Prevalence of parasites in Alabama poultry flocks with special emphasis on *Eimeria maxima*

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 2, 2020

Keywords: poultry, MLST, epidemiology, coccidia, *Eimeria*, backyard

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Abstract

Coccidiosis is a disease caused by *Eimeria* spp. and it is the most prevalent and economically important disease in poultry. Due to its economic importance, the control of coccidiosis is critical. Other parasites can cause disease in chickens but are not as relevant as coccidiosis. Some of these parasites are known zoonoses and can cause disease in humans.

It is thought that keeping chickens as backyard pets has become increasingly popular in the United States. Biosecurity is generally low in backyard flocks. As a consequence, they can serve as reservoirs for various pathogens that pose a risk for commercial poultry or human health. For the first part of the thesis, eighty-four fecal samples from 64 backyard chicken flocks throughout the state of Alabama were collected in the summer of 2017 and 2018. Coccidia oocysts were seen in 64.1% of flocks with oocyst counts in most samples below 10,000 oocysts per gram. Eggs of *Ascaridia* spp. or *Heterakis gallinarum* were seen in 20.3% of the flocks, and eggs of *Capillaria* spp. in 26.6% of the flocks. Egg counts were low, rarely exceeding 1,000 eggs per gram. DNA extracted directly from fecal samples was investigated by PCR for other relevant parasites. The results showed that 4.7% of flocks were positive for *Histomonas meleagridis*, 18.8% of flocks for *Tetratrichomonas gallinarum*, 18.8% of flocks for *Cryptosporidium* spp. and 87.5% of flocks for *Blastocystis* spp..These results will help to provide information that can be used to design outreach programs to improve health and wellbeing of birds in backyard flocks.

The aim of the second part of the thesis was to develop a MLST scheme for *E. maxima* and to compare strains from different farms. *Eimeria maxima* is a parasite of chickens that can cause coccidiosis and predispose its host to secondary infections. Typing *E. maxima* is important as some strains have been shown to provide cross protection to one another and have been linked

to low performance in broilers. *E. maxima* isolates have been typed by sequencing the internal transcribed spacer (ITS) region or sequencing the partial cytochrome oxidase I (COI) gene.

Multi-locus sequence typing (MLST) is a nucleotide sequence typing system that reflects the population and evolutionary biology of bacterial pathogens, but MLST schemes have also been developed for some eukaryotes.

Fecal and litter samples were collected from commercial broiler flocks in Alabama and Tennessee and three fecal samples from backyard flocks were included in the study. Oocysts were isolated from the feces and tested by PCR for *E. maxima*. Nineteen samples were found positive for *E. maxima*. Six gene loci that had been identified based on MLST schemes of several different parasites were amplified for these samples and sequenced. Sequences were compared to the reference genome in GenBank.

There was more than one allele in all six genes. In three of the genes, insertions and deletions were detected. Mutations were present in coding as well as in non-coding sequences in all genes except one. MLST was able to distinguish two commercially available vaccines and showed that most of the field isolates were not identical to these two vaccines. Backyard strains had different allele variants from vaccine and commercial strains of *E. maxima.*

The results show that MLST is a potential tool for epidemiologic investigation of *E. maxima*. The information gained from epidemiological investigations using the method will be useful in the development and evaluation of vaccine programs.

Acknowledgments

First, I would like to thank my parents, Mr. & Mrs. Pete and Julie Carrisosa. I would be nothing if it weren't for you. Thank you for allowing me to take a leap of faith and move across the country several years ago to attend Auburn University. Thank you for believing in me and pushing me to not only become the first person in the family to earn a 4-year degree, but now also a master's degree! This is not just my accomplishment, but yours as well. Thank you for everything, I love you both.

I would like to thank my boyfriend, Ford Gibbes, for being my greatest support system through undergrad and now graduate school. I cannot thank you enough for everything. Thank you for always pushing me to do my best and believing in my dreams. I feel that I can accomplish anything with you by my side. I love you!

To my advisor, Dr. Hauck, thank you for taking a chance on me and believing in me these past 2 and a half years. You have been a wonderful mentor and I couldn't be more grateful for the opportunity that you have given me. You have helped me become more confident as a scientist and in myself. I will truly miss being a part of your lab group, but I look forward to seeing what work will continue to come out of the lab.

