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Development of a Turkey Coccidiosis Vaccine Candidate: an Attenuated Line of the Protozoan Parasite, Eimeria meleagrimitis

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by

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ABSTRACT

Coccidiosis is an economically important enteric disease of poultry caused by protozoan parasites belonging to the genus *Eimeria*. Drugs are most commonly used to control coccidiosis. Widespread usage of anticoccidial drugs in the field has resulted in development of drug resistant strains and consequently decreased drug efficacy. An alternative control strategy using attenuated vaccines is desired. A series of experiments was designed to identify, isolate and attenuate a strain of a common and pathogenic species of *Eimeria* of turkeys, *E. meleagrimitis*.

Sensitivity of field isolates of turkey coccidia to the anticoccidials drugs amprolium, clopidol, diclazuril, and monensin was investigated. A total of thirty-four isolates comprising mixed species from farms across the country were tested. Of these, four isolates were sensitive to monensin, twelve sensitive to diclazuril, and seventeen sensitive to clopidol and none to amprolium. One isolate, designated MN2, was sensitive to three drugs tested and was selected for further investigation. The different *Eimeria* species present in the isolates were identified using polymerase chain reaction amplification of cox-1 gene and *E. meleagrimitis* was the most common species seen. A pure clone of *E. meleagrimitis* was developed and the pathogenicity and fecundity of the isolated *E. meleagrimitis* strain was tested and compared to a standard reference *E. meleagrimitis* strain. Data from the parameters tested showed that the strain had the same characteristics as that of the reference strain.

An attenuated line of *E. meleagrimitis* was developed by repeated propagation of the parasite in turkeys and collection of the very first oocysts produced following infection. After twenty generations of selection, a line with abbreviated life cycle of -16h was developed. Pathogenicity and immunogenicity of this line was tested and compared with the parent strain.

The attenuated line was proven to be non-pathogenic but was immunogenic. The developed line is a candidate for attenuated vaccine against turkey coccidiosis.

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DEDICATION

To my Dad,

A. Rathinam (1942-2005)

.

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INTRODUCTION

Coccidiosis is a disease of high economic importance infecting young turkeys. In most cases the losses are not due to mortality, but a low level infection which may result in reduced feed intake, lower body weights and consequently poor feed efficiency. The disease is currently controlled by prophylactic use of medication mixed into the feed. Several studies have shown that the parasites have developed resistance to all the drugs available in the market. Another method of control is by vaccinating the birds at early age, usually at hatch, with small doses of live oocysts which are sprayed on to the birds or incorporated in gels. A caveat in this method, however, is that there is a possibility of outbreak of clinical coccidiosis due to the virulent nature of the oocysts given. It would be desirable to have an attenuated vaccine which will contain strains that had lost their pathogenicity but maintain their immunogenicity. However, there are no such vaccines available for turkey coccidiosis.

Eimeria meleagrimitis is one of the most common and most pathogenic species of *Eimeria* infecting turkeys. The objective of this project was to identify a drug-sensitive strain of *E. meleagrimitis* and attenuate it by selection for precociousness. The dissertation is divided into five chapters:

Chapter I: Literature Review

Chapter II: Sensitivity of isolates of *Eimeria* from turkey flocks to the anticoccidials drugs Amprolium, Clopidol, Diclazuril, and Monensin

Chapter III: Molecular Detection of Field Isolates of Turkey *Eimeria* by Polymerase Chain Reaction Amplification of Cytochrome c Oxidase I Gene

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Chapter V: Attenuation of a drug-sensitive strain of a turkey protozoan parasite *Eimeria meleagrimitis* by precocious selection

CHAPTER I

REVIEW OF THE LITERATURE

INTRODUCTION

Coccidiosis is an enteric disease of high economic importance in poultry. It is caused by *Eimeria* spp., a group of protozoan parasites belonging to the phylum Apicomplexa. The organisms are present in almost every intensively reared poultry farm in the world. The disease is difficult to eradicate due to at least three factors: (1) the organism's resistant transmission stage of the life cycle (oocyst) that can withstand severe environmental conditions (Belli et al., 2006); (2) the high rate of multiplication in the intestine of the host (Levine, 1985) and (3) the environmental conditions in intensively reared poultry houses, namely moisture, temperature and high humidity (Williams, 1998).

The cost of coccidiosis to the chicken industry worldwide has been estimated as 3 billion dollars per annum (Williams, 1999). Coccidiosis is also a problem in the turkey industry due to intensification of production (Clarkson and Gentles, 1958; Joyner, 1978). Although the economics of turkey coccidiosis has not been calculated it is likely to be millions of dollars (Chapman, 2008) and most of this expenditure comes from the prophylactic use of anticoccidial drugs that involves inclusion of these agents in the feed. Anticoccidial drug resistance has developed to all anticoccidial drugs introduced for use in chickens and turkeys (Chapman, 1984, 1993, 1997; Chapman and Rathinam, 2007). Vaccination with small doses of live oocysts has also been carried out to control coccidiosis. Live vaccines available currently are either of wild-type origin or have been attenuated by selection for precocious development. Such attenuated vaccines incorporate species of *Eimeria* that have been undergone such selection and have reduced pathogenicity without losing immunogenicity (Williams, 2002a). Although attenuated vaccines are available for chickens, they have not been developed for the turkey. The objective

of this study was to identify a suitable isolate of a widespread species of *Eimeria* from the turkey, *E. meleagrimitis*, to characterize this parasite, and to attempt to attenuate it by precocious selection.

HISTORY AND TAXONOMY OF *EIMERIA*

The first recorded account of a parasite that may have been *Eimeria* was the discovery of "bodies" in the bile of rabbits by Antoni van Leeuwenhoek (1674). Two centuries later, these organisms were shown to be the rabbit species *Eimeria steidai* (Lindemann, 1865). In the last quarter of the 19th century, several researchers made independent discoveries of *Eimeria* by visualizing organisms in histological sections, observation of segmented cysts in feces (subsequently shown to be oocysts) and the presence of gross lesions in parasitized intestines. Initially, there was confusion in classifying the parasites observed in these early reports. Schneider (1875) named the genus *Eimeria* after Theodor Eimer (1843-1898) as organisms comprising an oocyst containing four sporocysts each with two sporozoites. A few years later, Leuckart (1879) coined a generic term "Coccidium" for these organisms. Labbe (1896) placed oocysts obtained from mice in the genus *Coccidium* and endogenous stages of the parasite observed in the intestines (later known as schizonts) in the genus *Eimeria*. A clearer picture was presented in 1900 when Schaudinn described the life cycle of a parasite obtained from a centipede. Based on his work, it was evident that organisms classified in two genera (*Coccidium* and *Eimeria*) based on their different morphology were in fact one and the same. Thus, they were reclassified into one genus, and by age of discovery, and precedence *Eimeria* Schneider 1875 replaced *Coccidium*. The current taxonomic classification of *Eimeria* sp. is given in Table

1.

Avian Eimeria

Credit for describing *Eimeria* in birds belongs to Rivolta and Silvestrini (1873) who identified a species they named *E. avium*, but Tyzzer (1929) argued that nowhere in their Italian text does the name "avium" appear. Railliet and Lucet (1891) first named a species isolated from the ceca of a chicken as *Coccidium tenellum*, which they later renamed *Eimeria tenella*. Smith (1895) was the first to report coccidiosis in the turkey. Later, in 1916, he stated that the coccidium in turkey resembled neither of the two known species of *Eimeria* of chicken at that time, and regarded it as a foreign species (Smith, 1916). Johnson (1923) reported that *Eimeria* of turkey resembled more of *E. steidei* seen in rabbits than *Eimeria* of chicken based on morphology of the oocysts. Johnson (1923/24) mentioned the probability of the existence of more than one species of *Eimeria* in the chicken based on oocyst morphology. All such hypotheses preceded the pioneering work of Ernest Tyzzer who studied the *Eimeria* of gallinaceous birds. He described 6 of the 14 known species of *Eimeria* in chicken and turkeys including *E. meleagrimitis*.

LIFE CYCLE

Eimeria is a parasite with a monoxenous (one host) and stenoxenous (one species of host) life cycle. It is predominantly a gastrointestinal parasite and is transmitted via the oro-fecal route. A typical life cycle of *Eimeria* starts with the host passing *Eimeria* oocysts in feces. These are unsporulated and are non-infective. A diagrammatic illustration of the life cycle of a typical

Eimeria species is shown in Fig. 1.

The first phase of the life cycle is sporogony, in which unsporulated oocysts undergo sporulation in the environment. Under ideal conditions (moisture, oxygen and temperature), they become sporulated oocysts and are then potentially infective (Williams, 1998). The sporulated oocyst contains four sporocysts, each containing two motile sporozoites. The life cycle proceeds when the infective oocyst is ingested by a suitable host. Upon reaching the gizzard the oocysts are subjected to mechanical grinding which releases the sporocysts (Farr and Doran, 1962). The sporocysts pass into the duodenum and are exposed to protease enzymes (trypsin, chymotrypsin), and bile salts (Chapman, 1978). The sporozoites are released from sporocysts (termed excystation) and then penetrate a host epithelial cell. Complex molecular mechanisms are involved in the recognition of host cells and penetration of the host cell membrane (Chapman et al., 2013).

Once inside an epithelial cell, a parasitophorous vacuole is formed around the sporozoite (Shi et al., 2009). The sporozoite enlarges, loses organelles involved in cell penetration, and becomes a life cycle stage known as a schizont. Nuclear division and cytokinesis then occur within the schizont to produce numerous motile stages known as merozoites. The number of merozoites produced varies depending upon the species of *Eimeria*. After the schizont matures, it ruptures and the merozoites are released; in the process killing the host epithelial cell. The merozoites then penetrate new host cells and the process of schizogony is repeated for several successive generations (usually 3-4, depending upon the species).

The final generation of merozoites penetrate epithelial cells and develop into either a macro or microgametocyte, a process known as gametogony. Microgametocytes undergo yet further division to produce many microgametes. The microgametes leave the host cell and

penetrate a mature macrogametocyte. This process of fertilization results in formation of a zygote. A thin layer is formed around the zygote, which is followed by the formation of a resistant oocyst wall (Belli et al., 2006). The host cell ruptures and the oocyst is excreted in the feces.

SPECIES IDENTIFICATION

Traditional methods

Various biological characters and structural features of the organism are used to identify and differentiate species of *Eimeria*. Traditional methods of identifying coccidia involve a combination of size and shape of oocysts and characteristic lesions seen in the host upon necropsy. However, due to the overlap in characteristics of different *Eimeria* species in chicken (Joyner and Long, 1974) and turkeys (Clarkson, 1960), the possibility of an inaccurate diagnosis cannot be ruled out. This is an important concern in turkey coccidiosis where there is a wide range in morphometry of oocysts and the lesions produced are not as distinct as seen in the chicken (Clarkson, 1960).

Biochemical and molecular methods

Starch Gel Electrophoresis. One of the early molecular techniques for the diagnosis and speciation of *Eimeria* was starch gel electrophoresis, a biochemical method utilizing electrophoretic properties of enzymes present in the parasite. Lactate dehydrogenase and

glucose phosphate isomerase were the most widely used enzymes to differentiate species (Rollinson, 1975; Shirley, 1975). This method has been superseded by more advanced procedures utilizing molecular characteristics of *Eimeria* parasites.

DNA Hybridization. This was the first true molecular method used to identify different species of *Eimeria* in chickens. Genomic DNA obtained from the parasite was digested using restriction enzymes, separated, blotted and hybridized using specific probes (Shirley, 1994). Apart from the need for large number of oocysts, and the time and labor involved, one limitation of the procedure is that mixed species show overlapping bands that cannot be separated (Chapman et al., 2013).

Polymerase Chain Reaction (PCR). Polymerase chain reaction is a widely used method in molecular biology that is used in various applications. The principle involves amplification of a small copy of DNA into several orders of magnitude by making multiple copies of the DNA fragment. In avian coccidiosis, the method was first utilized to identify *E. tenella* by amplifying its 5S tract of ribosomal DNA (rDNA) (Stucki et al., 1993). This paved the way for several studies involving development of assays to identify *Eimeria* using other regions of rDNA. The variability of Internal Transcribed Spacer (ITS) region of rDNA due to insertion, deletion and point mutations are used in identifying species-specific variations. Both ITS-1 (Schnitzler et al., 1998; Lew et al. 2003; Haug et al., 2007) and ITS-2 (Woods et al., 2000; Gasser et al., 2005) regions have been used as targets to identify all species of chicken *Eimeria*. Cook et al. (2010) developed an assay to identify turkey *Eimeria* targeting their ITS-1. Both ITS-1 and ITS-2 have undergone sufficient divergence to be a target for species-specific primer design (Lew et al.,

2003). In spite of this, intragenomic and intraspecific variation may limit its usage (Chapman, 2013). Cytochrome c oxidase subunit I (COI) gene, a mitochondrial gene, is now considered a much more reliable target for molecular species identification owing to its modest intraspecific variation, but sufficient DNA sequence variability strong enough for species identification (Ogedengbe, 2011).

A variation in the above-mentioned PCR method is called 'multiplex PCR' where multiple primers can be amplified in a single reaction tube. This allows identification of more than one species in one reaction mixture, thus minimizing reagents cost and the quantity of genomic DNA required. Recently, a multiplex PCR based on ITS-1 on a single assay for four species of chicken *Eimeria* has also been developed (You, 2014). Both the methods mentioned above are qualitative methods and do not quantify the amount of the specific species in a mixture. Quantitative PCR (qPCR) addresses this issue by calculating the expression of the target gene as an absolute value based on a calibration curve or a relative value (fold change). Quantitative PCR assay has been developed to identify species of chicken *Eimeria* using the ITS-1 (Kawahara et al., 2008) and ITS-2 regions as target (Kirkpatrick et al., 2009).

