
Vaccine 1
Vaccine 1
Vaccine 2
Vaccine 2
Non-Vaccinated
Non-Vaccinated

C


Day 18 Body Weight
a
a b
a b c
b c
b c
c
Body weight (grams)
800
600
400
200
0
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-Vaccinated/Challenged
Non-Vaccinated/Non-Challenged

D

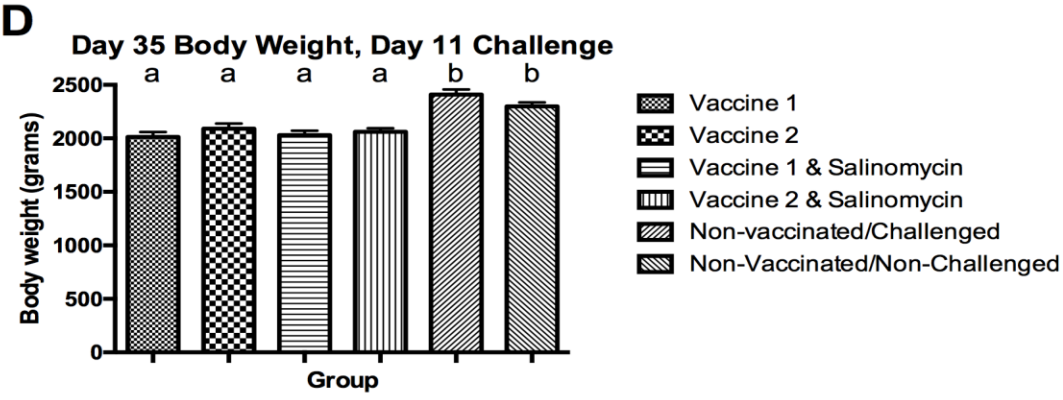

Day 35 Body Weight, Day 11 Challenge
a
a
a
a
b
b
Body weight (grams)
2500
2000
1500
1000
500
0
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-vaccinated/Challenged
Non-Vaccinated/Non-Challenged

Figure 5.2: Body weight in grams of early challenge birds. (A) Body weights of day one birds. (B) Body weights of day 11 birds, pre-challenge. (C) Body weights of day 18 birds, seven days post challenge. (D) Body weights of day 35 birds, end of grow out period.

