EIMERIA SPECIES IN COMMERICAL BROILER COMPLEXES IN THE UNITED STATES

by

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(Under the Direction of Michael Lacy)

ABSTRACT

Identifying Eimeria parasites has historically been done through biological characteristics such as pre-patent period, morphology and site of infection. All of these methods are time consuming and when parasites are in low numbers, species can easily be overlooked. PCR technology has provided a tool for rapid and accurate idenification. Eimeria species in commercial broiler facilities in the United States were identified using PCR. Results showed six species (*E. acervulina, E. brunetti, E. maxima, E. mitis, E praecox* and *E. tenella*) frequently in the forty-three complexes tested. Drug sensitivity of these Eimeria field isolates were tested using Anticoccidial Sensitivity Tests. Infections of isolates of *E. maxima* were also investigated. The reproductive indexes, patent period and pathogenicity of field isolates from the United States and from commercial vaccines. The identification methods described are important in accurately characterizing Eimeria and necessary in determining the best anticoccidial program.

INDEX WORDS: Eimeria, Coccidia, PCR, Anticoccidial Sensitivity Test, E. maxima,

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CHAPTER 1

Introduction

Eimeria are Apicomplexa parasites of significant economic importance to the poultry industry. Historically these parasites have been successfully controlled with chemotherapeutics, but overtime drug resistance has developed. The difficulty and costs associated with drug development and approval has severely limited the outlook for new chemical treatments. Without the introduction of new drugs to the market, alternatives such as live vaccines have been utilized.

E. maxima is the most immunogenic Eimeria species that infects domestic chickens (Smith et al., 2002). That said, different populations of *E. maxima* exhibit substantial immunological diversity (Allen et al. 2005). Because of this significant variation, it has been shown crucial to include more than one strain of *E. maxima* in vaccines (Barta et al 1998). Several studies have been conducted with *E. maxima* comparing pathogenicity (Fitz-Coy, 1992), but none have used all commercially available broiler coccidia vaccines in the United States. Vaccines incorporate several species of *E. maxima* due to the antigenic variation, and lack of immunological protection from only one strain.

With the growing availability of molecular tools such as Polymerase Chain Reaction (PCR), Eimeria species diagnostics have become much more accurate and are less laborious. Prior to PCR, Eimeria were speciated by determining the pre-patent period of oocysts, lesion location in intestines and oocyst morphology (Long and Joyner, 1984).

A limiting issue with vaccines is that immunity is only developed for the species included and any other Eimeria in the field can potentially to cause severe damage. To determine what species of Eimeria are currently in broiler complexes, we used PCR with species specific primers for *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*. The primers bind to species-specific regions of genomic DNA, and if particular species are present, the regions are amplified (Schwarz et al. 2009). Determination of Eimeria species in poultry facilities is important to properly manage parasite control measures that promote vaccine efficacy and limit drug resistance (Ogedengbe et al. 2011). Routine PCR diagnostics can provide insight to the fluctuations of Eimeria populations within poultry facilities (Carvalho et al. 2011). A current detailed survey of Eimeria in broiler facilities is lacking.

The main objectives of this study were to:

1. Detect Eimeria species from commercial broiler complexes across the United States using PCR.
2. Generate clonal populations of E. maxima from field isolates and broiler coccidia vaccines used in the United States.
3. Use these lines of E. maxima to compare pathogenicity, pre-patent period and micro-scoring variability between lines.

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Chapter 2

Literature Review

Coccidiosis, the disease caused by a number of species of Eimeria parasites, is of enormous economic importance, costing the world's poultry industry $3 billion dollars annually (McDonald and Shirley, 2009). Virtually all-commercial poultry production facilities have some amount of Eimeria.

The Eimeria that infect chickens are host specific and the different species have varied affinities for infection sites in the gastrointestinal tract. Eimeria in poultry do not have intermediate hosts (McDougald, 1998). Propagation of the parasite is dependent on birds ingesting sporulated oocysts shed in the feces of infected birds. Current poultry production practices with large flocks of birds raised in confined housing have created a niche for Eimeria that make it difficult to eliminate. Producers use anticoccidial drugs, live vaccines and good management practices to prevent significant losses from the ubiquitous parasite.

Coccidia are unicellular protozoan parasites that belong to the phylum Apicomplexa (Levine, 1970). This phylum is characterized by motile invasive stages that contain an apical complex (Morrison, 2008). Apical complexes are composed of electron-dense polar rings, a conoid formed by a spirally coiled filament inside the polar ring, micronemes that extend from the anterior region within the conoid and subpellicular tubules that extend from the polar ring region beneath the pellicle (Hammond, 1973). Figure 2.1. shows is a visual depiction of the apical complex in an Eimeria sporozoites or merozoite.

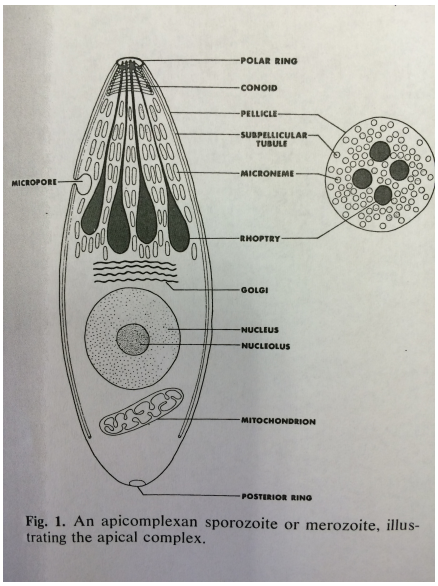

Fig. 1. An apicomplexan sporozoite or merozoite, illustrating the apical complex.

Figure 2.1. From The Coccidia, Hammond and Long, 1973.

There are two classes in the Apicomplexan subphyla, Sporozoasida and Piroplasmida. Sporozoasida contain Eimeria, which infect poultry, and this class is further divided into two subclasses, Gregarinasina and the Coccidiasina (Hammond, 1973). Coccidiasina are broken into two orders, Protococcidiorida and Eucoccidiorida, the latter is the main order infecting vertebrates. Eucoccidiorida is divided into three suborders: Adeleorina, Haemosporina and Eimeriorina. The Eimeriidae family (Minchin, 1903) is characterized by oocysts and schizonts lacking attachment organelles, oocysts with 0,1,2,4 or many sporocysts, and each sporocyst with 1 or more sporozoites. These parasites are monoxenous, with merogony in the host and sporogony typically outside host (Hammond, 1973). The genus Eimeria (Schneider, 1875) is characterized by oocysts with four sporocysts, each comprised of two sporozoites.

The genus contains eight species that have been identified to infect *Gallus gallus* var. *domesticus (*the domestic chicken): *E. acervulina, E. brunetti, E. hagani, E. maxima, E. mitis, E. mivati, E. necatrix, E. praecox,* and *E. tenella.* Seven of these species are most commonly associated with clinical coccidiosis in commercial chicken production and summarized in table

2.1: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*. *E. acervulina, E. brunetti, E. maxima, E. necatrix* and *E. tenella* are considered the most pathogenic. They disseminate throughout the intestinal tract and can cause simultaneous infection, representing a disease complex (Williams, 2002). *E. hagani* has been considered a doubtful species because of the incomplete original description, but nevertheless characterized by Oluleye in 1982, who described it as a distinct species, characterizing the pathogenicity, location of infection and lesions produced. In addition to varying in terms of infecting specific locations of host's intestines, Eimeria also vary in pathogenicity and symptoms.

Species	Pathogenicity	Prepatent period (hr)	Site of infection within tissue	Clinical Signs/Lesions
Acervulina	Moderate	97	Epithelial	Whitish round lesions, can appear ladder like, with heavy infections plaques coalesce and intestine walls thicken
Brunetti	High	120	2nd generation schizonts subepithelial	Coagulation necrosis, bloody enteritis in lower intestines
Maxima	Moderate	121	Gametocytes subepithelial	Thickened walls, orange-tinged contents, petechiae
Mitis	Mild-	93	Epithelial	No discrete lesions, pale exudate

	Moderate			
Necatrix	High	138	2nd generation schizonts subepithelial	Ballooning, white spots, petechiae, bloody feces
Praecox	Mild	83	Epithelial	No lesions, mucoid exudate
Tenella	High	115	2nd generation schizonts subepithelial	Hemorrhaging into lumen, whitish mucous, bloody cores, Bloody feces

Table 2.1: summarizes the pathogenicity, clinical signs and lesions, pre-patent period and site of infection of the seven most economically important Eimeria infecting chickens.

Eimeria have been differentiated by several characteristics: 1) morphology and dimensions of oocysts 2) host- and site- infection specificity 3) morphology of intermediate and endogenous stages of the parasites 4) pathogenicity 5) cross-immunity 6) pre-patent and patent periods (Joyner and Long, 1974). With the development of molecular diagnostics, PCR has been used to differentiate Eimeria species with relative ease and improved accuracy. Species-specific primers have been developed to diagnose accurately in a mixed population (Jenkins et al 2006).