CHEMOPROPHYLAXIS AND DRUG RESISTANCE

Inclusion of anticoccidial drugs in the feed (prophylaxis) is the most widely used method for the control of coccidiosis (Chapman et al., 2013). There is a long history of the use of drugs for this purpose. The antimicrobial of choice during World War II belonged to the sulfonamide family and one of these, sulfanilamide, was shown to be effective against *E. tenella* (Levine, 1939). Several other drugs were discovered shortly after, and were used to treat the disease following

outbreaks of coccidiosis. Another sulfonamide, sulfaquinoxaline, was shown to control coccidiosis when it was included continuously in the feed (Grumbles et al., 1948). This was the first demonstration of the use of drugs for the prevention of the disease (prophylaxis) rather than the treatment of clinical coccidiosis.

Nicarbazin, an equimolar complex of dinitrocarbanilide and hydroxydimethyl pyrimidine, was introduced in 1955 (Cuckler et al., 1955) and was the first true 'broad spectrum' anticoccidial to be introduced (McDougald, 1982). The mode of action is unknown but could be possibly inhibition of oxidative phosphorylation in the target organism (Wang, 1978). Despite having several side effects such as growth depression and excessive mortality due to heat stress, it is still used today as an anticoccidial since resistance to this drug appears to develop slowly (McDougald, 1982).

Just as new drugs were being developed and discovered, resistance to previously introduced drugs started emerging in the field. Cuckler and Malanga (1955) reported resistance to nitrofurazone, which was introduced less than a decade earlier.

Amprolium was introduced in 1960 and was the most popular anticoccidial for about 10 years (Chapman, 2014). The mode of action involves inhibition of thiamine uptake by second generation schizonts (James, 1980). The first report of resistance in the field to amprolium appeared just four years after its introduction (Chapman, 1997).

Clopidol, a drug belonging to a group of compounds called pyridones, was introduced in 1966. Its chemical structure is similar to another chemical group called the quinolones, and the mode of action is thought to be directed against the same metabolic pathway (Long, 1993). Quinolones are known to inhibit cellular respiration of the sporozoite by blocking mitochondrial

electron transport (Wang, 1976). Like amprolium, resistance to clopidol developed in the field within a short span of three years (Chapman, 1997).

A major breakthrough in coccidiosis control occurred in 1967 when a new class of drugs, the ionophorous antibiotics (ionophores) were discovered. Unlike the synthetic chemical drugs used until then, this group included fermentation byproducts of a bacterium *Streptomyces*. Although first isolated in 1951 (Berger et al., 1951), their use as anticoccidials was not recognized until more than a decade later when one of the drugs from this group, monensin, was shown to have an anticoccidial effect (Schumard and Callender, 1967). Monensin has the ability to enable movement of ions across cell membranes thus causing an increase in sodium ion influx into the sporozoite. This increase in sodium ion stimulates osmosis and allows water to enter into the sporozoite and consequently due to excessive water, the sporozoite swells and bursts (Chapman et al., 2010). Monensin was introduced in 1971 and since then has been the most used drug to control coccidiosis.

Resistance to monensin was relatively slow to develop compared to other drugs. Field isolates collected over a four-year period were tested for monensin resistance. Of the total 256 samples tested, only 2 samples were resistant to monensin, both being *E. maxima* (Jeffers, 1974a, b). Reduced efficacy was seen in the field a few years later (Chapman, 1982; Mathis et al., 1984) and resistance started building up a decade later resulting in reduced efficacy in the field (Chapman and Hacker, 1994).

A similar decline in the efficacy of monensin was seen in turkey coccidiosis. In a study conducted with field isolates collected from turkey farms, two groups of isolates were tested for monensin sensitivity. One group was collected from birds that were fed a monensin-incorporated diet and another from birds that were not exposed to monensin. Isolates previously

exposed to monensin turned out to be resistant to the drug (Jeffers and Bentley, 1980). In a battery and floor pen experiment, monensin was effective in controlling coccidiosis in turkeys when given a mixture of three laboratory species of *Eimeria* (Chapman and Saleh, 1999). However, in a recent battery study, of the 23 field isolates collected from commercial turkey farms tested for monensin sensitivity, only 6 turned out to be sensitive (Chapman and Rathinam, 2007).

The most recent anticoccidial drug introduced is diclazuril, an acetonitrile compound that was approved for use in the US in 2001. The mode of action of diclazuril is unknown. Kawazoe and Di Fabio (1994) were the first to report resistance to diclazuril in the field where 6 of the 12 field isolates tested emerged resistant to diclazuril.

IMMUNOPROPHYLAXIS

Immunity against coccidia

Immunogenicity is the ability of an organism (or a particular substance, such as an antigen or epitope present in the organism), to develop an immune response. *Eimeria* parasites are capable of imparting both humoral and cell mediated immunity in the host (Rose, 1987; Dalloul and Lillehoj, 2005). The extent or degree of immunogenicity is different for each species of *Eimeria*. In a study that ranked species of chicken *Eimeria* based on their immunogenicity *Eimeria maxima* was ranked as highly immunogenic, *E. acervulina* as moderately immunogenic and *E. necatrix and E. tenella* were poor in stimulating an immune response (Rose and Long, 1962).

Different strains of the same species were shown to have differing immunogenicity (Joyner, 1969).

Since immunity developed to one species does not provide protection against others, vaccines have to contain more than one species i.e. multivalent. The number of species incorporated in a vaccine depends upon the target market. Usually, vaccines intended for broilers contain *E. tenella*, *E. maxima* and *E. acervulina* and vaccines for breeders and layers will include *E. necatrix* and *E. brunetti* in addition to the three above-mentioned species. This is because the latter two species are not encountered in young birds (Chapman, 2000).

Vaccines

Johnson (1927) found that chickens could acquire immunity when given small repeated doses of oocysts. Other workers like Dickinson (1941) attempted to demonstrate the validity of vaccines by individual inoculation of birds. This process, however, was not realistic for large-scale commercial production due to the labor involved. Edgar was the first to bring out a commercial coccidiosis vaccine in 1952 against *E. tenella*, which was marketed by Dorn and Mitchel Labs as "DM Cecal Coccidiosis Vaccine" (Williams, 2002b). A few years later, other species were added and after various name changes, the vaccine became Coccivac®.

Live non-attenuated vaccine

Edgar's vaccine comprised live oocysts but the parasites were non-attenuated. This kind of vaccine incorporates one or more laboratory or field strains that have not been modified in any

way and that are potentially pathogenic. By giving a small dose of the vaccine, following initial ingestion, the bird 'cycles' the parasite and gets re-infected via the litter. After 3-4 cycles, birds develop solid immunity. Most coccidiosis vaccines currently sold belong in the non-attenuated category. Differences among the vaccines are the number of species present, the mode of inoculation or other technology involved in production.

There are several vaccines available for the chicken. Coccivac® (Merck) and ADVENT® (Huvepharma) are delivered in the hatchery via a spray where the vaccine is added to a dye and sprayed on chicks in boxes. The sprayed vaccine enters the alimentary system either by the eye via the lachrymal ducts or by ingestion as a result of preening (Williams, 2002a). Vaccines can also be given via drinking water at the farm. ADVENT also undergoes a DNA staining assay for viability (Shirley et al., 2005) and claims to have an accurate number of viable oocysts in each dose. Immucox® (Ceva Biomune) is delivered via bright green edible gel, which the birds feed on during transportation from the hatchery. Inovocox® (Zoetis) is another broiler vaccine that has wild-type strains of the three *Eimeria* mentioned above. It is the only *in ovo* coccidiosis vaccine (Weber et al., 2004). Eggs are injected on day 18 of incubation and the birds shed oocysts following hatch. Like other vaccines, the success of in ovo vaccination depends upon birds getting re-infected and 'cycling' the parasites in order to develop protective immunity.

Only two vaccines are available for turkey coccidiosis and they are Coccivac®-T, a 'tetravalent' vaccine containing *E. adenoeides*, *E. meleagrimitis*, *E. gallopavonis* and *E. dispersa* that recently has been withdrawn and Immucox®-T that is said to contain *E. adenoeides* and *E. meleagrimitis.*

Live attenuated vaccine

Attenuated vaccines contain strains that have lost or attenuated their virulence but maintain an active level of immunogenicity. Such strains are selected either by precociousness or by serial passage in embryonated eggs.

Attenuation by precocious selection. The first species to be attenuated by selection for precociousness was *E. tenella* (Jeffers, 1975). In this method, the earliest oocysts produced following an infection are collected, sporulated and used for a subsequent passage. At each successive passage, the time between infection and appearance of first oocysts (pre-patent period) decreases. This happens due to the parasites losing the last asexual generation and having a lower reproductive potential. As a consequence, they decrease in virulence, but are still effectively immunogenic. By selection for precociousness, the prepatent period of *E. tenella* has been reduced from 138 h to 102 h (Jeffers, 1975). Shirley and colleagues utilized this concept in the 1980s and they successfully attenuated all strains of chicken *Eimeria*. (Table 2; summarized in Shirley, 1989). These strains are marketed in Europe and other countries as Paracox® (MSD Animal Health) and Livacox® (Biopharm). The only attenuated vaccine available in the US is Hatchpak® Cocci III (Merial), a trivalent broiler vaccine. There is no attenuated vaccine for turkeys in any market. Based on published literature, there is only one report of an attenuated strain of turkey *Eimeria, E. meleagridis* (Matsler and Chapman, 2007), which was attenuated by selection for precociousness.

Attenuation by embryo-adaptation. Long (1965) showed that *Eimeria tenella* was able to complete its life cycle when injected into the chorioallantoic membrane of a developing embryo.

Serial passage of *E. tenella* in embryonated eggs resulted in attenuated pathogenicity while immunogenicity was retained. Embryo-adapted lines of *E. maxima* (Shirley, 1980) and *E. necatrix* (Gore et al., 1983) were also developed. However, this method was unsuccessful in species other than *E. tenella* due to instability of the attenuation (reviewed in McDonald and Shirley, 2009). The egg-adapted line of *E. tenella,* which was derived after more than 100 passages, is currently used in Livacox®.

Recombinant vaccine

The last two decades has seen several attempts at creating recombinant DNA constructs developed from various *Eimeria* proteins. At the time of writing this manuscript, the only successful recombinant vaccine is an affinity purified gametocyte antigen vaccine of *Eimeria maxima* (Wallach et al., 1989, 1995) which protects via maternal transfer of immunoglobulins to the young chick. Layers are vaccinated and the antibodies produced in them are passed on to the offspring thus giving them passive immunity. The *E. maxima* antigen has also shown to have partial protection against *E. tenella* (Smith et al., 1994). It is commercially sold as CoxAbic® (Phibro Vaccines) and is not available in USA.

Conceptual vaccines

DNA Vaccines. Recombinant vaccines have been developed with genes encoding sporozoite proteins, surface antigens and microneme proteins of sporozoites. Kopko et al. (2000) constructed a recombinant vaccine with gene encoding a sporozoite protein of *E. tenella* called

SO7 and expressed in the mammalian expression vector, pcDNA3. Birds were vaccinated intramuscularly and following a challenge with *E. tenella,* researchers were able to demonstrate protection, based on lesion scores and body weight data. Another DNA vaccine developed with a surface antigen of *E. tenella* sporozoite (TA4) protected chicken following a challenge based on a reduction in oocyst production and decreased weight loss (Wu et al., 2004). Other *Eimeria* sporozoite surface antigens used in DNA constructs are 3-1E (Song et al., 2001) and *E. tenella* microneme protein EtMic-1 (Qi et al., 2013), EtMic-2 (Ding et al., 2005; Sun et al., 2014).

Various expression vectors have been used in creating DNA vaccines including bacteria (*Salmonella*; Du and Wang, 2005), Fungi (*Saccharomyces*; Sun et al., 2014) and plants (tobacco; Sathish et al., 2011). Recombinant vaccines could be cheaper to produce *en masse* compared to the current live vaccines, as it does not include an expensive *in vivo* step in production. However, the biggest hurdle for these concepts to become reality is the variability in protection that they induce *in vivo* (Shirley et al., 2005) and the lack of reproducibility.

TURKEY COCCIDIOSIS

Introduction

Smith (1895) was the first to report that coccidiosis could occur in the turkey although he did not name the species involved. Later, he stated that the coccidium in the turkey resembled neither of the two known species of *Eimeria* in chickens at that time, and regarded it as a foreign species (Smith, 1916). The first species of *Eimeria* to be described from the turkey was *Eimeria meleagridis* (Tyzzer, 1927) and shortly after, two more species, *E. meleagrimitis* and *E. dispersa*

were reported (Tyzzer, 1929). Hawkins (1950) described *E. gallopavonis* and in the next three years three more - *E. adenoeides* (Moore and Brown, 1951), *E. innocua* (Moore and Brown, 1952) and *E. subrotunda* (Moore et al., 1954) were characterized. Of the seven species, *E. meleagrimitis*, *E. adenoeides* and *E. gallopavonis* are considered most pathogenic; *Eimeria dispersa* and *E. meleagridis* are moderately pathogenic and the other species are thought to be nonpathogenic.

Eimeria meleagrimitis

Eimeria meleagrimitis was first described by Tyzzer (1929) along with several other species of chicken, turkey and pheasant *Eimeria*. He regarded it as a tentative species since the oocyst morphology and its predilection site in the intestine was similar to that of *E. mitis* in the chicken. At the time of publication of his manuscript, he had not tested transmission of chicken *Eimeria* to turkeys and concluded that it might be *E. mitis* (Tyzzer, 1929). An extensive study was carried out 20 years later where the species was described in detail (Clarkson, 1959).