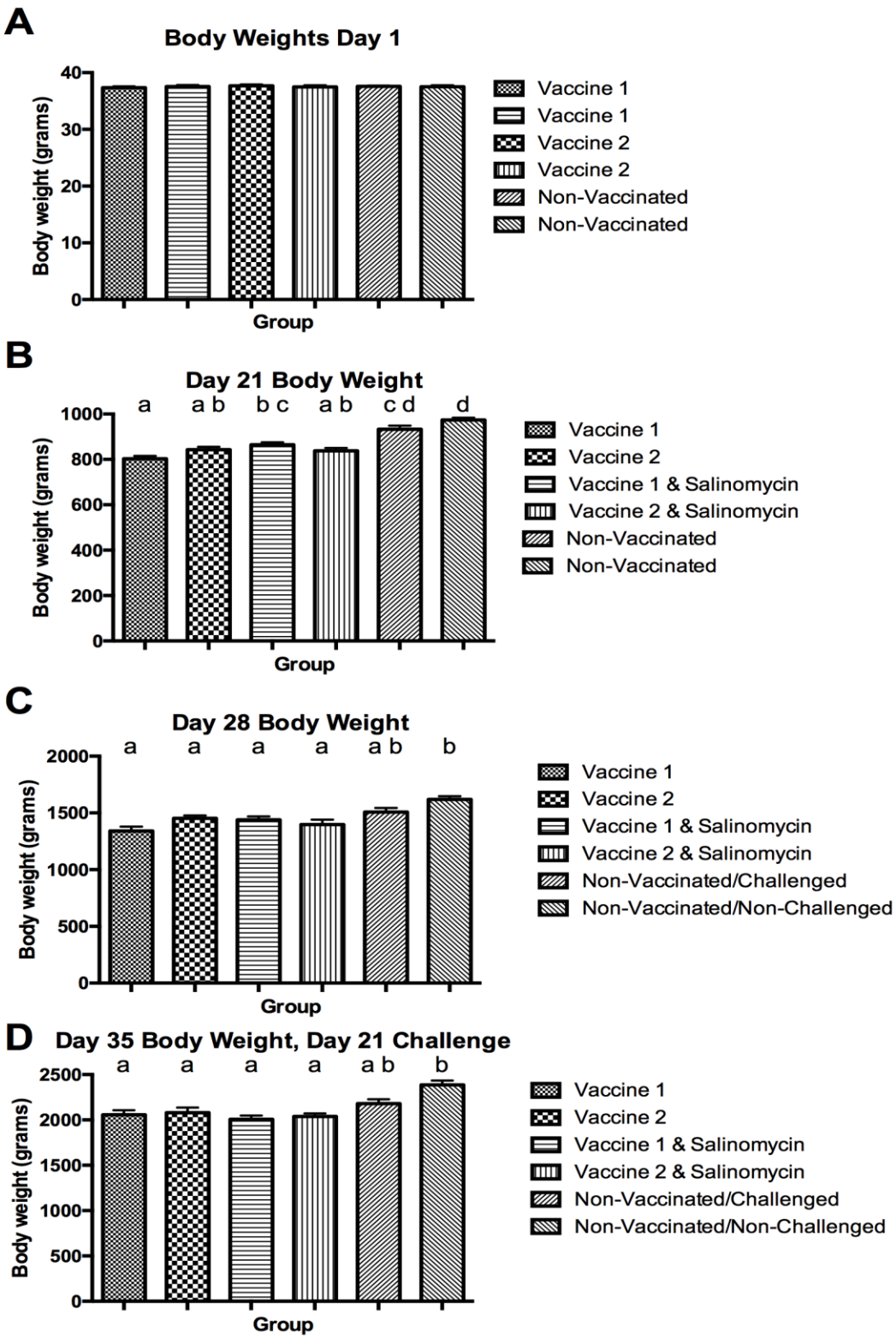
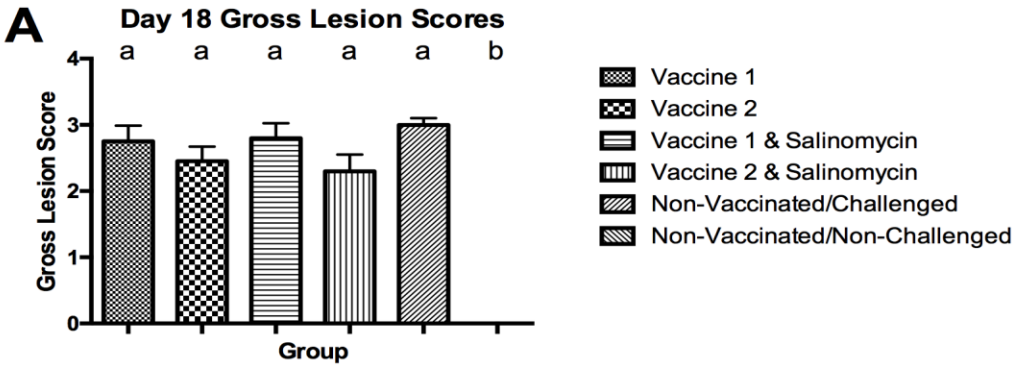
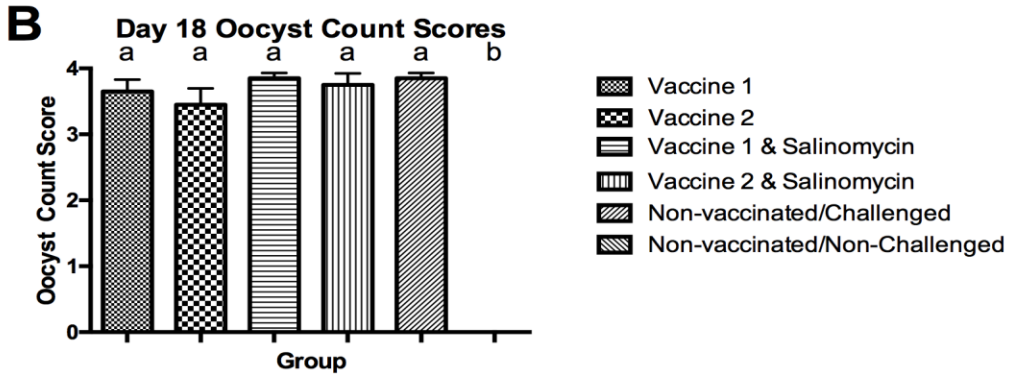
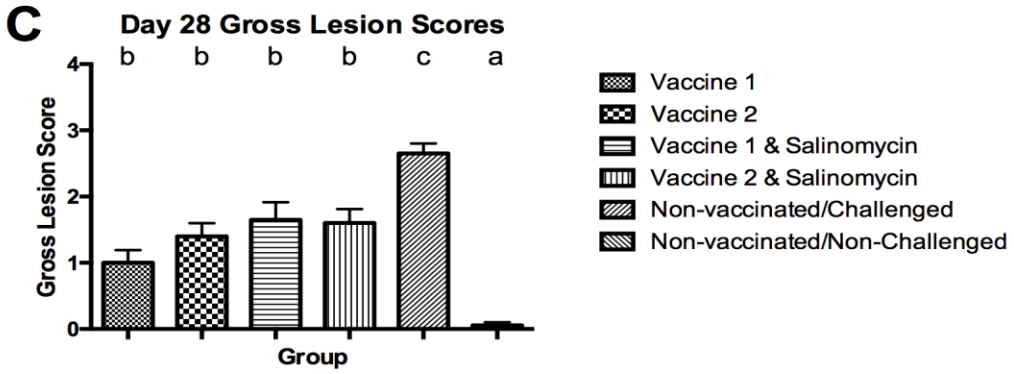