Unlike Apicomplexa parasites such as Toxoplasma, Eimeria are self-limiting. The life cycle of Eimeria passes through a series of stages, with a specific number of schizongenous generations (Hammond, 1973). When sporulated oocysts are ingested, the mechanical movement of the gizzard ruptures the oocyst wall, releasing sporocysts. Trypsin and bile in the duodenum promote excystation of sporozoites from the sporocysts. Depending on the species, sporozoites penetrate site-specific host mucosal cells in different segments of the intestinal tract. Some sporozoites of Eimeria (*E. brunetti and E. praecox*) develop within the cells in which they

initially penetrate (Tewari & Maharana, 2011). Other species' sporozoites (*E. acervulina, E. maxima, E. necatrix and E. tenella*) develop into trophozoites in other sites after being transported via the crypt epithelium (Lawn and Rose, 1982; Trout and Lillehoj, 1993). The sporozoites become trophozoites, and undergo schizogony, which is a rapid multiple fission. This stage is asexual, so daughter cells are mitotically produced. The daughter cells, or merozoites rupture the mucosa and individually penetrate more mucosal cells. The merozoites grow into second and depending on the species, third generation schizonts, each generation causing significant damage to the intestinal segment they infect. Clinical signs, such as bloody feces during *E. tenella* or *E. necatrix* infections, are associated with the schizont maturation, resulting in tissue damage.

As merozoites mature, they sexually differentiate, becoming either macrogamonts or microgamonts. Microgamonts multiply and divide to make the male form, microgametes. Microgametes are motile and after their release penetrate host intestinal cells to fertilize macrogamonts (Tewari and Maharana, 2011). Upon fertilization, a zygote is produced and after maturation, an oocyst. Each fertilization results in a new oocyst, so initial infection of the host with one oocyst has the potential to generate thousands of oocysts passed in the feces. Eimeria are not equal in their reproductive indices, for example *E. brunetti* has much higher reproductive potential than *E. maxima* (Williams, 2001). The unsporulated oocyst is shed in the feces. Unsporulated oocysts are not infectious until sporulation occurs. Sporulation of the oocyst is induced by several factors, but the most important ones are exposure to an environment containing free access to oxygen, a temperature range of 10°C- 30°C, with 30°C being optimal (Price, 2012). A saturated relative humidity is important for optimum sporulation time (Kheysin, 2013). Some species such as E. maxima however, have been shown to sporulate better at 16 %

than 42 and 62% moisture. (Waldenstedt et al, 2001). The sporulated oocysts are resistant to many disinfectants used to clean poultry houses and can remain infective for up to 602 days in an exogenous environment (Reid, 1990). Oocysts have no mode of locomotion, and so must rely on host ingestion to procreate. With broilers being raised in high-density numbers and coprophagy being a normal behavior in chickens, oocysts are frequently ingested.

To control coccidiosis, the poultry industry has used three main approaches: 1) anticoccidial drugs 2) vaccination 3) improving management practices. Each approach has shown to help production, but none are a silver bullet.

Anticoccidial drugs have been the treatment of choice for many commercial producers because of the treatment costs are small- less than $0.01/ bird (McDougald, 2003). These drugs vary in efficacy against each species and depending on the frequency of use as resistance has been observed over the years. In 1948, sulphaquinoxaline and nitrofurazone were the first anticoccidials approved by the U.S. Food and Drug Administration (FDA)(Peek and Landman, 2011). Approval and removal of anticoccidial drugs has varied around the world and even within a particular country depending on changes in efficacy or safety concerns (Peek and Landman, 2011).

There are three main categories of anticoccidial products according to their origin (Peek and Landman, 2011).

1. Synthetic compounds, also known as ‘chemicals’: These drugs have a specific action against parasite metabolism. Table 2.2 depicts specific chemical anticoccidials with their target in the parasite.
2. Ionophores or polyether antibiotics: These antibiotics are produced by the fermentation of Streptomyces or Actinomadura spp. They act by rupturing the

parasite via osmotic balance interference. Ionophores work by creating lipophilic complexes that transport ions into the parasite's cell, offsetting the cross-membrane gradient. They affect influx of ions with different charges and so are broken into three categories:

a. Monovalent Ionophores (monensin, narasin and salinomycin)
b. Monovalent glycosidic ionophores (maduramicin and semduramicin)
c. Divalent Ionophores (lasalocid)

Some ionophores possess Gram-positive antibacterial activity which has not only allowed for control of coccidia parasites but also clostridium perfringens, the causative agent of necrotic enteritis (Williams, 2005).

3. Mixed products: combination of chemical and ionophore or two chemicals (nicarbazin/narasin, meticlorpindol/methylbenzoquate respectively).

Based on their effect on the parasite, anticoccidials can be described as coccidiostats or coccidiocidals. Coccidiostats arrest the growth of intracellular life stages, but since the host is not able to develop proper immunity, development will continue after drug withdrawal. Coccidiocidals on the other hand, destroy the parasite during development, preventing continuation of development after drug removal. Some anticoccidials, such as ionophores, have both coccidiostatic and coccidiocidal properties. Different drugs are used in different grow out periods as well as in different production birds (layers vs. broilers; turkeys vs. broilers or layers).

Sulphonamides have a narrow toxicity safety margin, which makes it difficult to use for outbreaks (Peek and Landman, 2011). Some chemicals like nicarbazin, cause an increase in sensitivity to heat stress, therefore, its use is recommended in the cooler months of the year (McDougald and McQuistion, 1980).

Drug name	Trade Name	Mode of Action	Parasite life cycle target[4]
Robenidine	Robenz, Cycostat	Prevent asexual replication in *E. Tenella*	Multiple stages
Decoquinate	Deccox	Affect parasite metabolism- blocks electron transport in mitochondria[3]	Sporozoite
Amprolium	Amprol	Competes for absorption of thiamine (vitamin B1)	First generation schizont
Clopidol	Coyden	Allows parasite penetration into host cell but prevents further development- coccidiostat effect[2]	Sporozoite
Diclazuril	Clinacox	Mode of action unknown	Multiple stages
Halofuginone	Stenorol	Thought to inhibit parasite penetration and further development in host cells[1]	Asexual stages
Nicarbazin	Nicarb	Not defined but thought to affect parasite's ability to generate energy, directed against developing second-generation schizonts	Sporozoite
Nitrobenzamides	Zoalene	Thought to stop asexual replication of parasite	Asexual stages

1. (Kitandu, 2006)

2. (Wang, 1975)

3. (Ryley, 1967)

4. (Zhang et al, 2012)

Table 2.2: modified from Peek and Landman 2011 and Chapman 1997

A factor that has allowed the ionophores to continuously be effective is “leakage.” In other words, the drugs work on the majority of parasites infecting birds, but a few bypass the drug, and allow the bird to develop natural immunity (Hafez, 2008). Leakage is commonly seen in prophylactic or preventative drug use. Although resistance has been seen with all commercially available drugs, “leakage” has helped to alleviate the emergence of resistance and prolong the continual use of the drugs.

Drug resistance in coccidia has been described as “the ability of a parasite strain to survive and/or multiply in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication’ (Chapman, 1997). Resistance can be either complete or relative. Complete resistance is when increasing the drug dose is ineffective. Relative resistance on the other hand is when increasing doses tolerated by the host still show efficacy against parasite as seen in ionophores (Peek and Landman, 2011). Resistant parasites become dominant rapidly because the drugs immediately select for resistant parasites when the bird feeds (Chapman, 1997).

Drug resistance has been researched extensively, but an exact mechanism has not been elucidated. In a laboratory setting, resistance has been observed to be developed with drug selection by subsequent increased levels of drugs with each passage through birds (Ryley, 1980). Chapman showed in 1976 that resistance was produced when coccidia was passed through birds

medicated with drugs at increasing concentrations. He concluded that the selection of mutants resistant to suboptimal levels of drug is essential for subsequent selection of mutants resistant to high drug concentrations (Chapman, 1976). The reasoning behind this conclusion was that in the study, when drug concentrations were kept low, resistance to higher levels did not occur, suggesting that the degree of resistance was dependent upon drug selection pressure (Chapman, 1976).

Joyner and Norton showed in 1978 that resistance to methyl benzoate and Clopidol was not transferred between E. *maxima* lines that were propagated together, and simultaneous resistance to both drugs could not be induced. This potentiating or synergistic relationship between the drugs has been used as a method to prevent resistance. Drugs are added in combination, increasing the efficacy of both separately. It would be preferable to use potentiating drugs in hope to slow the occurance of drug resistance rather than introduce the drugs once resistance occurs (Ryley, 1980). In some instances, the opposite can happen, and parasites become doubly resistant. Jeffers (1974) showed that E. *tenella* strains resistant to Amprolium and Decoquinate could undergo genetic recombination, and result in parasites resistant to both drugs. Because of this response, it is important to remember that drug resistance can be transferred in the field between coccidia, without the presence of a drug. Drug resistance can be transferred within species of Eimeria but not between different species (Ryley, 1980).

Several methods have been proposed and carried out to combat drug resistance. Increasing the drug dosage is seldom feasible not only because of economic issues but also toxicity. The concentrations of drugs, such as monensin, are used in commercial feed at concentrations very close to those that could cause decreased weight gain (Ryley, 1980).