Prepatent period

Various prepatent periods have been reported for *E. meleagrimitis*. Unfortunately in most cases there is lack of information on the method of data collection. Tyzzer (1929), in the original description of the species, states that a development period of six days (144 hours) precedes the appearance of oocysts in intestinal discharges and Hawkins (1952) found that oocysts were passed in the feces 6 days after infection. Clarkson (1959) measured the prepatent period of *E.*

meleagrimitis by collecting feces from 25 birds at 2-hour intervals. The average prepatent period that he reported was 116 hr with a range of 114-118hr. According to Reid (1972) the prepatent period of *E. meleagrimitis* is 103 hours but no reference is given for this information. Long et al. (1977) also state that the prepatent period of *E. meleagrimitis* is 103 hours, an estimate that was apparently determined by measuring oocyst output "using a 24 hr collection of faeces". Levine (1985) states that the prepatent period of *E. meleagrimitis* is 3-6 days but no source is given for this information. El-Sherry et al. (2013) state a prepatent period of 120 hours, while Vrba and Pakandl (2014) report 120-126 hours.

Clinical signs

Eimeria meleagrimitis infection does not present any classical clinical signs. The bird exhibits droopiness, listlessness, palor, ruffled feathers, unthrifty appearance, huddling or looking chilled, mucus in the feces, diarrhea, death especially among young poults in heavy infection (Hinshaw, 1937). Birds also show dehydration, poor condition, and wasting. In severe cases, death is seen 6-8 days after initial infection. An infected bird's feces on 6 days after ingestion of oocysts are foul smelling, with the production of a dark brown liquid material in the feces with small streaks of blood, and pieces of mucosa (Clarkson and Gentles, 1958).

Pathology and lesions

According to Tyzzer (1929), the parasite develops throughout the intestine of turkeys although development in the small intestine is favored. The parasites penetrated deep into the epithelium

and developed below the nucleus of epithelial cells with no sub epithelial forms (Tyzzer, 1929). The site of infection is the duodenum and upper jejunum and the lesions include an edematous, congested and enlarged duodenum with red brown necrotic cores. Microscopically the tips of intestinal villi are disrupted by gametocytes on day 5 post-infection (Clarkson, 1959). Scanning electron microscopy shows extensive mucosal damage in the duodenum and jejunum but less destruction in the ceca. The villi were pitted and shriveled (Madden and Ruff, 1979). Pathogenicity is dose and age dependent and mortality can be up to 100% in 3-week old birds when infected with a high dose of 4×10^5 oocysts (Clarkson and Gentles, 1958).

Physiological effects

Eimeria meleagrimitis infection results in a decrease in pH in both the duodenum and the jejunum, but not in any other regions of the gut (Anderson et al., 1977). A decrease in plasma carotenoids, an increase in aspartate amino transferase (Augustine and Thomas, 1981), and a decrease in plasma and liver retinol has been reported (Augustine and Ruff, 1983). There was an increase in glutamic oxaloacetic transaminase (GOT) and decrease in total protein during an *E. meleagrimitis* infection (Augustine and Thomas, 1980).

Strain variation

Long et al. (1977) characterized a strain of *E. meleagrimitis* isolated in Yorkshire that differed from the Weybridge strain (Hein, 1969). The Yorkshire strain had relatively smaller oocysts

(16.15 x 14.75 μm compared to 20 x 17 μm of the Weybridge strain). The Yorkshire strain was confirmed to be *E. meleagrimitis* by cross immunity tests (Long et al., 1977).

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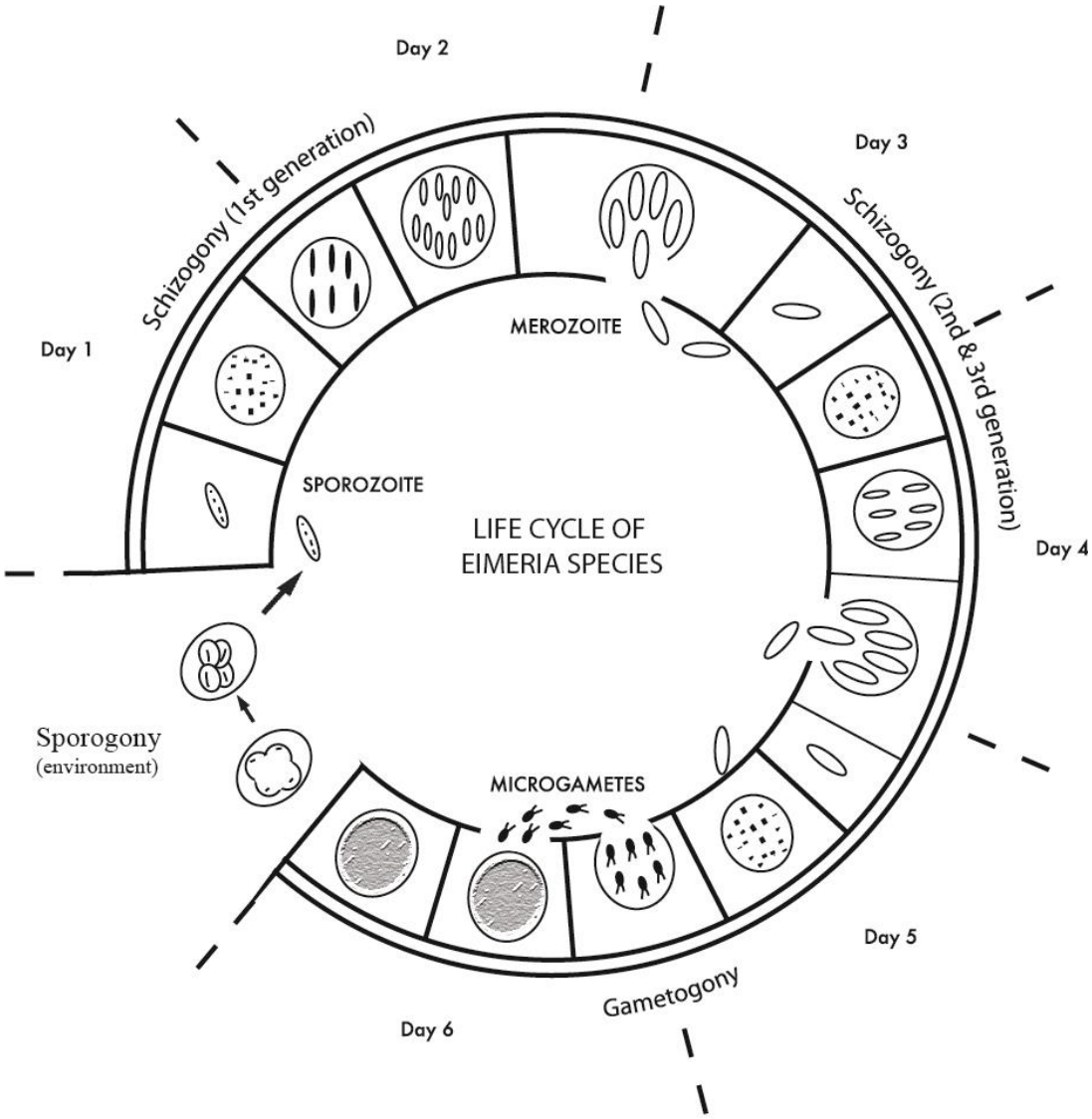

Fig. 1. Life cycle of a typical *Eimeria*

Table 1. Taxonomic classification of *Eimeria*

Taxonomic group	***Eimeria*'s classification**	**Taxonomist**	**Group characteristics**
Phylum	Apicomplexa	Levine, 1970	Presence of apical complex
Class	Conoidasida	Levine, 1988	Complete, hollow, truncated conoid
Order	Eucoccidiorida	Leger and Duboscq, 1910	Undergo asexual, sexual and spore formation during their life cycle
Family	Eimeriidae	Minchin, 1903	Direct life cycle
Genus	Eimeria	Schneider, 1875	Oocysts contain four sporocysts, each with two sporozoites

Table 2. Comparison of reduction in prepatent period (PPP) following selection for precocious development in chicken and turkeys

Species	Number of generations	Maximum reduction in PPP (h)	Reference
Chicken			
Eimeria acervulina	10	17	McDonald et al., 1982
Eimeria brunetti	25	36	Shirley et al., 1986
Eimeria maxima	12	14	McDonald et al., 1986a
Eimeria mitis	14	26	McDonald & Ballingal, 1983
Eimeria necatrix	15	18	Shirley and Bellatti, 1984
Eimeria tenella	15	20	McDonald et al., 1986b
Turkey			
Eimeria meleagridis	20	4-8	Matsler & Chapman, 2007

CHAPTER II

SENSITIVITY OF ISOLATES OF *EIMERIA* FROM TURKEY FLOCKS TO THE ANTICOCCIDIAL DRUGS AMPROLIUM, CLOPIDOL, DICLAZURIL, AND MONENSIN

Sensitivity of Isolates of *Eimeria* from Turkey Flocks to the Anticoccidial Drugs Amprolium, Clopidol, Diclazuril, and Monensin

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SUMMARY. The sensitivity of field isolates of turkey coccidia from the USA to the anticoccidial drugs amprolium, clopidol, diclazuril, and monensin was investigated. Clopidol and diclazuril were the most effective followed by monensin, and amprolium. Thirty one isolates were classified as resistant to amprolium, 23 resistant to monensin, 10 resistant to diclazuril, and 6 resistant to clopidol. Six isolates were partially resistant to monensin, 10 partially resistant to clopidol, and 11 partially resistant to diclazuril. Four isolates were sensitive to monensin, 12 sensitive to diclazuril, and 17 sensitive to clopidol.

Key words: Eimeria, turkey, resistance, amprolium, clopidol, diclazuril, monensin

Abbreviations: GSR = growth and survival ratio; OAA = optimum anticoccidial activity

Resistance to drugs used to control coccidiosis has been extensively documented in the chicken, but few investigations have been carried out in the domestic turkey (2). Anticoccidial agents currently approved for use in turkeys in the USA are commonly divided into two categories, ionophorous antibiotics that include monensin and lasalocid, and synthetic drugs (also known as "chemicals") that include amprolium, clopidol, diclazuril, and others. Resistance to monensin has been reported from turkey flocks, but recent studies have not been conducted with synthetic drugs (3, 5). In this study, the occurrence of resistance to three such compounds was investigated and their efficacy compared with monensin. Isolates were obtained from turkey farms throughout the USA.

MATERIALS AND METHODS

Animals and husbandry. Methods used, including animals and their husbandry, parasite collection and propagation, and evaluation of resistance, were similar to those previously described (3). Female Nicholas poults were obtained from a local hatchery and raised in brooder cages in an isolation building. At 10 days they were transferred to a test facility and randomly allocated to cages (four poults/cage). They were fed a medicated or unmedicated corn/soybean meal diet suitable for young turkeys. Amprolium, clopidol, and diclazuril (Amprol® 25%, Coyden® 25%, and Clinacox®; Huvepharma Inc., Peachtree City, GA) were included in the rations at concentrations of 125, 125, and 1 ppm respectively. Monensin (Coban® 60; Elanco Animal Health, Indianapolis, IN) was included in the ration at a concentration of 99.2 ppm.

Parasites and site of development. Isolates were obtained from litter samples collected from turkey growing units in Arkansas, California, Iowa, Kansas, Missouri, Michigan, Minnesota, North Carolina, Pennsylvania, South Dakota, and Virginia, USA. Some of the isolates had been used previously to determine their sensitivity to monensin (3). No attempt was made to purify the isolates, which in most cases comprised more than one species. They were characterized by recording whether gametocyte stages of the life cycle were present in different regions of the intestine. Two poults from each unmedicated infected group were euthanatized at experiment termination (6 days after infection) and their intestines removed for histological investigation. Samples of intestine (1 cm in length) were taken from the ascending limb of the duodenum, the mid jejunum, and mid cecum, fixed in 10% buffered formalin, stained with hematoxylin and eosin, and 5 µm paraffin sections examined for the presence of gametocytes.

Evaluation of resistance. Six experiments were carried out in each of which 5-7 isolates were investigated. Evaluation of resistance was based upon the method described by Jeffers and Bentley (5). Two replicates of four 10-day-old poults were weighed, allocated to cages, and

given either medicated or unmedicated feed. Two days later they were reweighed (day 0) and infected with a maximum of 100,000 oocysts. An additional two replicates were unmedicated and uninfected (controls). Poults were weighed 6 days after infection and the gain in weight calculated. Any mortality was recorded. These two parameters were expressed in a growth and survival ratio (GSR) defined as the cage weight at trial termination plus the weight of any dead birds, divided by the cage weight when infected. The percent optimum anticoccidial activity (% OAA) for each treatment was then calculated as follows: % OAA = [average GSR of infected medicated group – average GSR of infected unmedicated group] / [average GSR of uninfected controls – average GSR of infected unmedicated group] x 100. An isolate was considered resistant if the % OAA was ≤50%, partially resistant if the % OAA was 51-74%, and sensitive if the % OAA was ≥75%.

Analysis. GSR values for each experiment were combined and analyzed by one-way analysis of variance using the PROC ANOVA procedure of SAS software; means were compared using Duncan's multiple-range test.

RESULTS

Parasites. The site of development of each isolate is presented in Table 1. In most cases gametocytes were found in the duodenum, jejunum, and ceca suggesting several species were present. Identification of *Eimeria* in the turkey is difficult because species overlap in characteristics such as oocyst size and site of development (2). Common pathogenic species that develop in the duodenum/jejunum and cecum respectively include *E. meleagrimitis* and *E.*

adenoeides; isolates are tentatively identified as these although other species, such as *E. meleagridis* and *E. gallopavonis,* cannot be ruled out.

GSR. The GSR of medicated and unmedicated infected poults is presented in Table 2. Results for all experiments were combined and are illustrated in Fig 1. The mean GSR for isolates medicated with clopidol, diclazuril, and monensin was significantly greater than the unmedicated infected birds. The mean GSR for poults given clopidol and diclazuril was significantly greater than for birds given amprolium and monensin.