A
Body Weights Day 1
Body weight (grams)
40
30
20
10
0
Group
Vaccine 1
Vaccine 1
Vaccine 2
Vaccine 2
Non-Vaccinated
Non-Vaccinated
B
Day 21 Body Weight
a a b b c a b c d d
1000
800
600
400
200
0
Body weight (grams)
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-Vaccinated
Non-Vaccinated
C
Day 28 Body Weight
a a a a a b b
2000
1500
1000
500
0
Body weight (grams)
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-Vaccinated/Challenged
Non-Vaccinated/Non-Challenged
D
Day 35 Body Weight, Day 21 Challenge
a a a a a b b
2500
2000
1500
1000
500
0
Body weight (grams)
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-Vaccinated/Challenged
Non-Vaccinated/Non-Challenged

Figure 5.3: Body weight in grams of late challenge birds. (A) Body weights of day one birds. (B) Body weights of day 21 birds, pre-challenge. (C) Body weights of day 28 birds, seven days post challenge. (D) Body weights of day 35 birds, end of grow out period.


A
Day 18 Gross Lesion Scores
a a a a a b
Gross Lesion Score
0
1
2
3
4
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-Vaccinated/Challenged
Non-Vaccinated/Non-Challenged


B
Day 18 Oocyst Count Scores
a a a a a b
Oocyst Count Score
0
1
2
3
4
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-vaccinated/Challenged
Non-vaccinated/Non-Challenged


C
Day 28 Gross Lesion Scores
b b b b c a
Gross Lesion Score
0
1
2
3
4
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-vaccinated/Challenged
Non-vaccinated/Non-Challenged