Because drug sensitivity is correlated to exposure time, producers rotate anticoccidial treatment methods throughout the chicken's life as well as between flocks. Different seasons will also influence the efficacy of particular drugs, so they are rotated accordingly. Cross-resistance between drugs with similar modes of action is possible, so using distinctly different drugs is crucial within rotations and shuttle programs (Chapman, 1998).

Resistance in the field arises from prolonged drug exposure (McDougald, 2003). As described previously, it is common in the field to rotate anticoccidial drugs. Shuttle programs use different drugs throughout the birds life whereas rotation programs rotate drugs between flocks or seasonally (De Gussem, 2007). Shuttle programs commonly use one drug for the starter period and then another for the grower and finisher phase (Hafez, 2008). Rotation programs on the other hand, must use subsequent drugs with distinctly different mode of actions (Hafez, 2008). These programs work to prevent the coccidia population from developing complete resistance. Shuttle programs have not been shown to return sensitivity to particular drugs (McLoughlin and Chute, 1975). Despite the efforts to prevent resistance, it has been described in all of the currently available drugs, with prevalence varying between each (Chapman, 1997). With that in mind, different drugs also have different rates of resistance development. Some like Quinolones have rapid resistance, where others like Nicarbazin, are slow to develop resistance (Chapman, 1997).

Without the discovery and widespread use of drugs, the poultry industry would not have been able to reach the production capabilities it has today (Chapman, 2014). Drugs have allowed companies to effectively grow chickens intensively, and as a result, provided an affordable protein source available worldwide.

Although drug resistance has been shown in the laboratory setting, drug sensitivity in the field is much more difficult to gauge. In some instances, birds may be exposed to enough of a

challenge to cause death or bloody feces, but otherwise, the only indicators of exposure might be decreased bird performance (Ryley, 1980). Using a variety of methods, such as Anticoccidial Sensitivity Tests (ASTs), companies can be proactive in regard to possible drug resistance and potentially switch drugs before isolates become resistant.

ASTs were created to test the efficacy of a panel of drugs on a field strain of Eimeria. ASTs are a well-known technique to assess resistance of Eimeria isolates to a variety of anticoccidial drugs (Chapman, 1998). It is estimated that obtaining government approval may take 7-10 years and over $100 million dollars in the United States (McDougald, 2015). Due to this expense and extensive time line, it is very unlikely that any new anticoccidials will soon reach market. ASTs therefore have allowed companies to test a panel of already approved anticoccidial drugs against field isolates of Eimeria at their respective complexes. The World Association for the Advancement of Veterinary Parasitology has set guidelines for ASTs (Holdsworth et al, 2004). Unlike field exposures where there may be wide variation in oocyst challenge, AST's challenge birds with a given amount of oocysts, which makes measuring sensitivity more accurate. In the field, drugs may appear to be efficacious, but it is rather a low parasite load. The coccidia in a poultry house fluctuates over time, and may not always be a constant level.

Several criteria can be used to test anticoccidial efficacy. Measuring weight gain differences during the acute phase of infection is the most useful way to evaluate anticoccidial drug efficacy (Chapman, 1998). Lesion scores can be used to measure the pathology of coccidial infection. The most widely used lesion score system is the Johnson and Reid method (Johnson and Reid, 1970). Johnson and Reid developed a universal system that describes infections with six specific Eimeria species and severity of lesions. Intestines are grossly examined and scored at

particular locations (upper, mid, lower and ceca). Although the Johnson and Reid method is an excellent tool for research, it does have limitations. The method was developed with pure lab strains, and does not account for mixed field isolates that are used in ASTs.

E. *maxima* for example, is fairly difficult to score grossly, and so microscopic scores have been used as a supplement. Briefly, this method involves analyzing intestinal scrapings from the mid-intestinal region for any life stages of E. maxima. Depending on the number of parasites seen in a field of vision, scores are given accordingly with a 0 having none and a score of 4 having the most.

PCR is another method used to diagnose the species of Eimeria in field isolates. Using species-specific primers, mixed populations can be discerned rapidly and accurately. Prior to the molecular tools, diagnosis depended on location of gross lesions, pre-patent period of oocysts and morphology of oocysts. With mixed populations, gross lesions are not definitive. Determining the pre-patent period of oocysts is labor intensive and can be unreliable because of the overlapping properties of several of species in addition to intra-species variation (Haug et al., 2008). Using the morphology of sporulated oocysts to discern Eimeria species is relatively easy for the major three species (E. acervulina, E. maxima and E. tenella). Other lesser species are much more difficult to discern, because they overlap in size and shape and if any of the species are in low concentrations, they could be easily overlooked in a sample. Because of this, PCR has allowed accurate detection of Eimeria species in field isolates. Results allow insight into the epidemiology of Eimeria as well as potential vaccine species candidates.

Feed intake is also measured during ASTs in order to calculate feed conversion, a component of great importance for the producers' bottom line (Chapman, 1998). Since lesion

scores do not linearly correlate to infection, the weight gain and feed conversion measurements are crucial.

Although researchers differ in opinion of most efficacious indicator of sensitivity, most all agree that at least two methods should be used. Chapman in 1976 for example, used lesion scores, weight gain and oocyst production for measuring drug sensitivity. Reid (1975) described flaws in using oocyst counts for measuring anticoccidial efficacy. In that study, oocyst production was significantly higher in birds given anticoccidials in comparison to un-medicated birds (Reid, 1975). The weights of the birds however, were significantly greater than the un-medicated birds and similar to the uninfected controls, implying the importance of not solely using oocyst production for indicator of anticoccidial efficacy.

An alternative to anticoccidials is vaccination with live coccidia. The vaccines are administered to chicks on day of hatch; through oral ingestion and subsequent infection immunity is developed. Currently vaccines are the only practical alternative to anticoccidial drugs for control of coccidiosis in commercial poultry (Chapman et al. 2002). Birds are protected from infections later in production. In other words, birds should not pass oocysts and clinical signs of coccidiosis are absent. Full immunity is not developed from the first infection, and so vaccines rely on birds being re-infected several times with few oocysts to provide future protection and cause as little intestinal damage as possible (Reid, 1990).

Eimeria do not show cross protection, and so infection and subsequent immunity to *E. tenella* does not result in protection from any of the other species. *E. maxima* has been demonstrated to show antigenic variability and so cross protection immunity is not a definite for particular strains (Smith et al, 2002). E. *maxima* has great antigenic variability, but is highly immunogenic (Tyzzer, 1929). Although different populations exhibit immunological diversity,

the antigenicity appears to be stable with time (Shirley, 1980). Fitz-Coy (1992) showed variation in E. *maxima* pathogenicity from isolates collected across the United States and discussed the potential impact of biological variation in terms of drug response. Martin et al (1997) showed that five E. *maxima* isolates from across the United States, formed 3 distinct immunological groups that could not confer immune protection.

Because of the antigenic variation, some commercial coccidia vaccines have multiple strains of *E. maxima* to provide widespread immune development.

If vaccination is used properly, birds develop immunity to coccidia and resistance to a challenge. This can be observed by a reduction in macroscopic lesions, a decrease in oocyst production and acceptable performance of birds (Peek and Landman, 2011). The severity of coccidiosis in immunologically naïve birds is directly related to the number of infective oocysts that are ingested (Price, 2012). In other words, clinical signs of coccidiosis are a result from thousands of sporulated oocysts ingested whereas subclinical infections result from ingestion of only hundreds of oocysts (Reid, 1990). The low numbers of oocysts administered to birds from vaccines can make optimum cycling for development of immunity difficult (Allen and Fetterer, 2002).

Shown in Table 2.3 are the commercially available coccidia vaccines in the United States.

Name	Bird type	Species included	attenuation
Advent	Broiler	E.ac, E.ma, E.te	Non-attenuated
CocciVac-B	Broiler	E.ac, E.max, E.miv, E.te	Non-attenuated
CocciVac-D	Breeder/layer	E.ac, E.br, E.ma, E.miv, E.ne, E.pr, E.te	Non-attenuated
HatchPak III	Broiler	E. ac, E.ma, E.te	Attenuated
Immucox C1	Broiler	E.ac, E.ma, E.ne,	

		E.te	
Immucox C2	Breeder/layer	E.ac, E.br, E.max,E.miv, E.ne, E.pr, E.te	Non-attenuated
Inovocox (EM-1)	Broiler	E.ac, E.max, E.te	Non-attenuated

E.ac= E. acervulina, E.br= E. brunetti, E.ma=E. maxima, E.mit= E. mitis, E.miv= E. mivati, E.ne= E. necatrix, E. pr= E. praecox, E.te= E. tenella

Table 2.3. Commercially available coccidia vaccines for chickens in United States

There are three main types of vaccines being used in commercial production (Tewari and Maharana, 2011):

1. Vaccines with live virulent strains of coccidia
2. Vaccines with live attenuated strains of coccidia
3. Vaccines with live strains that are tolerant to ionophore compounds- these combine the early chemotherapeutic effect of ionophores as well as long term protection from vaccination

Virulent vaccines are able to induce immunity but a disadvantage is they offer a small margin of safety (Williams, 1999). The “crowding effect” or limit in available host intestinal cells for parasite development, reduces the reproduction of virulent Eimeria if infective doses reach a particular threshold, and subsequently reduces cell-mediated immunity (Williams, 2001). Attenuated parasites however, are better at optimally infecting the host in a way that minimizes intestinal cell damage while still stimulates immunity (Williams, 1998). In broiler production, where birds are harvested at young ages, virulent vaccines are often avoided. The weight loss induced from the vaccine is seldom recovered during the bird’s life (Williams, 1998).