OAA. The % OAA derived from these GSR values is presented in Table 3 and a summary of the resistance classification of isolates in Table 4. Thirty one isolates were classified as resistant to amprolium, 23 resistant to monensin, 10 resistant to diclazuril, and 6 resistant to clopidol. Six isolates were partially resistant to monensin, 10 partially resistant to clopidol and 11 partially resistant to diclazuril. Four isolates were sensitive to monensin, 12 sensitive to diclazuril and 17 sensitive to clopidol.

DISCUSSION

The sensitivity of 33 isolates of Eimeria from turkey farms in the USA to amprolium, clopidol, diclazuril, and monensin was investigated. In the USA, amprolium, diclazuril, and monensin are approved for the control of coccidiosis in turkeys but clopidol is only approved to control the blood protozoan *Leucocytozoon smithi*. The mean GSR for pooled isolates indicated that clopidol and diclazuril were the most effective followed by monensin and amprolium. The mean GSR for amprolium was not significantly different from infected unmedicated birds indicating that this drug was unable to control the isolates.

The extent to which these drugs had been used at the farms from which the isolates were obtained is not known. A general indication of their current usage can be obtained by analysis of monthly data for turkey rearing complexes compiled by AgriStats Inc., Fort Wayne, New Jersey, USA. During the three year period 2005 – 2007, monensin was used by 73.6% of complexes in at least one of the feeds given to tom turkeys. Amprolium, clopidol, and diclazuril were used by 2.6, 3.1, and 6.5 % of complexes respectively (Chapman, unpublished observations). Acquisition of resistance to *Eimeria* is thought to depend upon the type of drug, its mode of action, and the selection pressure it exerts on the coccidial population (4). The likelihood of resistance is greater the more extensively a drug is used. It is concluded that, in recent years, resistance has had less opportunity to develop to amprolium, clopidol, and diclazuril than to monensin.

Twenty-three of the 33 isolates investigated were considered resistant to monensin, 6 partially resistant, and 4 sensitive. Eleven of these isolates had been utilized in a previous study of the sensitivity of isolates of *Eimeria* from turkey flocks to monensin (3). Whereas isolates M1 and V2 were previously considered resistant and partly resistant, respectively, in this study their classifications were reversed. Isolate V5, previously considered partly resistant, was judged sensitive to the drug. Resistance to monensin was first reported from turkey flocks in Canada (5) but at that time isolates from the USA were found to be sensitive. Monensin has been used extensively since its first introduction in the 1970s and therefore resistance might be anticipated.

Thirty-one isolates were categorized as resistant to amprolium. Resistance was documented in the UK in 1970 (7) but there have been no subsequent studies with this drug. Amprolium was introduced in the 1960s and was used extensively in turkeys for about 20-30 years but is only occasionally used today (9). In addition to incorporation in feed, amprolium may be included in

the drinking water to treat birds diagnosed with coccidiosis, but the extent of such usage is unknown. It is possible that phenotypes resistant to amprolium can survive in the environment in the absence of intense selection pressure.

Twelve isolates were categorized as sensitive, 11 partially resistant, and 10 resistant to diclazuril. Diclazuril was first introduced in the USA in 2001. There have been no published studies of resistance, but in one report isolates obtained from farms where diclazuril had been used for six months produced a lower index score (a measure of pathogenicity) than isolates from farms where it had not been used in the year prior to collection. This suggests that at the latter farms a decrease in sensitivity to diclazuril had occurred (8).

Seventeen isolates were sensitive to clopidol, 10 partially resistant, and 6 resistant. Clopidol was introduced in the 1960s, and like amprolium, resistance was documented following its introduction in the UK (7). It is possible that exposure to this compound has been insufficient to result in the selection of many resistant strains.

Several laboratory studies have demonstrated that it is possible to induce resistance to amprolium, clopidol, diclazuril, and monensin in the chicken (1). In the turkey, however, there has been only one such study, in which resistance was induced to monensin (6). Numerous reports have documented that in chickens resistance is widespread (e.g. 10). Results of this study suggest that this is also true for the turkey.

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Table 1. Site of development of field isolates of turkey *Eimeria*

Isolate[A]	Region of gut[B]			Isolate[A]	Region of gut[B]		
	Duo	Jej	Cec		Duo	Jej	Cec
A7	+	-	+	M5	+	+	+
A8	-	+	-	M6	+	+	+
A9	+	+	-	M7	+	+	+
C1	+	+	-	MI1	+	+	+
C2	+	+	-	MN1	+	+	+
C3	+	+	-	MN2	+	+	-
C4	+	+	-	N1	+	+	+
C5	+	+	+	N2	+	+	+
I1	-	+	+	N3	+	+	+
K1	+	+	+	P2	+	+	+
K2	+	+	+	S1	+	+	-
K3	-	+	+	V1	+	+	+
K4	+	+	+	V2	+	+	+
M1	+	-	+	V3	+	+	+
M2	+	+	+	V4	+	+	+
M3	-	+	-	V5	+	+	+
M4	+	+	+				

[A]Isolates preceded with the capital letters A, C, I, K, M, MI, MN, N, P, S, and V were from Arkansas, California, Iowa, Kansas, Missouri, Michigan, Minnesota, North Carolina, Pennsylvania, South Dakota, and Virginia respectively.

[B]Regions of the gut parasitized were Duo - duodenum, Jej - jejunum, Cec - ceca.

Table 2. The effect of anticoccidial drugs upon the GSR of field isolates of turkey coccidia

Isolate[A]	GSR[B]				
	Medication[C]				
	None	Amp	Clo	Dic	Mon
	Experiment 1				
K3	1.685	1.621	1.802	1.758	1.703
M1	0.745	1.126	1.657	1.658	1.366
M3	1.284	1.357	1.735	1.625	1.381
N1	1.235	1.352	1.694	1.480	1.480
V4	1.056	1.311	1.611	1.529	1.365
	Experiment 2				
A7	1.390	1.432	1.754	1.619	1.779
M4	1.278	1.336	1.740	1.484	1.317
N2	1.508	1.470	1.643	1.643	1.513
V3	1.187	1.291	1.732	1.203	1.270
V5	1.196	1.277	1.540	1.628	1.637
	Experiment 3				
C2	1.062	1.003	1.362	1.058	1.103
M5	0.961	1.079	1.055	1.220	1.030
P2	1.004	1.107	1.339	1.271	1.082
S1	1.042	1.078	1.344	1.139	1.134
V2	1.127	1.072	1.287	1.414	1.143
	Experiment 4				
K1	1.039	1.200	1.479	1.524	1.418
K4	1.236	1.121	1.506	1.693	1.414
M2	1.075	1.081	1.101	1.736	1.477
MI1	0.832	1.289	1.352	1.740	1.434
MN1	1.321	1.203	1.469	1.555	1.366

Table 2 (Contd.).

Experiment 5					
A8	1.261	1.365	1.583	1.529	1.144
C1	1.298	1.392	1.702	1.685	1.233
K2	1.152	1.097	1.471	1.413	1.293
M6	1.213	1.160	1.502	1.597	1.214
N3	1.412	1.511	1.609	1.511	1.172
V1	1.269	1.276	1.669	1.564	1.224
Experiment 6					
A9	1.118	1.054	1.460	0.991	1.298
C3	1.321	1.801	1.810	1.937	1.776
C4	1.213	1.374	1.624	1.159	1.252
C5	1.402	1.559	1.820	1.833	1.621
I1	1.514	1.541	1.730	1.689	1.688
M7	1.213	1.223	1.706	1.759	1.598
MN2	1.577	1.769	1.666	1.761	1.749
Mean	1.219c	1.301bc	1.562a	1.527ab	1.384b

[A]Isolates preceded with the capital letters A, C, I, K, M, MI, MN, N, P, S, and V were from Arkansas, California, Iowa, Kansas, Missouri, Michigan, Minnesota, North Carolina, Pennsylvania, South Dakota, and Virginia respectively.

[B]Growth and survival ratio (GSR) = cage weight at termination / cage weight at initiation. Data are expressed as the average of two replicates / treatment. Mean values followed by different lowercase superscripts are significantly different ($P \leq 0.01$).

[C]Amp = amprolium, Clo = clopidol, Dic = diclazuril, Mon = monensin.

Table 3. The effect of anticoccidial drugs upon the % OAA and resistance category of field isolates of turkey coccidia.

Isolate[A]	% OAA[B] (resistance category in brackets[C])			
	Amp[D]	Clo[D]	Dic[D]	Mon[D]
Experiment 1				
K3	-66 (R)	122 (S)	78 (S)	22 (R)
M1	37 (R)	88 (S)	84 (S)	60 (PR)
M3	13 (R)	91 (S)	69 (PR)	20 (R)
N1	21 (R)	84 (S)	45 (R)	43 (R)
V4	36 (R)	75 (S)	65 (PR)	42 (R)
Experiment 2				
A7	12 (R)	99 (S)	63 (PR)	106 (S)
M4	12 (R)	97 (S)	43 (R)	8 (R)
N2	16 (R)	55 (PR)	55 (PR)	2 (R)
V3	18 (R)	96 (S)	3 (R)	14 (R)
V5	15 (R)	61 (PR)	77 (S)	79 (S)
Experiment 3				
C2	19 (R)	96 (S)	-1 (R)	13 (R)
M5	28 (R)	23 (R)	62 (PR)	16 (R)
P2	28 (R)	90 (S)	72 (PR)	21 (R)
S1	11 (R)	90 (S)	29 (R)	28 (R)
V2	-22 (R)	64 (PR)	115 (S)	7 (R)
Experiment 4				
K1	20 (R)	54 (PR)	60 (PR)	47 (R)
K4	19 (R)	44 (R)	74 (PR)	29 (R)
M2	1 (R)	3 (R)	85 (S)	52 (PR)
MI1	45 (R)	51 (PR)	89 (S)	59 (PR)
MN1	22 (R)	28 (R)	44 (R)	8 (R)

Table 3 (Contd.)

Experiment 5				
A8	24 (R)	73 (PR)	61 (PR)	-27 (R)
C1	23 (R)	100 (S)	93 (S)	-16 (R)
K2	-9 (R)	58 (PR)	48 (R)	26 (R)
M6	-11 (R)	59 (PR)	79 (S)	0 (R)
N3	34 (R)	68 (PR)	34 (R)	-83 (R)
V1	2 (R)	93 (S)	68 (PR)	-10 (R)
Experiment 6				
A9	-9 (R)	50 (R)	-19 (R)	26 (R)
C3	101 (S)	103 (S)	130 (S)	95 (S)
C4	28 (R)	70 (PR)	-9 (R)	7 (R)
C5	40 (R)	106 (S)	109 (S)	55 (PR)
I1	10 (R)	76 (S)	62 (PR)	61 (PR)
M7	2 (R)	84 (S)	93 (S)	66 (PR)
MN2	87 (S)	40 (R)	83 (S)	78 (S)

[A]Isolates preceded with the capital letters A, C, I, K, M, MI, MN, N, P, S, and V were from Arkansas, California, Iowa, Kansas, Missouri, Michigan, Minnesota, North Carolina, Pennsylvania, South Dakota, and Virginia respectively.

[B]% OAA is the percentage of optimum anticoccidial activity. % OAA = (GSR of infected medicated birds – GSR of infected unmedicated birds) / (GSR of uninfected unmedicated birds – GSR of infected unmedicated birds) x 100.

[C]An isolate was categorized as sensitive to a drug (S) when the % OAA was ≥ 75%, partially resistant (PR) when the % OAA was 51%-74%, and resistant (R) when % OAA was ≤ 50%.

[D]Amp = amprolium, Clo = clopidol, Dic = diclazuril, Mon = monensin.

Table 4. Number of isolates of *Eimeria* obtained from turkey flocks classified as resistant, partially resistant, or sensitive to drugs.

Drug	Resistance classification[A]		
	Sensitive	Partially resistant	Resistant
Amprolium	2	0	31
Clopidol	17	10	6
Diclazuril	12	11	10
Monensin	4	6	23

[A]An isolate was categorized as sensitive to a drug (S) when the % OAA was ≥ 75%, partially resistant (PR) when the % OAA was 51%-74%, and resistant (R) when % OAA was ≤ 50%.

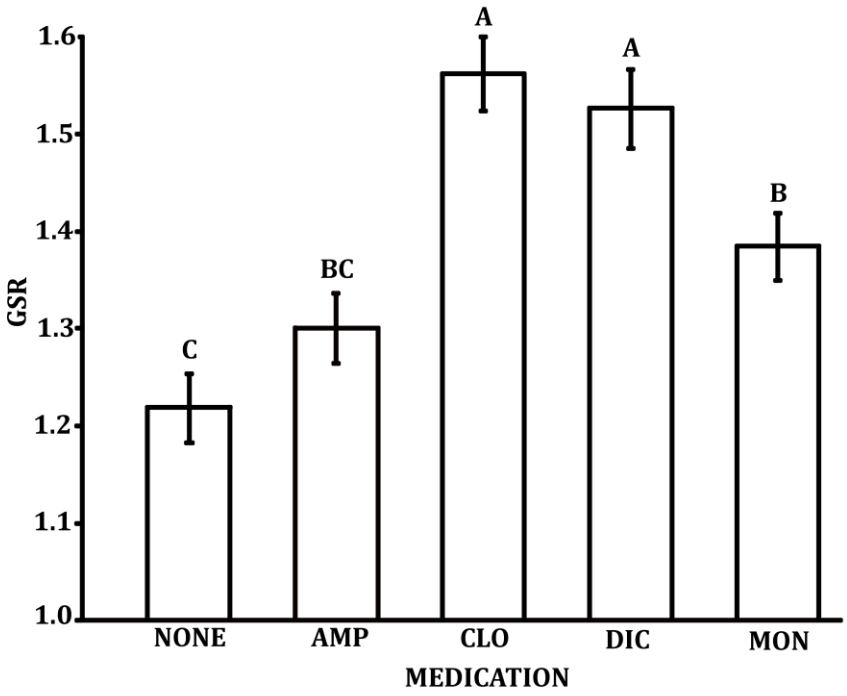

Fig. 1. The effect of anticoccidial drugs upon the growth and survival ratio (GSR) of field isolates of turkey coccidia. Data are the combined means of six separate experiments. Values with different uppercase letters are significantly different ($P \leq 0.01$). Amp = amprolium, Clo = clopidol, Dic = diclazuril, Mon = monensin.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: H.D. Chapman

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: September 12, 2012

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **October 31, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13005- "CONTROL OF COCCIDIOSIS IN TURKEYS**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **10-31-2015**, youmust submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at atime.