Thank you to the farm crew and the rest of the faculty and staff in the department. Each one of you has contributed to my time in Poultry Science. Thank you for taking in this molecular biologist and teaching her about chickens.

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List of Abbreviations

bp	base pair
COI	cytochrome oxidase I
cq	quantification cycle
DNAJ	dnaJ domain containing protein
epg	eggs per gram
HSP	70-kDa heat shock protein
ITS	internal trans
MLST	multi-locus sequence typing
NGS	next-generation sequencing
opg	oocysts per gram
PCR	polymerase chain reaction
PEP	prolyl endopeptidase
qPCR	quantitative polymerase chain reaction
RCC	regulator of chromosome condensation domain containing protein
SCP	serine carboxypeptidase
SNP	single nucleotide polymorphism
VPT	vacuolar proton-translocating ATPase subunit

Chapter 1. Introduction

Coccidiosis, a disease caused by the parasite *Eimeria*, is the most prevalent and economically important diseases in poultry (Shirley et al., 2005; USAHA Committee, 2019). Other parasitic diseases can occur in poultry but are often considered less relevant. Some of these parasites are zoonotic such as *Blastocystis* spp. and *Cryptosporidium* spp..

With Alabama being one of the largest producers of commercial broilers in the United States, control of coccidiosis is critical. Coccidiosis control is often done through the use of anticoccidial programs and vaccination. However, anticoccidials are not perfect and resistance to anticoccidials is increasing. The popularity of antibiotic free and organic flocks also limits the types of anticoccidials allowed.

Backyard flocks – which is a term used to describe a small flock of chickens at a private residence – are thought to be increasingly popular in the United States. People who do own backyard flocks state that they enjoy the fresh eggs and having them as pets but they also are not strict with their biosecurity practices (Elkhoraibi et al., 2014). This can potentially be an issue with the spread of disease to humans and chickens. But there is very limited information available on backyard flocks and even less on parasites in them.

This thesis aims at investigating the epidemiology of *Eimeria* spp. in commercial broilers from Alabama and Tennessee and backyard flocks from Alabama. Samples from commercial broiler flocks that contained *E. maxima* were used to develop a MLST scheme while samples from backyard flocks were used to determine prevalence of selected intestinal parasites in these backyard flocks: *Eimeria* spp. *Ascaridia* spp., *Capillaria* spp., *Heterakis gallinarum*, *Blastocystis* spp., *Cryptosporidium* spp., *Histomonas meleagridis*, and *Tetratrichomonas gallinarum*.

This information can be valuable to understand if backyard strains of *E. maxima* are present in commercial flocks and vice versa. The results from this study can be used to further classify strains of *E. maxima* in all types of chicken flocks.

Chapter 2. Literature Review

2.1 *Eimeria* spp.

Eimeria spp. are the causative agents of coccidiosis, which is one of the most economically important and prevalent diseases in the poultry industry (Shirley et al., 2005; USAHA Committee, 2019). The genus has several species that infect the gastrointestinal tract of the chicken, and *Eimeria* spp. that infect chickens do not infect other animals, so there are no reservoirs in the environment (Cervantes et al., 2020). *Eimeria* spp. are considered ubiquitous in chicken flocks and due to its high host specificity transmission only occurs mechanically through personnel moving between chicken houses and farms.

Coccidiosis is one of the most important threats to poultry due to the fact that *Eimeria*, its causative agent, has a direct life cycle and does not need an intermediate host. *Eimeria* oocysts are highly resistant, can multiply to high numbers in a flock quickly, and they are able to build up resistances to drugs and anticoccidials.

Description & Prevalence

There are nine recognized species of *Eimeria* that infect chickens: *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria hagani*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria mivati*, *Eimeria necatrix*, *Eimeria praecox*, and *Eimeria tenella* (Cervantes et al., 2020). Of those the most prevalent in commercial broilers are *E. acervulina*, *E. maxima*, and *E. tenella* – with *E. acervulina* being the most common (Jeffers, 1974a; Haug et al., 2008; Györke et al., 2013; Hauck et al., 2019a). Concurrent infection with multiple species of *Eimeria* is also very common (Aarthi et al., 2010; Györke et al., 2013; Hauck et al., 2019a). *E. hagani* was described by Levine

(1938), but there is question to whether it is a valid species or not due to the fact that the original description of the species was incomplete (Chapman, 2003).