Three methods have been used to attenuate Eimeria (Fetterer et al, 2014):

1. Passage through embryonated chicken eggs
2. Selection of genetically stable precocious coccidia lines

3. Use of gamma irradiation to induce mutations

Precocious lines of Eimeria are the only attenuated coccidia used in commercial vaccines. Fetterer et al (2014) showed that a vaccine with irradiated oocysts conveyed protection to birds that were challenged with Eimeria *acervulina*, *maxima* and *tenella*. In the previous study, irradiated-oocyst vaccinated birds still produced gut lesions post-field challenge. It was not shown that irradiated oocysts provided a significant advantage, but producing irradiated oocysts is quicker than attenuating pathogenic strains via isolating precocious parasites.

Attenuated Eimeria are characterized by precociousness, or a shorter pre-patent period. Attenuation comes from the loss of the second-generation schizont stage and is more superficial in the mucosa (Long, 1973). This characteristic provides several benefits because of the parasites' low reproductive potentials such as minimal tissue damage and crowding, giving rise to optimal immunity (Long, 1973). But a potential issue arises in that the asexual stage of Eimeria have been shown to be the most likely source of antigens capable of inducing protective immunity (Rose and Long, 1980). Shirley and Millard (1986), showed that vaccination with attenuated Eimeria resulted in subsequent immunity to virulent parent strains.

Subunit or recombinant vaccines have been developed and used in laboratory settings, but a major limiting factor has been the small number of antigens that have been isolated capable of inducing sufficient protective immunity (Peek and Landman, 2011). Subunit or recombinant vaccines are different from vaccines using live oocysts. These vaccines use specific antigens from the parasite that are able to induce immunity (Meeusen et al, 2007). One subunit vaccine has been produced commercially, CoxAbic®, which used primarily to protect broiler breeders (Ziomko et al, 2005). A huge limiting factor of spread of the vaccine is the costly and laborious

production (Kitandu, 2006). With 40 billion broilers produced each year for food around the world, a vaccine that is widely accessible and affordable is important.

Determining the keystone protective antigen or antigens has proven difficult, with proteins tested thus far providing various degrees of protective immunity (Allen and Fetterer, 2002). Another aspect that has proven difficult in finding and effective recombinant vaccine is that there are differences in immunity between species (McDonald and Shirley, 2009).

Cycling vaccines with drugs has been shown to reinstate coccidia populations that are sensitive to anticoccidial drugs (Mathis and Broussard, 2006). The exact mechanism of this restoration of drug sensitivity has not been determined but may be a result of drug-sensitive strains having higher reproductive potential and outgrowing resistant strains or interbreeding between the resistant and sensitive vaccines resulting in more sensitive offspring (Peek and Landman, 2011). Unlike vaccination, changing the drugs being administered to a flock has not been shown to reintroduce coccidia sensitivity. The intermittent use of live vaccines can provide a unique benefit by reintroducing drug sensitive Eimeria populations to poultry farms. It has been demonstrated that in a mixed population of sensitive and resistant Eimeria *tenella*, the sensitive parasites tended to dominate in the absence of medication (Long et al, 1985).

Coccidia vaccines are widely used in broiler breeders because developed immunity is crucial in protecting birds kept for long periods of time. Commercial broiler farms have not been as quick to abandon drugs because broilers harvested at very young ages(typically 6-8 weeks) and thus long term immunity isn't as important. However with broilers grown to an older age and turkeys, vaccines are cheaper than in feed anticoccidials until withdrawal. Vaccine administration in mass at the hatchery of chicks soon after they are placed in chick boxes and

before they are delivered to broiler farms has made the use of vaccines more practical in the broiler industry.

It has been shown that oocysts that do no excyst after being ingested by chicks can pass through the feces and remain infective (Williams, 1995). These oocysts can infect another chick, and can promote early uniform infection in broilers. This early immunity is crucial for broilers because of their short grow-out periods (Williams, 2002). "Trickle down infection" has been the term developed to describe this phenomena, where protective immunity is effectively stimulated by small doses of coccidia challenge (Joyner and Norton, 1973, 1976). Use of attenuated Eimeria has also helped to disseminate concerns of adverse reactions post-infection (Chapman et al, 2002). In ovo injection of oocysts into 18-day-old chick embryos has also been used in broilers for vaccination (Weber and Evans, 2003). Whole house brooding is much more efficacious in evenly spreading oocysts, resulting in the best immune development (Williams, 2002, Weber and Evans, 2003). If birds ingest relative equal amounts of oocysts, the flock will show greater flock uniformity in immunity, and subsequently performance. Half-house brooding allows higher numbers of oocysts to be concentrated in one area, thus making birds less likely to ingest equal amounts.

Lesions in vaccinated birds, have few to no endogenous parasites in them, indicating a success in immunization (Williams and Andrews, 2001). Several others have described the presence of lesions in vaccinated birds, but the lack of adverse effects on the birds (Schetters et al, 1999).

Bird performance is the deciding factor in most decisions regarding preventing coccidiosis. Anticoccidial drugs have been shown to improve bird performance as well as providing relatively inexpensive coccidia control. Nevertheless, consumers are increasingly

indicating a preference for “antibiotic free” poultry. Although producers may be able to produce birds at a lower cost with antibiotics, the market may require antibiotic-free production, which places more emphasis and pressure on vaccination and chemical anticoccidials (Cervantes, 2014; 2015). Studies have shown that vaccinated birds do not vary significantly in weight at slaughter compared to medicated birds (Chapman et al, 2002). In some instances after compensatory gain, vaccinated birds have performed better than medicated birds (Mathis, 1999).

Vaccinated flocks in the European Union have also been shown to have lower mortality than those on medication (Williams, 2002). The success of vaccination requires proper management of vaccine administration and housing that is optimized for oocyst cycling. It is not likely that a live vaccine will perform better than a non-ionophore anticoccidial drug, unless there is a drug resistant population of coccidia on the farm (Williams, 2001). On the other hand, vaccinated birds should not perform worse than drug-treated birds if the drugs have no other therapeutic properties i.e. antibiotic properties of ionophores (Williams, 2001). Ionophores have been shown to improve feed conversion ratio (FCR) in disease-free birds (Marusich et al, 1977). That said, ionophores are technically antibiotics and thus there is concern their use may be limited in the future as the trend toward antibiotic free poultry increases. Improvements in availability, costs and administration efficacy of vaccines will allow more broiler producers to use a viable alternative to anticoccidials.

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Chapter 3

Diagnosis of coccidiosis in poultry under field conditions: The use of PCR in a survey to augment conventional clinical methods[1]

1. Kimminau, E.A., Fuller, L.A., and McDougald, L.R. To be submitted to *Avian Diseases*

Summary: To determine the prevalence of Eimeria species in commercial broiler complexes, oocysts were harvested from litter samples. DNA was extracted and species-specific primers were used to test for presence of *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*. Forty-three complexes from across the United States were sampled and species of Eimeria were determined using PCR diagnostics. The prevalence of each was as follows: *E. acervulina* (95%), *E. brunetti* (80%), *E. maxima* (87%), *E. mitis* (54%), *E. necatrix* (26%), *E. praecox* (85%), and *E. tenella* (77%). The seven mentioned species vary in pathogenicity, but have all been shown to cause decrease in performance, and in the case of *E. brunetti* and *E. necatrix*, mortality. The ubiquity of all of the Eimeria species warrants further investigation, as historically *E. acervulina*, *E. maxima* and *E. tenella* were the biggest concern for broiler farmers.

Key words/Index terms: coccidia, Eimeria, United States, PCR

Abbreviations: AST- Anticoccidial Sensitivity Test, PCR- Polymerase Chain Reaction

Introduction:

Diagnosis of Eimeria species has depended largely on biological traits of individual species (7, 8, 18, 24). Due to the site-specific infections, gross lesion scoring (10) has allowed fairly accurate detection of some Eimeria species by veterinarians in the field, but difficulty in accuracy arises in mixed species infections or in subclinical coccidiosis where lesions may not be as pronounced. The size and shape of the sporulated oocyst has also been used to differentiate Eimeria species, but due to extensive overlap in dimensions of several species (16), morphology cannot be used as a sole means of speciation. Pre-patent period has been used in combination with oocyst morphology, but this technique is laborious and time consuming (16).