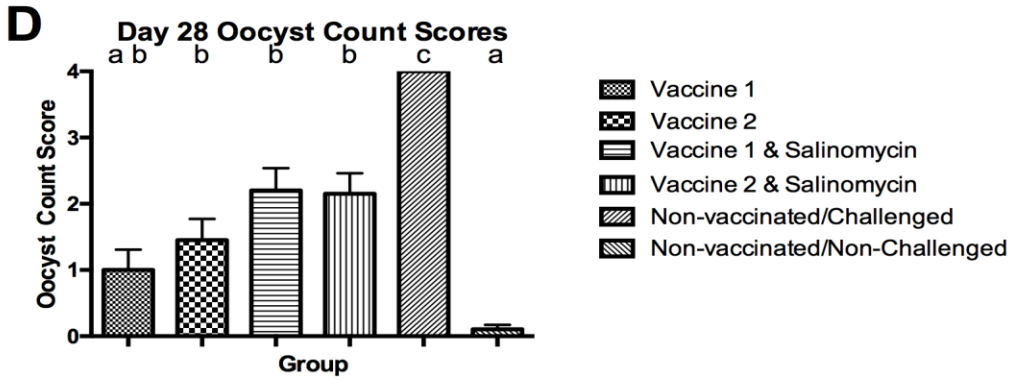

D
Day 28 Oocyst Count Scores
a b b b b c a
Oocyst Count Score
0
1
2
3
4
Group
Vaccine 1
Vaccine 2
Vaccine 1 & Salinomycin
Vaccine 2 & Salinomycin
Non-vaccinated/Challenged
Non-vaccinated/Non-Challenged

Figure 5.4: Gross lesion scores and oocyst count scores seven days post challenge. (A) Gross lesion scores of early challenged birds at day 18. (B) Oocyst count scores of early challenged birds at day 18. (C) Gross lesion scores of late challenged birds at day 28. (D) Oocyst count scores of late challenged birds at day 28.

Chapter 6

SUMMARY AND CONCLUSIONS

As coccidiosis is a disease of major economic impact for the poultry industry, there are many different mechanisms of prophylaxis control and treatment available. The focus of this body of work was to determine effective vaccination strategies, combined with other measures of control, to determine protection from challenges with pathogenic field isolates of multiple species of *Eimeria*.

The first part of work was to determine the application parameters of different diluents utilized in the mass application of coccidiosis vaccines at the hatchery with different vaccines. The two vaccines varied by the number of oocysts per dose, and species present in the vaccine. Water and gel diluents were compared in settling, application pattern, and oocysts per droplet between the two vaccines. Between the high and low dose vaccine, there was an approximately sixfold difference in oocyst counts. When the vaccines were mixed with each diluent according to manufacturer's protocols, no settling of oocysts occurred. When assessing application parameters, spray droplets occurred in five categories of sizes, and the pattern of spray droplets was even from front to back of the area evaluated, though the largest droplets were found on the left and right edges. The gel droplets were uniform in size, and the pattern of gel droplets was even across the application field, as it was applied via gel drop bar. Oocysts per droplet increased as the water droplet size increased but remained the same in the uniform gel droplet. There was no difference in oocysts per droplet based on location in either diluent. When using a water diluent, there is a general decrease in oocysts per dose reaching chick level. This is due in part to shearing of oocysts

and aerosolization of the vaccine during application. The gel diluent had a consistent number of oocysts present throughout the administration process, indicating no loss of oocysts.

The next trial used the same four vaccine and diluent combinations as did the application trials. Chicks were vaccinated at day of hatch and placed on litter to allow for vaccine cycling. Oocyst shedding for cycle one was variable, with a low percentage of birds vaccinated with the high oocyst dose vaccine in either diluent shedding *E. maxima*. By cycle two, all birds from all vaccinated groups were shedding all oocysts. Vaccine cycling produced the most severe lesion scores at 14 days post vaccination, when looking at both gross and microscopic lesion scores. All vaccinated groups had decreased gross lesion scores compared to a non-vaccinated challenged control post challenge, and there was no significant difference between any of the vaccinated groups.

The final experiment examined protection of vaccinated birds, birds on a bioshuttle program, and birds on an ionophore only against an early and late *E. tenella* challenge. Birds received one of two vaccines or were left unvaccinated day of hatch. None of the groups had a decrease in gross lesion scores after an early challenge at 11 days, including the groups that had salinomycin added to the grower diet at 14 days. All birds that received the vaccine had a significant decrease in gross lesion score following a late challenge at 21 days. All vaccinated groups had a significant decrease in body weight at the end of the 35 days grow out period compared to the non-vaccinated/non-challenged group.

When taken together, this data shows that, regardless of vaccine or diluent used, there are no inherent flaws in application with either method tested. Also, with enough time allowed for vaccine to cycle and for birds to develop immunity, all vaccines utilized were protective against challenge from pathogenic field isolates of multiple *Eimeria* species.