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

cnc/car

cc: Animal Welfare Veterinarian

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UofA UNIVERSITY OF ARKANSAS
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Attn: University of Arkansas Graduate School

October 14, 2014

Dear Sir,

I attest that Thilakar Rathinam was first author of the manuscript cited below and completed at least 51% of the work for the paper.

T. Rathinam and H. D. Chapman (2009) Sensitivity of Isolates of *Eimeria* from Turkey Flocks to the Anticoccidial Drugs Amprolium, Clopidol, Diclazuril, and Monensin. Avian Diseases: September 2009, Vol. 53, No. 3, pp. 405-408.

Yours Sincerely,

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CHAPTER III

MOLECULAR DETECTION OF FIELD ISOLATES OF TURKEY *EIMERIA* BY POLYMERASE CHAIN REACTION AMPLIFICATION OF CYTOCHROME OXIDASE I GENE

Molecular Detection of Field Isolates of Turkey *Eimeria* by Polymerase Chain Reaction Amplification of Cytochrome c Oxidase I Gene

ABSTRACT Detection of various species of *Eimeria* in samples collected from 30 commercial turkey farms using PCR was attempted. Genomic DNA was extracted from clean oocysts from the field samples. Polymerase chain amplification of species-specific cytochrome c oxidase subunit I (COI) gene was performed for five species of turkey *Eimeria*. The species tested were *Eimeria adenoeides*, *E. meleagrimitis*, *E. meleagridis*, *E. dispersa* and *E. gallopavonis*. Of the 30 samples tested, all were positive for *E. meleagrimitis*, 9 were positive for *E. adenoeides*, 2 for *E. dispersa* and none for the remaining two species. *E. meleagrimitis* occurred as a single infection in 70% of the farms while 30% of the farms had a mixed infection with *E. meleagrimitis* and *E. adenoeides* and 7% were triple positive with *E. meleagrimitis, E. adenoeides* and *E. dispersa*. This is the first account of the field prevalence of turkey *Eimeria* species using molecular methods.

INTRODUCTION

Coccidiosis is an important enteric disease of turkeys and is caused by apicomplexan protozoan parasites of the genus *Eimeria*. Infection with *Eimeria* is known to cause significant economic losses to the poultry industry (Williams, 1999). Control of this disease relies upon the use of in-feed anticoccidials and vaccination of birds at hatch. Continuous usage of the drugs in the field has resulted in decreased susceptibility of the parasites to anticoccidials. Rathinam and

Chapman (2009) showed that the field isolates of turkey *Eimeria* were resistant to majority of the anticoccidial drugs used.

Identification of different species of *Eimeria* and their prevalence is important in designing strategies for the prevention and control of coccidiosis. Traditionally, epidemiological distribution and prevalence studies of turkey coccidiosis were based on several parasite characteristics (oocyst morphometry, pre-patent period, development and pathology in the host) and host responses (Tyzzer, 1932). But, these methods are labor intensive, time consuming and less reliable due to the shared features among various species (Clarkson, 1959; Long and Joyner, 1984). Molecular based identification methods are gaining popularity and several assays have been developed for chicken (Schnitzler et al., 1998, 1999; Lew et al., 2003) and turkey *Eimeria* (Cook et al., 2010). This study was aimed at identifying the prevalence of different *Eimeria* species in turkey field isolates using species-specific polymerase chain reaction (**PCR**) amplification of mitochondrial cytochrome c oxidase subunit I (**COI**) gene.

MATERIALS AND METHODS

Parasites

Isolates were obtained from litter samples collected from turkey farms in Arkansas, California, Iowa, Kansas, Missouri, Michigan, Minnesota, North Carolina, Pennsylvania, South Dakota, and Virginia, USA. Some of the isolates had been used previously to determine their sensitivity to anticoccidials drugs (Rathinam and Chapman, 2009). Oocysts were collected from the litter samples, sporulated and propagated in young poults. The procedures for litter sample collection

and methods for propagation have previously been described (Chapman and Rathinam, 2007). All live animal protocols followed FASS (2010) guidelines for agricultural research and were approved by the Institutional Animal Care and Use Committee. Upon propagation and sporulation, oocysts were cleaned in sodium hypochlorite and following repeated washing in water, were collected in Hank's Basal Salt Solution and stored at -20°C until further use.

Genomic DNA extraction

Genomic DNA (**gDNA**) was extracted from oocysts using QiaAMP DNA Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol with minor modifications as detailed below. The samples were pelleted by centrifugation in a 1.5 mL micro centrifuge tube at 1500 x g, for 2 minutes at 4°C. The pellet was resuspended in 500 µL ATL buffer (Qiagen) and 20 µL Proteinase-K (Qiagen) was added. The suspension was then transferred to 1.5 mL screw cap tubes with 200 mg of sterile glass beads (~0.5 mm diameter). The oocysts were disrupted using a mini-BeadBeater (Biospec Products, Bartlesville, OK) twice for 2 minutes with 1 minute incubation on ice in between. The supernatant was collected into a new collection tube and the gDNA was extracted according to the kit instructions. The eluted DNA was stored at -20°C for further use.

Polymerase chain reaction

Identification of *Eimeria* sp. in each sample was performed by PCR amplification of the species specific COI gene. Amplification was performed using a Veriti® 96-well Thermal Cycler

(Applied Biosystems, Foster City, CA). The primers used were kindly provided by Dr. John R. Barta (University of Guelph, ON, Canada) and their sequences are shown in Table 1. The reaction mixture for each sample comprised 5μL of gDNA, 12.5 μL of PCR master mix (Promega Corporation, Madison, WI), 400 nM forward primer, 400 nM reverse primer, and nuclease-free water to make up a final volume of 25 μL. The cycling conditions used were one cycle at 95°C for 10 min, and 39 cycles at 94°C for 30 s, 50-62°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Negative controls were included to check for nonspecific amplification of primers. Genomic DNA isolated from single oocyst lines of turkey *Eimeria* species characterized in our laboratory (*E. adenoeides*, *E. meleagrimitis*, *E. meleagridis* and *E. dispersa*) were used as positive control. No positive control was included for *E. gallopavonis* due to unavailability of a pure line, but the primer had been tested for amplification of *E. gallopavonis*-specific COI gene (Barta, pers. comm.).

Gel electrophoresis

The amplification products were analyzed on 1% agarose (Promega) gel electrophoresed at 100 V in TBE buffer (Life Technologies, Grand Island, NY). A GeneMate Quanti-Marker 100 bp (BioExpress, Kaysville, UT) DNA ladder was used as the size standard. Ethidium bromide was incorporated into the gels as the nucleic acid staining agent and the gels were analyzed under UV light using Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA) (Fig 1).

RESULTS

Of the five turkey *Eimeria* species investigated, only three species were seen in the samples (*E. adenoeides*, *E. meleagrimitis*, and *E. dispersa*). *E. meleagridis* or *E. gallopavonis* were not identified (Table 2). The most prevalent species was *E. meleagrimitis* (30/30; 100%), followed by *E. adenoeides* (9/30; 30%) and *E. dispersa* (2/30; 7%).

Presence of only one species (*E. meleagrimitis*) was seen in 21 isolates (70%), whereas mixed species with two or more species was seen in 9 isolates (30%). Frequencies of various species in a mixed isolate are given in Fig 2. The most prevalent mixture was *E. adenoeides* and *E. meleagrimitis* with 7 isolates (24%) testing positive for this mixture. Two isolates (7%) were positive for three species (*E. adenoeides*, *E. meleagrimitis*, and *E. dispersa*) (Fig 2).

DISCUSSION

Knowledge of the distribution and prevalence of *Eimeria* species in the turkey is lacking and such information would play an important role in identifying appropriate control tools and strategies. Several reports have been published showing the prevalence of chicken *Eimeria* species in the field (Lee et al., 2010; Ogedengbe et al., 2011), but no such reports exist for the turkey. In this study, the prevalence of different species of *Eimeria* in turkey field isolates was investigated. We used a species-specific COI based PCR assay to identify five species of turkey *Eimeria* (*E. adenoeides*, *E. meleagrimitis*, *E. meleagridis*, *E. dispersa*, and *E. gallopavonis*).

Over the years, several species-specific PCR diagnostic assays have been developed to identify chicken *Eimeria*. These assays target different loci like internal transcribed spacer-1 (**ITS**) (Schnitzler et al., 1998, 1999; Lew et al., 2003; Su et al., 2003; Haug et al., 2007, 2008), ITS-2 (Woods et al., 2000; Lien et al., 2007), 5s RNA and small subunit rRNA (Stucki et al.,

1993; Tsuji et al., 1997). Cook et al. (2010) developed ITS-1 based PCR for the diagnosis of turkey *Eimeria*. Though ITS based assays are being used widely for species identification, the intragenomic and intraspecific variations in these sequences may limit its usage (Chapman et al., 2013). COI gene is now considered a much more reliable target for molecular species identification owing to its modest intraspecific variation, but sufficient DNA sequence variability strong enough for species identification. The presence of this gene in multiple copies in *Eimeria* genome also makes it a good PCR target (Ogedengbe et al., 2011). Considering the various advantages of COI over ITS-1 for species identification, we have used a COI based PCR assay to determine prevalence in our experiment.

E. meleagrimitis was identified as the most prevalent species in the field and was detected in all of the single infections and co-infections observed in the study. The second most prevalent species was *E. adenoeides* (30%) and was found in 100% of the mixed infections. Only 7% of the total samples tested were positive for *E. dispersa*. *E. meleagrimitis* and *E. adenoeides* are considered as the two most common pathogenic species in turkeys. This supports an earlier finding that *E. meleagrimitis w*as the most prevalent species (Jeffers and Bentley, 1980). Of the 38 isolates they tested, a majority comprised two species *E. meleagrimitis* and *E. adenoeides*, 10 contained predominantly *E. meleagrimitis* and one *E. adenoeides* (Jeffers and Bentley, 1980). A survey was conducted on the prevalence of turkey *Eimeria* in 13 turkey producing states in the 1960s and showed that *E. meleagrimitis* was identified in 22 farms while *E. adenoeides* was seen in 34 farms. However, when the survey was repeated in 1982-83, of the 39 isolates tested, the number of samples containing *E. meleagrimitis* was 33 while *E. adenoeides* was 32 (Edgar, 1986) suggesting an increase in prevalence of *E. meleagrimitis* over the years.

This is the first report of the field prevalence of turkey *Eimeria* species using molecular methods. This report identifies *E. meleagrimitis* as the most prevalent species. Currently there are only two vaccines available for turkey coccidiosis, both of which are live, wild-type vaccines. Possibility of vaccine failure and risk of an outbreak of clinical coccidiosis is quite high in wild-type vaccines (Williams, 2002). It would be desirable to have an attenuated vaccine which will contain strains that had lost their pathogenicity but maintain their immunogenicity.

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Table 1. DNA sequence, annealing temperatures of COI primers for turkey *Eimeria* Sp.

***Eimeria* species**	**Primer**	**Sequence (5' to 3')**	**Annealing temperature (°C)**	**Size (bp)**
E. adenoeides	E.ad.CO1_427F E.ad.CO1_1186R	CCAACCTCAGTAGATCTAATTGTA GTGGAAGTGAGCAATGACA	62	713
E. dispersa	E.disp.CO1_577F E.disp.CO1_1028R	ACAGCTATTATGTTAATTGGT GCATACCAAGTATCTAATGAA	55	451
E. gallopavonis	E.gal.CO1_292F E.gal.CO1_1153R	AGAGTGAATTGTGTATCACTATTA GAGATAATACGAAATGGAAGTGG	62	861
E. meleagridis	E.md.CO1_431F E.md.CO1_1443R	CCTCAGTAGATTTAATTGTC TTAGAAGATTAGGGAATATAA	58	1012
E. meleagrimitis	E.mel.CO1_474F E.mel.CO1_1028R	CTCAAGTTTCCTATCCTCAG GCGTACCAGATATCTAAGGAG	50	554

Table 2. Results from the COI PCR of turkey field isolates

Isolate[a]	Species tested[b*]			Single infection	Mixed infection
	ADE	MEL	DIS		
A7	-	+	-	+	-
A9	-	+	-	+	-
C2	+	+	-	-	+
C4	-	+	-	+	-
C5	-	+	-	+	-
C6	+	+	-	-	+
C7	-	+	-	+	-
C8	+	+	+	-	+
IA1	-	+	-	+	-
IA2	-	+	-	+	-
K1	-	+	-	+	-
K3	+	+	-	-	+
K4	+	+	-	-	+
M1	-	+	-	+	-
M2	+	+	-	-	+
M4	-	+	-	+	-
M6	-	+	-	+	-
M8	-	+	-	+	-
M9	+	+	-	-	+
M10	-	+	-	+	-
M11	-	+	-	+	-
MI1	-	+	-	+	-
MN1	-	+	-	+	-
MN2	-	+	-	+	-
N3	+	+	+	-	+
P1	-	+	-	+	-
P2	-	+	-	+	-
S1	-	+	-	+	-
V1	-	+	-	+	-
V2	+	+	-	-	+
Total	**9**	**30**	**2**	**21**	**9**

[a]Isolates preceded with the capital letters A, C, I, K, M, MI, MN, N, P, S, and V were from Arkansas, California, Iowa, Kansas, Missouri, Michigan, Minnesota, North Carolina, Pennsylvania, South Dakota, and Virginia, respectively.