In contrast to the above methods, polymerase chain reactions (PCR) allow researchers to rapidly and accurately diagnose Eimeria species and eliminate much of the guesswork associated with the similarities of these parasites. PCR techniques utilize species-specific variations in the parasites' genome to discern between the seven major species infecting domestic chickens: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis, E. praecox* and *E. tenella*. Primers complementary to each of the mentioned species are used to anneal and amplify the variable regions, and these amplified fragments can be visualized to detect presence or absence of the Eimeria species targeted by the particular primers (22). PCR has also been used to clarify long-standing issues of speciation among chicken Eimeria (23) and helped coccidiologists understand unique characteristics of turkey Eimeria which facilitate accurate diagnosis (5). Diagnostic and epidemiological studies of Eimeria have become routine using PCR (13, 3, 19, 9) and allow for detection under field conditions where only low numbers of particular species are present. Field surveys utilizing these techniques have been done in several countries including Brazil, Canada, China, India, Japan and Norway (4, 20, 23, 1, 12, 6). However, surveys of this type have never

been conducted in the United States. In fact, the last survey of chicken Eimeria in the United States, was conducted by Jeffers in 1974 (7), a time well before the development of PCR. As a result, Eimeria species compositions in U.S. broiler production facilities have not been evaluated for over forty years. The purpose of this study was to survey the current Eimeria species in broiler complexes in several states across the United States using PCR.

Materials and Methods:

Parasite Collection:

Litter samples were collected from forty-three broiler complexes across the United States and sent to the University of Georgia for conducting Anticoccidial Sensitivity Tests (ASTs). Litter samples from six farms with flocks between three and six weeks old. The complexes were located in: Alabama, Arkansas, California, Georgia, Louisiana, Missouri, Mississippi, North Carolina, Tennessee, Texas and Washington. Samples of litter with fresh excreta were collected from the middle of each house, near the feed lines, and near the water lines, ensuring that the litter collected from each farm was similar in composition as possible.

Upon receipt in the laboratory, litter samples were immediately processed. A full description of the procedure to collect oocysts has been previously described by Long et al (17). Briefly, litter was soaked in water, drained through double-layer cheesecloth for oocyst collection and the oocysts were subsequently passed once through naïve birds. Feces were collected from the birds a week later, cleaned, and oocysts were collected by salt (NaCl) floatation. Fifty mL of oocyst/water solution was centrifuged, supernatant discarded and re-suspended in one mL of water. DNA was extracted from the oocysts/water solution using Zymo© fecal DNA kit, and followed manufacturer's instructions.

PCR:

DNA primers developed by Schnitzler (21) and Lew (13) were used to amplify the ITS-1 region of specific Eimeria species. Components of each PCR reaction: 50 ng of template DNA, 0.5 uM of forward and reverse primers for respective species (Table 3.1) and Ready-to-Go PCR beads (GE scientific) were used to make a 25 uL reaction. Laboratory strains of each species were run as positive controls and were used to optimize annealing temperatures. Eimeria spp. primer set was run with water to test for contamination. The PCR parameters were as follows: 1 cycle- 95 °C, 5 min; 40 cycles- 95°C, 15 sec, 45-65°C, 30 sec, 72°C, 30 sec; 1 cycle- 72°C 15 min. PCR amplicons were separated by gel electrophoresis using 1% agarose gel stained with ethidium bromide and visualized with UV light. GeneRuler^{TM} 1kb Plus DNA Ladder was used to measure band size, and positives were considered when band size matched corresponding species-specific amplicons.

Results

Forty-three broiler complexes were sampled from 2013-2014. Figure 3.1 shows the prevalence of each species in the samples, with *E. acervulina* identified in the most samples (93%). Conversely, *E. necatrix* was found in the fewest number of samples (27%). With the exception of E. *necatrix*, all species were seen in at least 50% of the complexes tested, and *E. necatrix* (27%), was still not infrequent. The other species were seen frequently: *E. brunetti* in 72%, *E. maxima* in 86%, *E. mitis* in 53% and *E. tenella* in 72% of the samples tested.

All samples tested contained atleast two species of Eimeria (Table 3.2). There was only one complex with two species, *E. acervulina* and *E. maxima*. Three complexes (7% of complexes) from Georgia, Mississippi and North Carolina had all seven species. The most frequent combination of Eimeria species (16% of complexes) was: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. praecox* and *E. tenella*. The second most frequent (9% of the complexes) was: *E.*

aceruvlina, *E. brunetti*, *E. maxima*, *E. mitis*, *E. praecox* + *E. tenella*. All other frequencies of species combinations were 2 & 5 %, and covered all geographic areas sampled.

Alabama and Texas had the lowest prevalence of E. *brunetti* (33%), where all other states had prevalence above 50% (Table 3.3).

E. *acervulina*, E. *maxima* and E. *praecox* were the only species seen at the high prevalence (>50%) in each state.

Discussion

This PCR diagnostic survey covering most broiler production states is the largest for commercial complexes in the United States since 1974. However, Jeffers (6) used conventional methods of species identification in his study. More recently in 2009 Schwarz (22), sampled litter from fourteen broiler farms in Arkansas and sixteen from North Carolina to survey Eimeria with PCR and compare genetic composition of the species on a farm with broiler performance (cost of production vs. pound-of-meat-produced). High performance farms were classified by low cost of production versus pound-of-meat-produced and low performance farms were high cost of production versus pound-of-meat-produced. High and low performance farms in North Carolina were found to have: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis, E. praecox* and *E. tenella*. The presence of these six species is in line with what our lab saw in the samples we tested. Arkansas on the other hand had *E. acervulina*, *E, maxima, E. praecox* and *E. tenella* present on both high and low performance farms. *E. mitis* was unique high performance farms and *E. brunetti* to the low performance farms. We did not detect *E. necatrix* on the Arkansas complexes we tested and saw *E. necatrix* in only one complex in North Carolina.

Our study is the largest survey of coccidial species in the Untied States in the last forty years, and represents the first attempt to apply PCR methodology to a production area as broad as the United States.

Eimeria species and complex species composition:

E. aceruvlina, E. maxima and *E. tenella* have been recognized as the most common species in broilers, and are all considered pathogenic. These three are relatively easy to identify in the field because of the gross lesions or with *E. maxima*, the distinct oocyst size. They are also contained in all commerically avaiable broiler vaccines.

E. acervulina was identified in 93% of the complexes sampled, a fact with which was similar to the results of Jeffers (7). *E. maxima* was seen in 86% of the complexes tested, which was also similar to the 1973 Jeffers (7) survey. In 2010, Jenkins (9) showed a *E. maxima* 83% prevalence and 100% for *E. aceruvlina* prevalence in broiler complexes sampled from Arkansas, Delmarva and North Carolina.

Jeffers (7) also observed single species infections with *E. acerulina* and *E. maxima* at 12% and 8% respectively. None of the complexes we tested had single species infection, showing either the increased commonality of Eimeria variation or the increased speciation capabilities that have come with using PCR.

Jeffers (8), described *E. tenella* prevalence at 28% in 1973. Our findings of *E. tenella* (72%), show a drastic change. Jenkins (9) compared species of Eimeria in vaccinated and farms using anticoccidial drugs and observed *E. tenella* only on vaccinated farms. In Arkansas, one of the major broiler producing states, for example, prevalence of *E. tenella* was 16% in 1973 and 86% in 2013/2014 (8). Georgia on the other hand, showed a decrease in prevalence, 45% and 33% respectively (8). Aside from Georgia, all other states showed a prevalence of *E. tenella*

above 50%. Since our findings showed *E. tenella* so frequently, it would seem advantageous to further investigate the prevalence of this pathogenic species in relation to coccidiosis control strategies.

The big three (*E. aceruvlina, E. maxima* and *E. tenella*) are contained in all commericially avaiable broiler vaccines, which may explain the consistant prevalence in *E. aceruvlina* and *E. maxima* and increased prevalence in *E. tenella*.

E. brunetti and *E. necatrix* are both extremely pathogenic species, historically disregarded as a problem to broiler production and considered rare. *E. necatrix* is considered an issue in older birds such as broiler breeders, because of the long pre-patent period.

Jeffers observed 2.3% prevalence of *E. brunetti* in litter from the major broiler-producing regions of the United States. Our survey showed a prevalence of 78% of the pathogenic Eimeria species. Schwarz (22) compared Eimeria species in birds of high and low performance flocks and observed the presence of *E. brunetti* in only low-performing farms. Because of the frequency we observed, monitoring this pathogenic species in relation to broiler performance would be beneficial.

When Jeffers (7) surveyed Georgia broiler farms, the prevalence of *E. necatrix* was 0.4%. Our survey showed a 50% prevalence of *E. necatrix* in Georgia. Jeffers (7) hypothesized the lowered prevalence of the species may be due to the introduction of highly effective broad-spectrum anticoccidials: Bonaid, Coban, Coyden, Deccox and Robenz. The higher frequency of this pathogenic species currently in this particular geographic region warrants further monitoring, especially if the species have decreased sensitivity to the previously mentioned anticoccidials. *E. necatrix* is only included in one commercial vaccine for broilers, which seems unlikely to be the source of the relatively frequent prevalence seen. When Haug (5) surveyed Norwegian

commercial broiler facilities for composition of Eimeria species, *E. necatrix* was found in only one flock, and the birds were harvested at sixty-nine days. It would therefore be useful to investigate the prevalence of this pathogenic species in relation to age of harvested flocks in the United States.

E. praecox and *E. mitis,* unlike the other species, do not cause distinct lesions. Because of this, it has been thought that identification of infection with these species is grossly underestimated. With the availability of molecular diagnosis, recent research has shown a relatively high prevalence of the two species (9).

E. praecox has historically been recognized as a non-pathogenic Eimeria species (14,15). Williams (26) showed that effects of infection with field strains of *E. praecox* were clinically similar to *E. acervulina* and in some instances *E. praecox* was more pathogenic than *E. acervulina*. Due to the prevalence of *E. praecox* (79%) seen in our survey and the proven pathogenicity of the species (2), *E. praecox* could be more of a production problem in the US than previously thought.

E. mitis has been shown to cause significant reduction in performance, which leads to substantial economic loss (11). Despite the proven economic losses, there is a lack of distinct lesions in *E. mitis* infections, which make field diagnoses difficult. Jenkins (9) observed *E. mitis* only at a farm using a live coccidia vaccine. *E. mivati*, a contested species included in the particular commercial vaccine, has been molecularly shown to be a variant of *E. mitis* (22). This may explain the high prevalence of *E. mitis* in the samples tested.

E. brunetti, *E. necatrix* and *E. praecox* are not included in any of commerically available broiler coccidiosis vaccines in the United States, and all were not rare (72%, 27% and 79% prevalence respectively). Due to these speices frequency and lack of inclusion in commerically

available vaccines (with the exception of *E. necatrix* in one vaccine), further investigation in exposure in the field is warranted. Poorer performance associated with commerical vaccine use may be due to the lack of immunity developed for *E. brunetti, E. necatrix* and *E. praecox.* It would seem benefical to measure bird performance when all or the most prevalent (*E. brunetti* and *E. praecox*) are included in a vaccination regime and birds are raised in field conditions with endogenous Eimeria isolates.

In conclusion, using molecular diagnostics have allowed identification of species that may be overlooked in the field. All of the seven major species of Eimeria were frequently seen in the tested complexes. Since only *E. acervulina, E. maxima, E. tenella* and *E. mitis* (variant *E. mivati*) are included in commercial vaccines, it seems of great importance to evaluate inclusion of the other species. Speciating Eimeria populations is a valuable tool and may provide insight to drug sensitivity and a farm's bird performance.

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Table 3.1: Primers used for this survey, including annealing temperatures modified for optimum non-specific amplification using laboratory strains of specific *Eimeria* spp

Species	Forward Primer	Reverse Primer	Annealing Temperature °C	Amplicon Size (bp)
E. spp[a]	AAG TTG CGT AAA TAG AGC CCT C	AGA CAT CCA TTG CTG AAA G	50	Variable
E. acervulina[b]	GGC TTG GAT GAT GTT TGC TG	CGA ACG CAA TAA CAC ACG CT	50	321
E. brunetti[b]	GAT CAG TTT GAG CAA ACC TTC G	TGG TCT TCC GTA CGT CGG AT	45	311
E. maxima[a]	GTG AT/AT CGT TC/TG G/AG/AA GTT TGC	CT/AC ACC ACT CAC AAT GAG GCA C	55	145
E. mitis[a]	GGG TTT ATT TCC TGT CC/GT CGT CTC	GCA AGA GAG AAT CGG AAT GCC	55	328
E. necatrix[b]	TAC ATC CCA ATC TTT GAA TCG	GGC ATA CTA GCT TCG AGC AAC	65	383
E. praecox[a]	CCA AGC GAT TTC ATC ATT/C GG GGA/G	AAA A/GCA A/CAG CGA TTC AAG	45	116
E. tenella[b]	AAT TTA GTC CAT CGC AAC CCT	CGA GCG CTC TGC ATA CGA CA	55	278

a from Lew et al 2003 (13)
b from Schniztler 1998 (20)

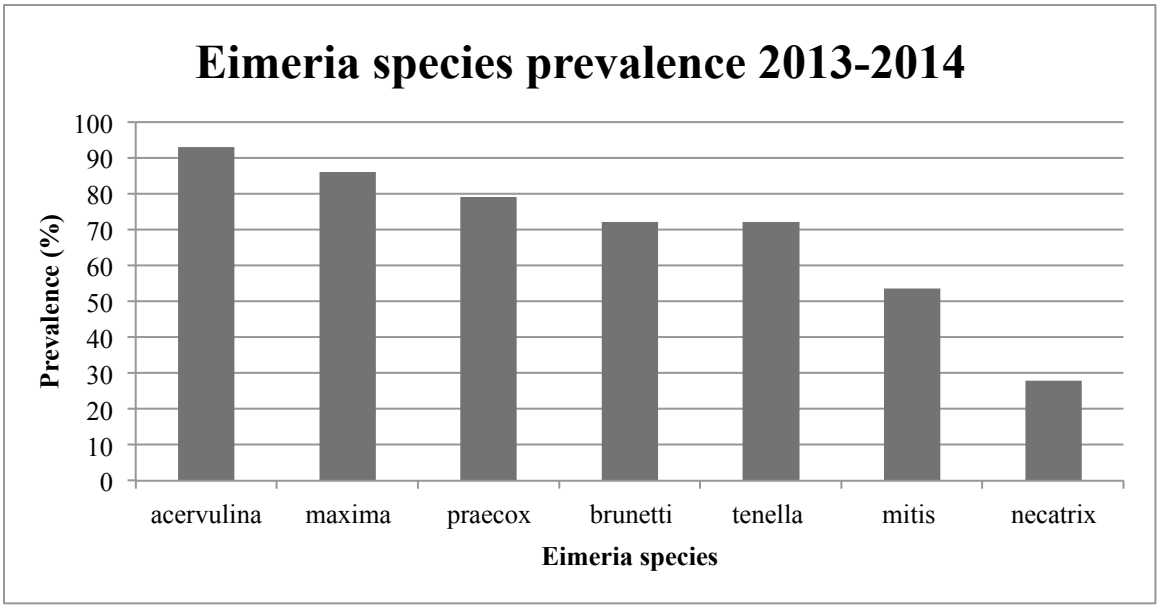

Figure 3.1: The prevalence (%) of Eimeria species from sampled complexes.

Table 3.2: Distribution of Eimeria species combinations from sampled complexes

Number of species identified per complex	Species Combinations	Number of complexes	% of complexes
2	*E. acervulina* + *E. maxima*	1	2
3	*E. acervulina* + *E. maxima* + *E. tenella*	1	2
3	*E. acervulina* + *E. brunetti* + *E. praecox*	1	2
3	*E. maxima* + E. necatrix + *E. tenella*	1	2
3	*E. acervulina* + *E. mitis* + *E. praecox*	2	5
4	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. tenella*	1	2
4	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. necatrix*	1	2
4	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. praecox*	2	5
4	*E. acervulina* + *E. maxima* + *E. praecox* + *E. tenella*	2	5
5	*E. acervulina* + *E. brunetti* + *E. necatrix* + *E. praecox* + *E. tenella*	1	2
5	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. mitis* + *E. necatrix*	2	5
5	*E. brunetti* + *E. maxima* + *E. mitis* + *E.*	2	5

	praecox + *E. tenella*		
5	*E. acervulina* + *E. maxima* + *E. necatrix* + *E. praecox* + *E. tenella*	2	5
5	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. mitis* + *E. praecox*	2	5
5	*E. acervulina* + *E. maxima* + *E. mitis* + E. praecox + *E. tenella*	2	5
5	*E. acervulina* + *E. brunetti* + *E. mitis* + *E. praecox* + *E. tenella*	2	5
5	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. mitis* + *E. tenella*	2	5
5	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. praecox* + *E. tenella*	7	16
6	*E. acervulina* + *E. maxima* + *E. mitis* + *E. necatrix* + *E. praecox* + *E. tenella*	1	2
6	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. mitis* + *E. necatrix* + *E. praecox*	1	2
6	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. mitis* + *E. praecox* + *E. tenella*	4	9
7	*E. acervulina* + *E. brunetti* + *E. maxima* + *E. mitis* + *E. necatrix* + *E. praecox* + *E. tenella*	3	7

Table 3.3: The prevalence (%) of Eimeria at respective locations.

Location	# of complexes	*E. acervulina*	*E. brunetti*	*E. maxima*	*E. mitis*	*E. necatrix*	*E. praecox*	*E. tenella*
Alabama	6	83%	33%	83%	67%	50%	67%	50%
Arkansas[A]	7	100%	100%	86%	29%	0%	71%	86%
California[B]	3	100%	67%	100%	67%	0%	67%	67%
Georgia	6	100%	83%	83%	67%	50%	67%	33%
Mississippi	13	84%	77%	85%	62%	23%	92%	85%
North Carolina	2	100%	50%	100%	50%	50%	100%	100%
Tennessee	3	100%	100%	100%	67%	0%	67%	100%
Texas	3	100%	33%	67%	0%	67%	100%	67%

Only one sample was collected from [A]Missouri and so was combined with Arkansas.

[B]Washington was combined with California.