[b]Species of *Eimeria* tested were ADE (*E. adenoeides*), MEL (*E. meleagrimitis*), DIS (*E. dispersa*).

[*]None of the samples tested were positive for MED (*E. meleagridis*) and GAL (*E. gallopavonis*) and the data is not shown in the table.

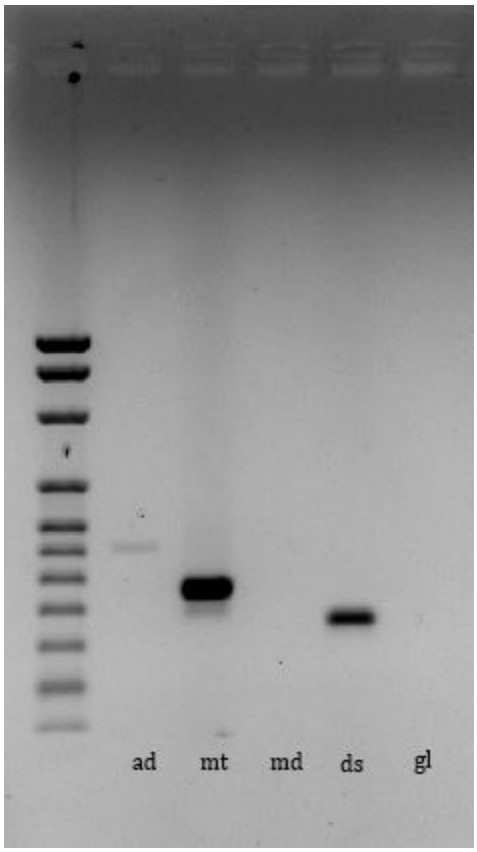

Fig 1. Agar gel electrophoresis of PCR product obtained by amplification of gDNA from N3 isolate using species specific COI primers against turkey *Eimeria*. ad-*Eimeria adenoeides*; mt-*E. meleagrimitis*; md-*E. meleagridis*; ds-*E. dispersa*; gl-*E. gallopavonis*. First lane on the left represents 2.5 Kb DNA ladder.

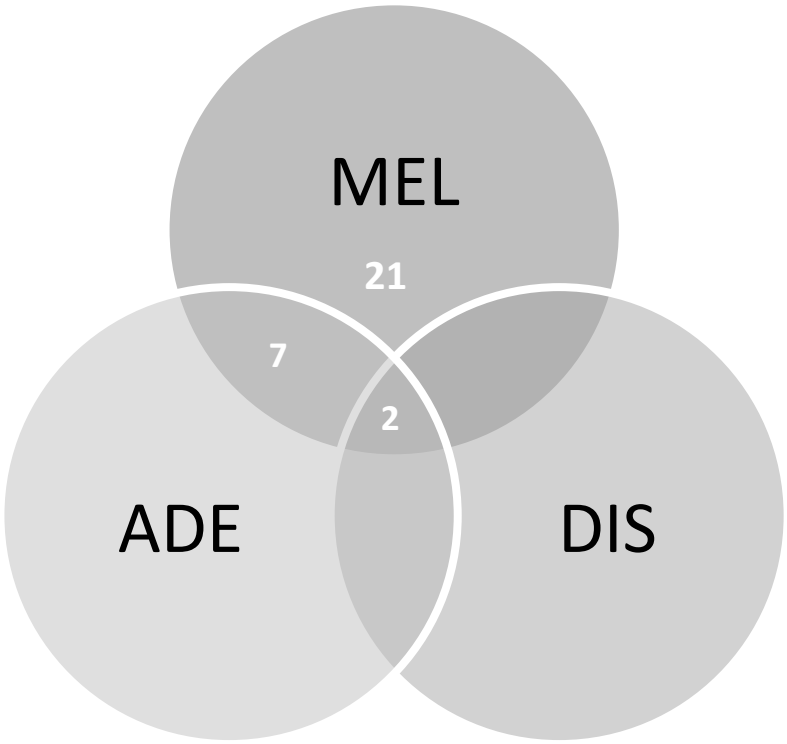

Fig 2.Frequency distribution of *Eimeria meleagrimitis* (MEL), *E. adenoeides* (ADE) and *E. dispersa* (DIS) co-infection in litter samples collected from 30 turkey farms in the USA. *Eimeria* species were identified by PCR amplification of cytochrome c oxidase subunit I gene

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: H.D. Chapman

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: September 12, 2012

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **October 31, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13005- "CONTROL OF COCCIDIOSIS IN TURKEYS**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **10-31-2015**, youmust submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at atime.

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

cnc/car

cc: Animal Welfare Veterinarian

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CHAPTER IV

A COMPARISON OF THE PATHOGENICITY OF TWO STRAINS OF *EIMERIA MELEAGRIMITIS* ISOLATED FROM THE TURKEY

Comparison of the Pathogenicity of a Newly Isolated Field Strain of Turkey Coccidium *Eimeria meleagrimitis* and a Reference Strain

ABSTRACT. The pathogenicity of a strain of *Eimeria meleagrimitis* (Minnesota strain) was evaluated in the current study by comparing it to a reference strain (Weybridge strain). Two-week old poults were infected with 1 x 10^4, 5 x 10^4 or 1 x 10^5 sporulated oocysts of each strain. Body weight gain, feed consumption and feed conversion were estimated from days 0 to 6 post-infection. Lesions in the intestines and fecal droppings were scored on day 6 following infection based upon a new system that was established. The Minnesota strain showed significantly lower pathogenicity at the lowest dose tested (1 x 10^4 oocysts) At higher levels of infection, both strains exhibited significantly high pathogenicity. The Weybridge strain was more pathogenic based on mortality, lesion and dropping scores.

INTRODUCTION

Protozoan parasites of the genus *Eimeria* cause the disease coccidiosis in poultry; an infection that can result in a variety of adverse pathological effects in the host (McDougald, 2003). One of the most important coccidia of the turkey is *Eimeria meleagrimitis* that infects the duodenum and jejunum (Tyzzer, 1929; Clarkson, 1959). An infection with *E. meleagrimitis* can cause an edematous and congested duodenum, erosion of large areas of epithelium, a brownish mucoid exudate, scattered hemorrhages and watery diarrhea (Joyner, 1973).

PCR identification of various turkey field isolates conducted in our laboratory showed *E. meleagrimitis* to be the most prevalent species (Rathinam et al., unpublished obs.). Previously, a

pure line of *E. meleagrimitis* was established from a drug sensitive isolate by single oocyst isolation (Shirley and Harvey, 1996). The objective of this experiment was to determine the pathogenicity of the recently isolated *E. meleagrimitis* strain and compare it with a known reference (Weybridge) strain.

MATERIALS AND METHODS

Birds and husbandry

Female Nicholas turkey poults were obtained from a local hatchery and raised in a brooder in an isolation building at a stocking density of 257 cm^2 / poult. They were fed an unmedicated corn /soybean meal diet suitable for young turkeys and provided with water ad libitum. At 13 days of age poults were weighed and transferred to a test facility and randomly allocated to cages in battery units that had been thoroughly cleaned and fumigated with concentrated ammonia solution prior to use.

Parasites

A pure isolate of *E. meleagrimitis* was kindly provided by Dr. R. N. Marshall of the Veterinary Laboratories Agency (Weybridge), Surrey, UK. This strain had originally been isolated from turkey poults in 1965 in the UK and is referred to as the Weybridge strain (Hein, 1969).

A field isolate of *E. meleagrimitis* was isolated from a litter sample received from a turkey farm in Minnesota, USA (Rathinam and Chapman, 2009). A pure line was obtained using the

method described by Shirley and Harvey (1996) and was subsequently propagated turkeys reared in isolation. Various criteria were used to establish the identity of the pure strain including the presence of macrogametocytes in histological sections of the jejunum, and the size of the oocysts (length x width approximately 20 x 17 µm). For each experiment, cultures were less than a month old when used. Laboratory procedures for all propagations followed guidelines for techniques in coccidiosis research (Shirley, 1995).

Experimental design

The experiment comprised seven treatments, each with four replicate cages containing six birds / cage. Treatments 1-3 comprised poults that were orally inoculated one day after placement in cages (14 days of age) with 1 x 10^4, 5 x 10^4, or 1 x 10^5 oocysts of the Weybridge strain. Treatments 4-6 comprised poults given the same doses of the Minnesota strain. Treatment 7 comprised poults that were sham inoculated with water (uninfected controls).

Weight gain, feed intake, feed conversion, and mortality

Bodyweight gain of poults was determined from day 0 to 6 following infection. The amount of feed consumed from day 0 to 6 was determined and feed conversion calculated. The intestines of any birds that died during the course of study were examined to determine if coccidiosis was the cause of death and the % mortality calculated.

Lesions

Two birds from each cage (8 poults / treatment) were killed on day 6, their intestines removed, and the jejunum examined for characteristics indicative of coccidiosis. It proved difficult to provide a subjective score for the severity of the host response to infection. A variety of pathological effects were observed which were inconsistently present in poults from the same treatment. These included congestion of blood vessels, edema, pale coloration, and a thin intestinal wall and occasionally an enlarged intestine, the presence of necrotic cores, and petechiae in the intestinal wall. A lesion scoring system was developed by utilizing the first four criteria (congestion, fluid, pale coloration, and a thin intestinal wall). A point was allocated for each of these criteria so that for each poult examined a maximum of four points was possible. The mean value for the eight poults in each group was then calculated. Birds that died due to coccidiosis received a score of 4.

Fecal droppings

Feces were scored subjectively on a scale of 0-3 based upon the form of the droppings, their color, the extent of diarrhea, and the presence of any mucus or blood. A score of zero was given if individual droppings were normal in appearance (short and rounded in shape, green in color often with a covering of white urate crystals), and dry with no evidence of diarrhea, blood, or mucus. A score of 1 was given if the droppings were discrete but loose in structure, often sausage-like in shape, green to brown in color, with scattered patches of diarrhea in the tray. A score of 2 was given if half of the droppings had no shape or form, they were brown to orange in color, and diarrhea occupied up to 50 % of the tray. A score of 3 was given where droppings

were minimally formed with no discrete structure, were orange-brown in color with red regions, and diarrhea occupied most of the tray.

Statistical analysis

Cages were assigned by a completely randomized design with four replicates per treatment. All data were analyzed by one-way ANOVA using SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC). Mean separation was done by Student-Newman-Kuel's test and significance was set at $P \leq 0.05$.

RESULTS

Weight gain and Mortality

Significant reductions in weight gain compared with uninfected poults were evident from days 0-6 following infection with both strains of *E. meleagrimitis* (Table 1). No significant differences in weight gain were found in poults whether they were given 5 x 10^4 or 10^5 oocysts of either strain. However, poults given 10^4 oocysts of the Minnesota strain gained more weight from days 0-6 than poults given 10^4 oocysts of the Weybridge strain. One bird died on day 6 after infection with 10^5 oocysts of the Weybridge strain but no deaths occurred in other treatments.

Feed intake and Feed conversion

Feed consumption was significantly reduced compared with uninfected poults from days 0-6 following infection with both strains of *E. meleagrimitis*. No significant differences in feed intake from days 0-6 were found in poults given 5 x 10^4 or 10^5 oocysts of either strain. Poults given 10^4 oocysts of the Minnesota strain consumed more feed than poults given 10^5 oocysts. With the exception of poults given 10^4 oocysts of the Minnesota strain, infected poults had significantly higher feed conversions than uninfected poults (Table 1).

Lesions

Mean lesion scores are presented in Table 1. No lesions were found in uninfected birds and poults given 5 x 10^4 and 10^5 oocysts of the Minnesota strain had a significantly greater lesion score than poults given 10^4 oocysts. A significant increase in lesion score was seen in poults given higher doses of the Weybridge strain. Poults given 10^5 oocysts of the Weybridge strain had a significantly higher lesion score than poults given this dose of the Minnesota strain.

Fecal droppings

Poults infected with the Minnesota strain showed an increase in dropping score as the number of oocysts given increased (Table 1). On day 6 after infection the severity of score was proportional to the dose of oocysts administered for the Minnesota strain of *E. meleagrimitis* but not for the Weybridge strain of that species. Poults given the Weybridge strain had higher dropping scores overall than those given the Minnesota strain.

DISCUSSION

E. meleagrimitis is a pathogenic species that infects the turkey *Eimeria* (Hawkins, 1950; Clarkson, 1959). The pathogenicity of the reference strain (Weybridge strain) used in this study has been established (Hein 1969; Long et al., 1977). A lesion and fecal dropping score system was developed in the current study in an attempt to standardize subjective study criteria. Based on body weight gain, the Minnesota strain was less pathogenic at the lower dose (10^4) compared to the Weybridge strain. Nonetheless, the Minnesota strain suppressed body weight significantly even at the lowest dose tested confirming that it was pathogenic. While no mortality was seen in the birds infected with the Minnesota strain, there was slight mortality (4 %) in poults infected with the Weybridge with a dose of 10^5 oocysts. Feed consumption and feed conversion data support the findings seen with body weight gain.

Existence of strain variation among various species of *Eimeria* has been reported earlier and differences in such strains have been based on physical characteristics, immunogenicity and pathogenicity. Joyner reported difference in immunogenicity of two strains of *E. acervulina* (Joyner, 1969) and *E. tenella* (Joyner and Norton, 1969). In the latter study, comparison of the Weybridge and Houghton strains of *E. tenella* showed that the Weybridge strain was more pathogenic (as seen by cecal lesions) than the Houghton strain (Joyner and Norton, 1969). In turkeys, Long et al. (1977) reported a strain of *E. meleagrimitis* (Yorkshire strain) which had smaller oocysts than previously described for that species. The strain was confirmed to be *E. meleagrimitis* by cross protection test and enzyme analysis.