Chapter 4

Research Note- Eimeria species identified from two broiler complexes

1. Kimminau, E.A., Fuller, L.A., and McDougald, L.R. To be submitted to *Avian Diseases*

Summary:

Broilers from two complexes consisting of ten farms were necropsied and fecal material was collected from the bird. Oocysts were harvested from individual birds and diagnostic polymerase chain reaction (PCR) was run to determine the species of Eimeria from each farm.

Introduction:

Coccidiosis, caused by Eimeria parasites, is one of the most expensive and common diseases in poultry production (9). Subclinical coccidiosis, the more common form of the disease, can be more difficult to diagnose (2, 3). Diagnosis can be through a variety of ways such as oocyst morphology, pre-patent period and gross intestinal lesions (8). These biological traits have been used to identify Eimeria populations in the field, but are extremely laborious and time-consuming. Frequently veterinarians sample broilers from farms, grossly and microscopically examine the intestines for coccidia. PCR diagnosis in concert with field necropsies would allow accurate detection of Eimeria species. Identifying Eimeria species cycling through the grow-out period is important to understanding the epidemiology of the parasites. Particular presence of species may elucidate bird performance (16) or drug sensitivity (8). Eimeria are seldom found as single-species populations on commercial farms, and because of this, the combination of species may vary the pathogenicity of one another (5, 8, 9, 14). This interaction between the species is important for the field application, and may be contributing to decreases in flock performance.

Research Note:

The two complexes examined were on the same anticoccidial program: nicarbazin for starter, nicarbazin for grower and diclazuril for finisher diets. Birds were necropsied for routine flock monitoring and fecal material from individual birds was collected and sent to the University of Georgia. Samples were processed immediately upon arrival.

The fecal contents were soaked in water over night. Contents were centrifuged and the supernatant discarded. Remaining material was floated in a saturated salt (NaCl) solution. Oocysts were removed from the supernatant, were used for DNA extraction using the Zymo Fecal kit © per manufacturer' instructions. Polymerase Chain Reaction (PCR) was used to amplify extracted DNA. Previously developed Eimeria specific primers were used (7) and (11). PCR reactions comprised of Ready-to-go PCR beads (GE Scientific, Piscataway, New Jersey), 20 pM of Eimeria specific primers and 50 ng of template DNA to a final reaction of 25 ul. Thermocycler parameters were as follows: 1 cycle- 95 °C, 5 min; 40 cycles- 95°C, 15 sec, 45-65°C, 30 sec, 72°C, 30 sec; 1 cycle- 72°C 15 min. PCR amplicons were separated by gel electrophoresis using 1% agarose gel stained with ethidium bromide and visualized with UV light. A GeneRuler^{TM} 1kb Plus DNA Ladder was used to measure band size, and positives were considered when band size matched corresponding species-specific amplicons. Results from the two complexes are shown in Table 4.1 and Table 4.2 respectively.

With the exception of E. *necatrix* which was not seen in any of the birds, all other species were detected in >50% of the birds. E. *acervulina* and E. *praecox* were seen in 100% of the birds. E. *mitis* was the least frequent and was still seen in 60% of the samples.

Discussion:

Litter samples have been tested with PCR diagnostics by Jenkins et al (8), but data from individual birds through out the grow-out period is lacking. Oocysts counts in feces are higher than litter, and may be a more sensitive indicator of Eimeria epidemiology on farms (Hodgson). Data from birds is important because it can help establish specific cycling times and oocyst output. It can also give insight to the variability of species seen under different anticoccidial programs.

Six Eimeria species that all cause negative effects on bird performance were identified in both complexes. Several of these species may be over looked in the field, but never the less cause depression in performance.

E. acervulina, *E. maxima* and *E. tenella* have been of greatest interest in broiler production. These species are all considered pathogenic, cause economic losses to producers and as a result are in all commercially available vaccines. The other three Eimeria may have been overlooked in the past because of difficulty in lesion scoring or low parasite number. With the advent of molecular diagnosing, these lesser species have been identified more frequently in the field.

Allen (1) and Williams (19) have shown *E. praecox* to cause significant weight loss in birds, and in some cases, more pathogenic than *E. acervulina*, a species included in all commercially available vaccines. *E. praecox* has also been shown to significantly increase the negative effects of *E. acervulina* infections (10). In both complexes the two species were seen in 100% of the birds and as a consequence may be causing reduction in performance.

E. *mitis*, was identified in 50 and 60% of the farms tested from each complex. It has been shown to be pathogenic and cause significant adverse effects on performance (2). Using

molecular analysis, *E. mivati* and *E. mitis* have been shown to be one species (12). Due to this close phylogenetic relationship, accurate detection of *E. mivati* has not been possible using PCR.

Light infection with *E. Brunetti* has been shown weight loss and poor feed conversion in infected birds, and gross lesions can be difficult to diagnose (9). Because of the high prevalence, 80% and 100% in the two complexes, future monitoring is warranted.

Because of the great genetic variability of *E. maxima*, there arises a potential for false negatives (Kumar 2014). Since *E. maxima* can be characterized by its distinctly large size and yellow color, it is important to microscopically look at oocysts, lesions and PCR to effectively determine presence in a sample.

Monitoring Eimeria species in farms is of interest to producers to help evaluate anticoccidial programs. *E. mitis* and *E. praecox* for example do not cause distinct gross lesions, and as a result are more difficult to diagnose. Using PCR allows for detection of Eimeria that may be over looked in the field. PCR in concert with farm necropsies can more accurately diagnose subclinical coccidiosis and identify all species of Eimeria that cause performance losses.

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Table 4.1: Complex 1: Identification of E. species from flocks of respective ages

Flock Age	E. acervulina	E. brunetti	E. maxima	E. mitis	E. necatrix	E. praecox	E. tenella
21 d	X			X		X	X
26 d	X	X	X			X	X
28 d	X	X	X			X	
32 d	X	X	X	X		X	X
36 d	X	X	X	X		X	X
39 d	X	X	X			X	X
43 d	X	X	X	X		X	X
46 d	X		X			X	X
49 d	X	X		X		X	
53 d	X	X	X	X		X	X

Table 4.2: Complex 2: Identification of E. species from flocks of respective ages

Bird Age	E. acervulina	E. brunetti	E. maxima	E. mitis	E. necatrix	E. praecox	E. tenella
18 d	X	X				X	X
21 d	X	X	X			X	X
25 d	X	X	X	X		X	X
28 d	X	X		X		X	X
35 d	X	X	X	X		X	X
39 d	X	X	X			X	X
42 d	X	X	X			X	X
46 d	X	X	X	X		X	X
49 d	X	X	X	X		X	X
53 d	X	X	X	X		X	X

Chapter 5

Eimeria maxima Variability

Introduction:

Eimeria maxima, one the Eimeria species known to infect chickens, is of significant importance to broiler production and included in all commercially available coccidia vaccines. The parasite was historically considered relatively non-pathogenic because of the life cycles taking place in superficial intestinal cells and the ability to develop rapid immunity (11). These characteristics have actually been shown to cause serious losses. The development of the parasite in the superficial intestinal cells pre-disposes birds to subsequent infections of *Clostridium perfringens*, the causative agent of necrotic enteritis (12). It is known that *E. maxima* lesions are seen during the sexual development of the relatively large parasite in epithelial cells, and this stage induces pathogenicity (3, 9, 14). Although immunity can develop rapidly with infection as few as 100 oocysts (2), species-wide protection has been shown to be difficult to obtain. Because of the high immunovariability (1), several distinct strains of *E. maxima* are included in a single vaccine.

With the great variability of the *E. maxima* seen in vaccines and the field, developing reliable ways to accurately measure infections in the field is of the upmost importance. Because of the difficulty that can arise in gross-lesion scoring *E. maxima* (5), microscopic lesion scoring has been offered as a supplement to diagnosis. Goodwin et al (4) showed that microscopic oocyst counts of *E. maxima* detection was the most efficacious for parasite detection in the field in comparison with classical gross lesion scoring. Idiris et al (7) used laboratory strains of *E. maxima* to quantify pathogenicity, but little has been done to measure microscopic lesion scores

of a variety of *E. maxima* isolates and bird performance. Determination of the economic impacts resulting from microscopic scores is important to understanding the impact of *E. maxima* infections. The goal of this project was to investigate the variation in microscopic oocyst counts and performance parameters when infected with different *E. maxima* isolates.

Materials and Methods:

Parasites: E. *maxima* were collected from field isolates and several commercial vaccines. Coccidia free chickens were infected with single oocysts, and positive infections were collected and propagated through naïve chickens. Oocysts collected from feces were harvested through NaCl-floatation method as previously described by (8).

Each line was tested with species-specific PCR primers to confirm the purity of each sample and ensure the presence of *E. maxima*.

Chickens: Male Cobb 500 chicks were obtained on day of hatch from a Cobb hatchery in Cleveland, Georgia. They were placed in coccidia free wire floor cages and raised there for 10 days with ad libitum non-medicated feed and water. Birds were then placed in sterilized batteries.

Experiment 1-

Five coccidia free chickens per *E. maxima* isolate were infected with 5,000 oocysts. Starting 100 hours post infection, feces were collected for each battery. Oocyst concentrations were measured by methods previously described by (8). Briefly, ten grams of feces from each isolate dissolved in 40 mL of saturated salt solution. The solutions were transferred to a McMasters chamber for counting.