Pathogenicity of the Minnesota strain is compared to the Yorkshire strain (Long et al., 1977) and Weybridge strain (present study) in Table 2. Based on body weight gain data from in

Long et al. (1977), the Yorkshire strain appears to be less pathogenic at 5×10^4 compared to both Weybridge and Minnesota strains. The higher doses did not have a dose dependent body weight decrease in birds infected with any strain.

The criteria used for evaluation of pathogenicity include body weight gain, feed consumption, feed conversion, lesion score and fecal dropping score. A system was developed for lesion and fecal scoring of *E. meleagrimitis* with an objective of describing each score. A lesion scoring system has been used extensively in chickens to evaluate the efficacy of anticoccidial drugs (Johnson and Reid, 1970). Thus, for the cecal parasite *Eimeria tenella*, lesions in the ceca were graded from 0 to 4 based upon a literature review of pathological signs of infection. Lesion scores have been used to evaluate *Eimeria* infection in the turkey but no description of the actual pathological signs associated with scores of different values has been provided (e.g. Mathis, 1993; McDougald, 1976, 1986; Mitrovic, 1968). Thus, a score of 0-4 was assigned based upon visual evaluation of intestinal damage (Mathis, 1993). In another study, intestinal lesions were scored on a scale of 0-3, where scores of 1, 2, and 3 indicated any detectable lesions, moderately severe lesions, and most severe lesions, respectively, (McDougald 1986). Jeffers and Bentley (1980) concluded that the characteristics of the lesions produced by turkey coccidia do not readily permit the use of a definitive lesion scoring system such as that used in chickens.

Fecal dropping scores have been used to evaluate *Eimeria* infections in the turkey but the actual methods employed have not been described in detail. For example, McDougald (1986) utilized a scoring system from 0 to 3 that was a subjective assessment of the extent of diarrhea and presence of mucus and blood in the droppings. In this study, we use a similar system and define the criteria used for allocating scores from 0 to 3 according to the form of the droppings,

their color, the extent of diarrhea, and the presence of any mucus or blood. We show that on day 6 after infection the severity of score is proportional to the dose of oocysts administered for the Minnesota strain of *E. meleagrimitis* but not for the Weybridge strain of that species (Table 1).

The Minnesota strain of *E. meleagrimitis* showed significantly less pathogenic effects at the lowest dose tested (1×10^4 oocysts) based on all the parameters tested and at higher levels of infection, both strains exhibited significantly high pathogenicity. The Weybridge strain was more pathogenic at 1×10^5 oocysts based on mortality, lesion and dropping scores.

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Table 1. Bodyweight gain, feed intake, feed conversion, mortality, lesion score, and dropping pan score of poults infected with different doses of two strains of *E. meleagrimitis*.

Infected	Oocyst dose[1]	Weight gain (g)[2]	Feed intake (g)[3]	Feed conversion[4]	Mortality %	Lesion score[5]	Dropping score[6]
Uninfected	0	237±6 ^{a}	342^{a}	1.45^{b}	0	0^{c}	0 ^{d}
Minnesota strain	1 x 10^4	141±5 ^{b}	277^{b}	1.96^{b}	0	0.5^{c}	1.0^{c}
	5 x 10^4	96±9 ^{c}	254^{bcd}	2.84^{a}	0	2.375^{b}	2.0^{b}
	1 x 10^5	92±4 ^{c}	248^{cd}	2.70^{a}	0	2.75^{b}	3.0^{a}
Weybridge strain	1 x 10^4	102±9 ^{c}	273^{bc}	2.72^{a}	0	0.75^{c}	2.75^{ab}
	5 x 10^4	85±4^{c}	244^{d}	2.89^{a}	0	2.75^{b}	2.75^{ab}
	1 x 10^5	79±5 ^{c}	253^{bcd}	3.21^{a}	4	3.75^{a}	2.0^{b}

Values in columns with no common superscript differ significantly ($P \leq 0.05$). Each observation is the mean for four cages each containing six poults.

[1]Number of oocysts administered / poult.

[2]Mean bodyweight gain / poult ± SEM from day 0-6 after infection.

[3]Mean feed consumption / poult from day 0-6 after infection.

[4]Feed conversion was calculated by dividing feed intake by bodyweight gain.

[5]Lesion scores were based upon four criteria (congestion, edema, pale coloration, and a thin intestinal wall) for which a point was allocated (maximum of four points / poult). The mean value for eight poults (two poults from four cages of each treatment) was then calculated.

[6]Dropping scores are the mean for four cages each containing six poults.

Table 2. Body weight gain (in % compared to uninfected controls) from days 0-6 (present study) or 0-7 (Long et al., 1977) following an infection with various strains of *E. meleagrimitis*

Dose	Body weight gain (%)		
	Yorkshire strain[1]	Weybridge strain[2]	Minnesota strain[2]
0	100	100	100
1×10^4	Not done	59	43
5×10^4	63	34	40
1×10^5	35	33	39

[1] Data from Long et al., 1977

[2] Data from present study

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: H.D. Chapman

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: September 12, 2012

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **October 31, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13005- "CONTROL OF COCCIDIOSIS IN TURKEYS**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **10-31-2015**, youmust submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at atime.

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

cnc/car

cc: Animal Welfare Veterinarian

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CHAPTER V

ATTENUATION OF A DRUG-SENSITIVE STRAIN OF A TURKEY PROTOZOAN PARASITE *EIMERIA MELEAGRIMITIS* BY PRECOCIOUS SELECTION

Attenuation of a Drug-sensitive Strain of a Turkey Protozoan Parasite Eimeria meleagrimitis by Precocious Selection

ABSTRACT An attenuated line of *Eimeria meleagrimitis* was established by repeated propagation of the parasite in 9-day old turkey poults and subsequent selection for precocious development. Following 20 passages, the prepatent period decreased from 120 hours to 104 hours. A series of experiments were conducted to evaluate the pathogenicity, immunogenicity and fecundity of the newly selected attenuated line. Fourteen-day old poults were infected with $5x10^4$ oocysts of either the parent strain or the attenuated line to ascertain pathogenecity. Judged by body weight gain, feed consumption and feed efficiency following infection, the attenuated line proved to be non-pathogenic. The immunogenicity of the attenuated line was tested by infecting poults with incremental doses of 10^2, 10^3 and 10^4 oocysts at 0, 7, and 14 days of age respectively with either the parent strain or the attenuated line. Twelve days following the last inoculation, all birds were challenged with 5×10^2 oocysts of the parent strain, and oocyst production from 96-168 hours following challenge was determined. No oocysts were seen in both the groups following challenge, indicating that the attenuated line is as immunogenic as the parent strain. The fecundity was assessed by infecting two-week old birds with $5x10^2$ oocysts of either parent or attenuated strain, and oocysts produced in the feces were counted at 12-hour intervals from 96 to 240 hours post-infection. Oocyst production showed that the attenuated line had a patent period that started earlier and was shorter compared to the parent line.

INTRODUCTION

Coccidiosis is an important disease of domestic livestock caused by protozoan parasites of the genus *Eimeria*. The disease is particularly important in the poultry industry where large-scale confinement at high stocking densities has provided ideal conditions for transmission. Seven species of *Eimeria* have been described from the turkey and several of these are considered pathogenic (Chapman, 2008). Infection can result in reduced feed intake, lowered weight gain, impaired absorption of nutrients and death.

At present, control of coccidiosis in the turkeys is achieved by administering anticoccidial drugs in the feed and millions of dollars are spent annually for this purpose. Several compounds are approved but their efficacy has been compromised by the development of drug resistance (Chapman and Rathinam, 2007; Rathinam and Chapman, 2009). An alternative to chemotherapy is immunization of turkeys against coccidiosis. This is feasible since infection with the species of *Eimeria* that parasitize the turkey can induce a protective immune response. Vaccines are available for immunizing turkeys against coccidiosis but the strains used are virulent and can, by recycling in susceptible birds, can cause the disease the vaccine is intended to prevent. *Eimeria meleagrimitis* is one of the most pathogenic species of *Eimeria* that infect turkeys (Clarkson, 1959; Ruff et al., 1980; El-Sherry et al., 2013; Vrba and Pakandl, 2014).

The objective of this study was to develop a line of *Eimeria meleagrimitis* that has lost its pathogenicity but is capable of protecting the turkey against challenge with virulent strains. This was done by selecting for 'precociousness', which involves serial passage following collection of the earliest shed oocysts and passaging in fresh birds, each time attempting to collect at an earlier time than before. By doing so, the time between the infection and appearance of oocysts in the feces (prepatent period) decreases at every passage, with which the parasite loses its

pathogenicity. The term was first coined by Jeffers (1975) when he successfully demonstrated the technique with *Eimeria tenella*.

MATERIALS AND METHODS

Experiment 1: Isolation of precocious E. meleagrimitis

Birds and husbandry. Female turkey poults (Nicholas breed) were obtained from a local hatchery, transferred to an isolation building, and reared in brooder cages at a stocking density of 257 cm^2/poult until they were 9 days of age. Husbandry and management followed guidelines for the care and use of agricultural animals in agricultural research (FASS, 2010) and all experiments were approved by the University of Arkansas Animal Care and Use Committee. At 9 days of age poults were transferred to a test facility and randomly allocated to clean grower cages (five poults/cage). Poults were fed a corn/soybean meal diet formulated according to starter feed requirements for turkey poults.

Parasites. Parasites were isolated from field samples received from commercial turkey farms as described previously (Chapman and Rathinam, 2007). Parasites collected from such field samples comprised a mixture of species and no attempts were made to purify them. They were subjected to a drug sensitivity test (Rathinam and Chapman, 2009) using anticoccidial drugs commonly used in turkey farms. One strain that was sensitive to the tested drugs and showed parasite development in the small intestine (MN2; Rathinam and Chapman, 2009) was selected as a candidate for isolation of *E. meleagrimitis*. A pure line of *E. meleagrimitis* was

established following a single oocyst isolation using established methods (Shirley and Harvey, 1996). This was designated the "parent" strain and used in the current study.

Selection procedure. Selection of precocious line followed procedures similar to those that were successful in the chicken (reviewed in McDonald and Shirley, 2009) and turkey (Matsler and Chapman, 2007). This involved collecting the very first oocysts produced following infection with the parent strain and serial propagation of successive generations in a new flock of turkeys. Groups of 5, 9-day-old poults were infected orally with 5×10^3 sporulated oocysts of the parent strain of *E. meleagrimitis*. Based on available literature, the prepatent period (**PPP**) of *E. meleagrimitis* could be as early as 103 hours (Reid, 1972). Hence, feces produced by infected birds from 0–100 hours post-inoculation (**HPI**) were discarded and subsequently collected from each group at 4-hr intervals.

Oocysts recovered from each 4-hr fecal collection were counted (minimum number detectable was approximately 0.3×10^3 oocysts/bird) and sporulated by methods described by Long et al. (1976). Viable oocysts collected from the earliest time point were then given to a second group of poults. This process was repeated for 20 successive generations in further groups of birds (Table 1). For each generation, an attempt was made to obtain oocysts at an earlier time interval than in the previous passage. At each passage, collections at later time intervals were also made as a backup in the event of failure of oocysts collected from an earlier time point. The intended dose of 5×10^3 oocysts/bird was given when possible except in passages where the number of oocysts produced were less than required to dose the next batch of birds with 5×10^3 oocysts. In such cases, the available oocysts were used for dosing the subsequent batch of birds for the next passage.

Experiment 2: Evaluation of precocious E. meleagrimitis

Pathogenicity. Four groups of 5 poults were inoculated with $5x10^4$ oocysts of either the precocious line or parent strain at 14 days of age. They were weighed on days 0, and 6 after infection, and weight gain was determined. A further four groups of five birds were uninfected and served as controls. The pathogenic dose was selected based on the dose titration study. A dose of $5x10^4$ oocysts of the parent strain caused a significant reduction in weight gain 6 days after infection without significant mortality (Chapter 4, page 79). Feed consumption for the study period (Day 0-6) was measured and feed conversion estimated. On Day 4 post-infection, fecal trays were cleaned out. A dropping score (Chapter 4, page 79) was given on day 6.

Immunogenicity. Thirty poults were placed in six cages (five birds/cage) and inoculated on a weekly basis (at 0, 7, and 14 days of age) with 10^2, 10^3, and 10^4 oocysts of the precocious line or the parent strain of *E. meleagrimitis* respectively. Another set of 15 poults were raised in a separate facility but not infected (uninfected controls). At 25 days, all birds were challenged with 500 oocysts of the parent strain of *E. meleagrimitis*, and the number of oocysts produced in the feces from day 4–8 was recorded.

Fecundity. On Day 14 of age, forty poults were placed in eight cages (five birds/cage) and inoculated with $5x10^2$ oocysts, respectively, of the precocious line or the parent strain of *E. meleagrimitis* (4 cages per treatment). Fecal samples were collected at 12-hour intervals starting

at 96 HPI and continued until 240 HPI. Oocysts were counted by standard techniques and the oocysts produced per bird was calculated.

Statistical analysis

Cages were assigned by a completely randomized design with four replicates (three in immunogenicity study) per treatment. All data were analyzed by one-way ANOVA using SAS Enterprise Guide 4.3 (SAS Institute, Cary, NC). Mean separation was done by Student-Newman-Kuel's test and significance was set at $P \leq 0.05$.

RESULTS

Experiment 1: Isolation of precocious E. meleagrimitis

The prepatent period of the 'parent' strain was 120 hours. The successive passages and reduction in PPP are shown in Table 1. After 20 passages, the PPP was 104 hours. The earliest shed oocysts in some generations did not produce progeny (Table 1; Passages 4, 9, 10, 13, 16). Progeny of oocysts collected from the next time point (4 hours later) of that passage were used in subsequent passages.

Experiment 2: Evaluation of precocious E. meleagrimitis

Pathogenicity. Results of the pathogenicity study are given in Table 2. The birds that were infected with the precocious line had significantly higher body weight gain compared to birds that received the parent strain. Poults that received the precocious line consumed more feed than those that received the parent strain, but significantly less than controls. Feed conversion of birds infected with precocious line was no different from controls. Birds that received the parent strain had a significantly poor feed conversion. Birds infected with the parent strain had a high dropping score while the other groups in the study received no score.