Calculations were as follows: $(y \times 6.67) \times V \times \text{dilution}$

Where y = the number of oocysts counted in four columns

V= the volume of the mixture= 40 mL

Dilution = 4

Experiment 2-

10 coccidia free chickens per *E. maxima* isolate were individually weighed, wing-banded and infected with 5,000 oocysts. Birds were weighed every two days until 7 days PI when the study was terminated. Feed consumption was measured for feed conversion. Birds were then sacrificed by cervical dislocation. Intestines were examined for gross lesions of *E. maxima* according to the Johnson and Reid method (5). Scrapings were taken from the mid-intestine, by the Meckel's diverticulum and placed on a slide. The slide was analyzed for any parasite life stages at X 10 objective. The following measurements were assigned to the corresponding scores: 0= no oocysts, 1= 0-20 oocysts per field, 2=21-50 oocysts per field, 3=51-100 oocysts per field and 4 – too numerous to count per field.

Results:

Experiment 1: Overall oocyst outputs per bird and the isolate reproductive index for each of the trials are shown in table 6.1. Reproductive indexes were calculated by: oocysts produced by bird / oocysts input (5,000/bird). Figure 5.1 and 5.2 show a graphical depiction of the oocyst output between the different strains for trial 1 and 2 respectively.

Experiment 2: Comparisons were made of means of weight gain (D0-7) and FCR using Student t-tests on each isolate using JMP 11, results are shown in Table 5.2 and 5.3 for Trial 1 and 2 respectively. For the First experiment, the feed conversions of the uninfected controls were significant ($p<0.05$). Least squares were run to see if the micro or gross lesions were able to predict the weight gain. Except for Isolate 3 micro-score predicting weight gain, there were no other significant correlations (Table 5.3).

Discussion:

Experiment 1: The second peak of oocysts shed was not common to previous work. Al-Badri and Barta (2) measured the pre patent period and shedding patterns of two laboratory strains of E. maxima. Both laboratory strains had a pre-patent period of 120-126 hours, which was earlier than our isolates with 128-134 hours. A repeat of the study is therefore required, and extra precautions to prevent oocyst contamination and re-infection will be taken.

Experiment 2: The lack of correlation between gross lesions and weight gain has been shown previously. Because microscores are more accurate to indicate E. maxima presence, we want to further investigate E. maxima infections at different levels to see if microscores vary. For veterinarians and producers to have the most accurate measure of E. maxima infection and decreased bird production, it seems beneficial to reevaluate the micro-score system.Idris et al. (6) showed that the greatest weight difference after E. maxima infection was after day 7, E. maxima doesn't usually cause the greatest weight reduction compared to the other coccidia- we see this, the weights for one isolate for example wasn't significant from the controls. Stephens et al showed significant weight gain differences on male chicks weren't seen until day 6 post infection and the greatest differences were seen day 10-12 and then the weights became more similar.- this study however infected birds with 50,000 oocysts where we only used 5,000 and 15,000. E.maxima causes more of the damage for paw discoloration and tendency for necrotic entertitis. Although E.maxima is the most immugenic, the great genetic variability make protection not as simple.

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Table 5.1 Oocyst output per individual birds over the allotted time

Isolate	OPG/ bird
Isolate 1	2.6×10^5
Isolate 2	1.24×10^5
Isolate 3	3.15×10^5
Vaccine 1	1.79×10^4
Vaccine 2	6.78×10^4

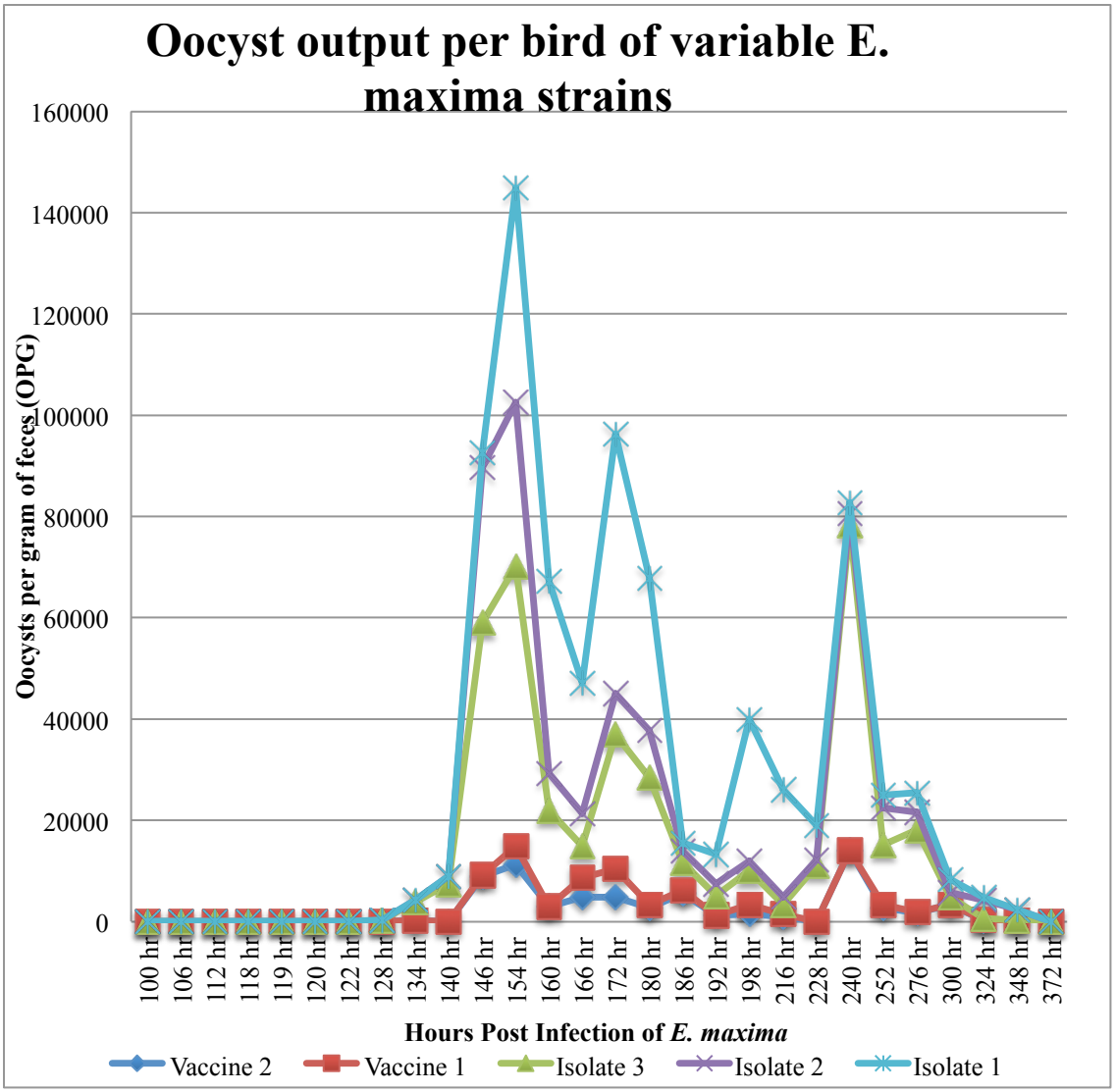

Figure 5.1 – Oocyst output per bird of E. maxima strains

Table 5.2 Means of FCR and Isolate

Isolate	Mean FCR (D0-7)	Mean Weight gain (D0-7)
Un-infected	1.44^{C}	313.7^{A}
1	1.58^{BC}	285.7^{A}
2	2.01^{A}	170.9^{D}
3	1.78^{ABC}	229.2^{BC}
Vaccine 1	1.82^{ABC}	266.1^{AB}
Vaccine 2	1.97^{AB}	209.0^{CD}

Table 5.3 – Correlation between Isolate and Micro-score- Fit Least Squares First Study

Isolate	Microscore/weight gain
1	0.7550
2	0.8225
3	**0.0215**
Vaccine 1	0.8837
Vaccine 2	0.3044

Chapter 6

Conclusion

This study was done to develop a better understanding of the Eimeria species currently infecting broiler facilities. The use of PCR has allowed for rapid and accurate detection and provides a useful tool for profiling the species on a farm within a complex. In concert with traditional species identification, PCR offers a unique tool to monitor Eimeria epidemiology. The *E. maxima* results show the variability of the species causing variable micro and gross-lesion scores and potentially confounding results. This variation must be considered when analyzing infections and performance. The late peak in *E. maxima* seen in the study is also something to investigate and testing those oocysts compared to the early oocysts may shed light into host immunity.

Anticoccidial drugs have been important to the poultry industry in regard to the success it enjoys today. Customer preference shifts toward antibiotic and drug free animal production will likely make vaccine use more prevalent in the future. From our survey, the species of vaccines are not the only ones in the field. The other species may be contributing to the suboptimal performance seen with vaccination. As a result, research into the biology of Eimeria and species currently in broiler complexes may contribute to vaccine improvement.