Immunogenicity. Poults that were not immunized and challenged with 500 oocysts of the parent strain produced oocysts (31.2 x 10^6 oocysts per bird). Birds that were immunized with the parent strain and the precocious line and challenged with the parent strain did not produce any oocysts (Table 3).

Reproduction. The earliest time point g that oocysts were observed in the feces was 108 hours for the precocious group and 120 hours for the parent line (Fig. 1). Oocyst production of the parent strain peaked at 156 hours with 158 x10^4 oocysts produced per bird. The spike in birds infected with the precocious line, however, was earlier at 120 hours with 68 x10^5 oocysts produced per bird. By 240 hours following infection, the oocyst output in precocious line had come down to 7 x10^5, while it was still high in the parent strain with 52 x10^5 per bird.

DISCUSSION

An attenuated line of a pathogenic species of turkey parasite *E. meleagrimitis* was developed and

its pathogenicity, immunogenicity and fecundity was evaluated by comparing it with the parent strain. The PPP of *E. meleagrimitis* has been reported to be as short as 103 h (Reid, 1972; Long et al., 1977) to as long as 144 HPI (Tyzzer, 1929; Hawkins, 1952). Other reports have mentioned time points in between including 116 (Clarkson, 1959), 120 (El-Sherry et al., 2013), 120-124 (Vrba and Pakandl, 2014) hours after infection. Levine (1985) states a wide range of 3-6 days of PPP. In our study, based on a four-hour collection procedure, the PPP of the parent strain was 120 hours.

Selection of a strain for precociousness was first described by Jeffers (1975) who attenuated the pathogenic chicken species, *E. tenella*. By collecting the very first oocysts that were produced and subsequently propagating those oocysts, there was a reduction in PPP of 12-18 hours (Jeffers, 1975). Other species of chicken *Eimeria* have also been attenuated by this method following several generations of propagation. The PPP of *E. brunetti* was reduced by 36 h following intense selection for 25 generations (Shirley et al., 1986) while that of *E. maxima* was reduced by 14 h after 12 generations (McDonald et al., 1986a). After 15 generations of selection, the PPP of *E. necatrix* (Shirley and Bellatti, 1984) and *E. tenella* (McDonald et al., 1986b) reduced by 18 and 20 h respectively. There was a reduction of 17 h following 10 generations of selection with *E. acervulina* (McDonald et al., 1982) and 26 h after 14 generations with *E. mitis* (McDonald and Ballingal, 1983). In turkey *Eimeria*, the only available record of attenuation is that of *E. meleagridis* where there was an 8 h decrease in PPP after 20 generations of selection (Matsler and Chapman, 2007). In the current study, PPP of *E. meleagrimitis* decreased from 120 h to 104 h after selection for 20 generations (Table 1).

Attenuation of a strain occurs when the parasite loses an asexual generation during its life cycle and/or because of its low reproducibility due to smaller number of merozoites produced

during schizogony (Jeffers, 1975). In all the chicken studies mentioned above, the parasites that were attenuated by precocious selection had lost at least their last generation of schizogony (Long, 1993). In the current study modification in life cycle of the attenuated line was not investigated. The production of oocysts following a low dose of infection showed that the reproducibility of the attenuated line was low and that it peaked earlier compared to the parent strain (Fig. 1).

Birds infected with *E. meleagrimitis* has been shown to develop solid immunity following daily inoculation with high dose of oocysts daily for 18 days (Augustin and Ridges, 1963). In the present study, around two weeks after the last inoculation dose, birds were challenged with a low dose of the parent strain. Oocyst production was studied following the course of infection. The birds inoculated with the parent strain and precocious line did not produce oocysts proving that the birds had developed a solid immunity. Evaluation of pathogenicity of the precocious line was performed by analyzing body weight gain, feed consumption, feed conversion and dropping score. A dose of 5×10^4 oocysts decreased body weight significantly compared to uninfected controls and such pathogenicity has been observed earlier (Clarkson, 1959; Hein, 1969; Long et al., 1977; Ruff et al., 1980). The precocious line did not suppress body weight and the birds that received the line were comparable to uninfected controls.

E. meleagrimitis is one of the most prevalent (Jeffers and Bentley, 1980; Edgar, 1986; Rathinam et al., unpublished observations) and pathogenic species of turkey *Eimeria*. Inclusion of a line that has been attenuated by selection for precocious development in live vaccines for turkeys will result in better control of coccidiosis.

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Table 1. Oocysts of *Eimeria meleagrimitis* recovered from turkey feces during selection for an attenuated strain based on precocious development

Passage	Oocysts/bird (x10^4) collected at time period indicated (hours after infection)								
	100	**104**	**108**	**112**	**116**	**120**	**124**	**128**	**144**
1	0	0	0	0	0	**1.2**	2.3	2.1	65.36
2					0	**1.7**	4.6	7.5	
3					**0.9**	2.2	3.1	6.5	
4				0.4[#]	**1.21**	1.9	2.37	4.45	
5			0	0	**1.41**	2.73			
6				**0.14**	0.9	1.46	4.31		
7			0	0	**0.75**	3.25	2.90		
8				**0.37**	1.18	4.01	6.35		
9			0.15[#]	**0.41**	0.93	2.17	8.22		
10		0	0.19[#]	**1.27**	1.78	4.69			
11		0	**0.63**	2.45	9.98	4.23			
12		0	**0.48**	1.26	4.70	11.12			
13		0	0.23[#]	**1.13**	3.02	6.55			
14		0	**0.55**	1.29	1.31	6.19			
15		0	**0.94**	4.21	2.17	7.93			
16		0.06[#]	**1.03**	3.02	3.54	6.22			
17	0	**0.23**	1.09	4.45	7.21				
18	0	**0.76**	2.65	5.24	5.91				
19	0	**1.37**	3.01	6.08	9.63				
20	0	**2.08**	1.78	5.91	12.22				

* Values shown in bold were used for next passage

[#] Infection with these oocysts did not produce oocysts in the subsequent passage

Blank cells indicate that samples were not collected at that time point

Table 2. Body weight gain, feed consumption, feed conversion (Day 0-6) and fecal dropping score (Day 6) following infection with $5x10^4$ oocysts of parent or precocious line of *Eimeria meleagrimitis*

Treatment	Body weight gain/poult (g)	Feed consumption/poult (g)	Feed conversion	Dropping score
Uninfected control	159 ± 7^a	257^a	1.62^b	0^b
Infected precocious	146 ± 7^a	210^b	1.44^b	0^b
Infected parent	65 ± 4^b	175^c	2.73^a	1.75^a

Values shown are mean± standard error of mean. Values along a column with different superscript are significantly different ($p\leq0.05$)

Table 3. Oocyst production of 4-week old turkeys immunized with either the precocious line or parent strain of *Eimeria meleagrimitis* following a challenge dose of $5x10^2$ oocysts of the parent strain

Immunization*	**Challenge**	**Oocysts per bird ($x10^6$)****
Non immunized control	Yes	31.2 ± 7
Precocious line	Yes	0 ± 0
Parent strain	Yes	0 ± 0

*Birds were immunized with 10^2, 10^3 and 10^4 oocysts of *E. meleagrimitis* parent strain or precocious line on Day 0, 7 and 14 respectively

**Mean±SEM. Values with different superscript are significantly different ($P \leq 0.05$)

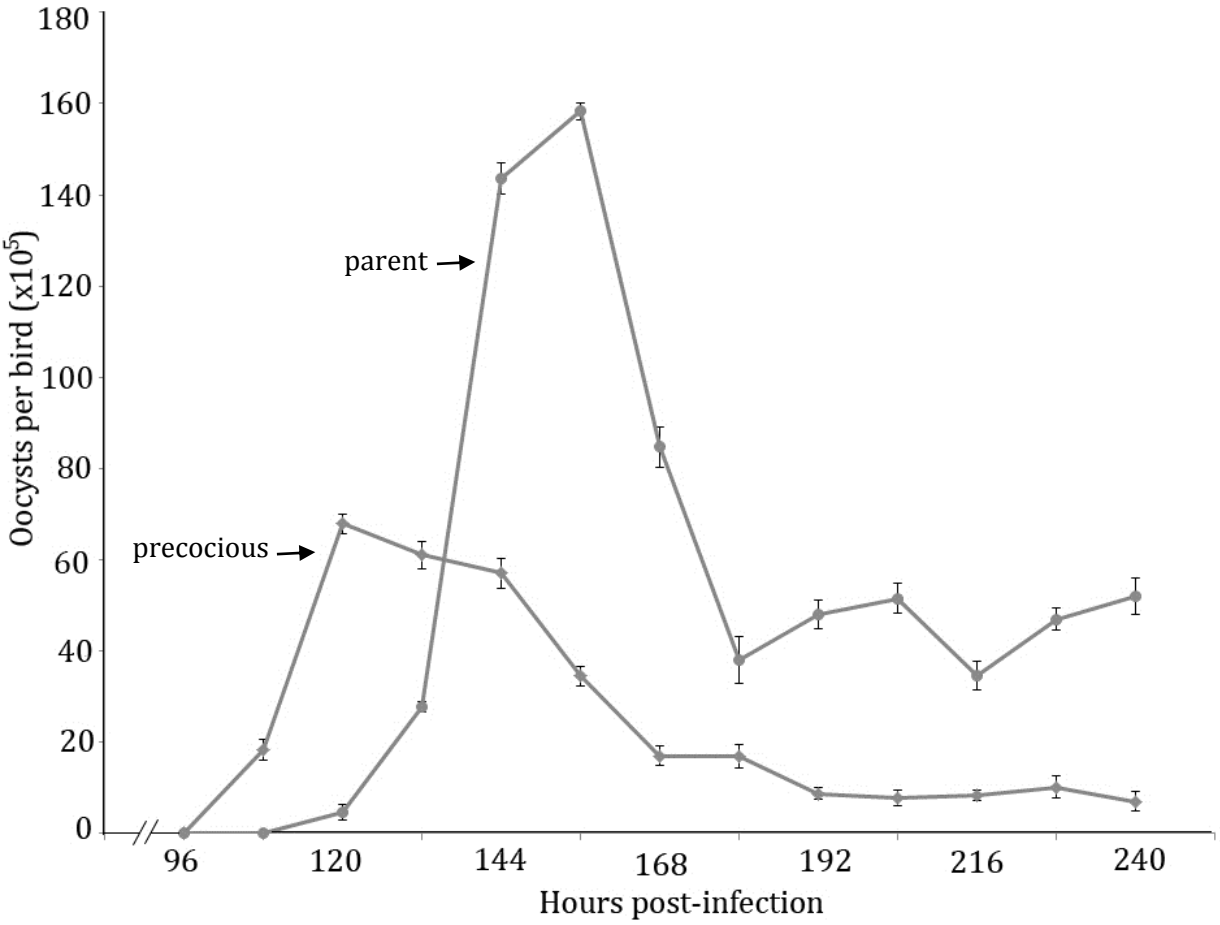

Fig. 1. Oocysts produced in feces per bird (mean±SEM) from 96 to 240 hours following an infection with $5x10^2$ oocysts of *Eimeria meleagrimitis* parent strain or precociously selected line.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: H.D. Chapman

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: September 12, 2012

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **October 31, 2015**

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The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **10-31-2015**, youmust submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at atime.

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

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cc: Animal Welfare Veterinarian

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CONCLUSION

Prolonged prophylactic usage of medication has resulted in *Eimeria* developing resistance to the anticoccidials drugs. Vaccines currently available to control turkey coccidiosis contain virulent wild-type strains and pose a risk of clinical coccidiosis outbreak. Hence, it would be beneficial to have vaccines with strains that are attenuated.

The current study was aimed at identifying a drug-sensitive strain of a pathogenic species of *Eimeria* of turkey, *E. meleagrimitis* and attenuating it. In the first part, *Eimeria* isolated from samples collected from turkey farms across the US were subjected to a drug sensitivity trial. They were tested for sensitivity to four commonly used anticoccidials drugs viz., amprolium, clopidol, monensin and diclazuril. Most of the isolates were resistant to the drugs tested (Chapter 2). In the next phase of the experiment (Chapter 3), identification of various species in samples collected from various farms was attempted using a polymerase chain reaction targeting the cytochrome c oxidase gene. It was shown that *E. meleagrimitis* was the most prevalent species in all the isolates examined. Following this, *E. meleagrimitis* was purified from a drug-sensitive isolate (Minnesota strain) by isolating a single oocyst and propagated in live birds. Subsequently, the pathogenicity of the newly isolated strain was compared to a reference strain of *E. meleagrimitis* (Weybridge) and based on various performance parameters, was shown to be pathogenic (Chapter 4). In the final chapter (Chapter 5), an attenuated line of *E. meleagrimitis* (Minnesota) was selected based on precocious development where the earliest oocysts produced following gametogony were collected, sporulated and propagated in a new batch of birds. After 20 such selections and propagations, the prepatent period had decreased from 120 hours to 104 hours. The newly developed line of *E. meleagrimitis* was compared with the originally isolated

strain (designated the ‘parent’ strain) for pathogenicity, immunogenicity and fecundity. It was shown that the attenuated line had significantly decreased pathogenicity and produced less number of oocysts and produced them earlier than the parent strain. When birds were vaccinated with the attenuated line and challenged with the parent strain, no oocysts were produced indicating that the birds had developed protective immunity against *E. meleagrimitis*.

In the present study, a drug-sensitive strain of *E. meleagrimitis* was identified and an attenuated line was developed from it and was shown that the new attenuated line was less pathogenic but maintained its immunogenicity. Inclusion of a line that has been attenuated by selection for precocious development in live vaccines for turkeys will result in better control of coccidiosis.