To be infective, *Eimeria* oocysts must be sporulated. Sporulation of poultry *Eimeria* spp. typically occurs within 24 to 48 hours under proper conditions (Norton and Chard, 1983). For sporulation to occur three factors are necessary: temperature, humidity, and oxygen. Waldenstedt et al. (2001) found that *E. maxima* in lower moisture environments sporulated quicker than in high moisture environments. Graat et al. (1994) found that sporulation rate of *E. acervulina* oocysts in litter and feces was not influenced significantly by temperature and relative humidity but found it highest in dry litter. However, outbreaks with coccidiosis in a flock most often occur in moist conditions – so survival of oocysts rather than sporulation rate contributes to the outbreak (Reyna et al., 1983).

Clinical signs

While most infections are mild or subclinical, coccidiosis can result in outbreaks that can result in major financial losses. Clinical signs include decreased weight gain, bloody diarrhea, morbidity, and in some cases mortality (Cervantes et al., 2020).

In experimental infections with *E. acervulina*, body weight gain decreased during the acute phase of the infection and increased during the recovery. Additionally, it was found that feed intake decreased on day 4 and 5 post infection (Adams et al., 1996). Another experimental infection with *E. acervulina* and *E. maxima* showed an adverse effect on growth and feed conversion for 2-3 weeks, followed by compensatory growth during the recovery period – another 2-3 weeks (Voeten et al., 1988).

A study conducted by Pinard-van der Laan et al. (1998) looked at different lines of chickens for resistance to *E. tenella*. They found that all lines, when experimentally infected, showed a reduction in growth and the white leghorns had 27% mortality.

Lesions

The following lesion descriptions are based on Cervantes et al. (2020), Johnson and Reid (1970), and Conway and McKenzie (2007).

Eimeria acervulina

Lesions caused by *E. acervulina* are white and round in shape, often in ladder-like streaks. These lesions are typically seen in the duodenum. In heavy infections, lesions can extend to the jejunum and ileum.

Eimeria brunetti

Lesions caused by *E. brunetti* present as petechiae in the mucosa of the lower small intestine and thickening of the intestinal wall. There may also be loss of color and watery intetestinal contents. In heavy infections, the mucosa will be damaged and necrosis appearing 5-7 days postinfection. These lesions can be seen in the lower intestine and rectum. Early stages of infection with *E. brunetti* are in the midintestinal region.

Eimeria maxima

E. maxima is the species with the largest oocysts of the *Eimeria* that infect chickens and has an egg-shaped morphology. It is typically seen in the jejunum on either side of the diverticulum and causes the lumen to be filled with yellow or orange mucus and sometimes blood. In heavy infections, lesions can extend from the duodenum to the ileo-cecal junction.

Eimeria mitis

There are no distinct lesions caused by *E. mitis*, in mild infections it could resemble lesions caused by *E. brunetti*. Infection with *E. mitis* will adversely affect performance of the chicken and skin pigmentation.

Eimeria necatrix

Infection with *E. necratrix* can cause lesions such as ballooning, white spots (schizonts), petechiae, and mucoid blood-filled exudate in the jejunum. Later development of *E. necatrix* oocysts occurs in the ceca which can help with species diagnosis.

Eimeria praecox

There are no distinct lesions caused by infection with *E. praecox*, there may be mucoid exudate in the duodenum and watery contents. *E. praecox* is not considered pathogenic.

Eimeria tenella

E. tenella infects and causes lesions in the ceca of the chicken. At onset of infection hemorrhaging occurs, later there will be thickening of the mucosa and clotted blood. In heavy infections, lesions can be seen above and below the cecal junction.

Diagnosis

Diagnosis of coccidiosis can be made immediately upon necropsy by identifying gross lesions in the intestines. If gross lesions alone are not enough to make a diagnosis, microscopy can be used to identify oocysts from intestinal scrapings or fecal samples. Speciating *Eimeria* under a microscope can be difficult as each species has similar oocyst morphologies and size of their oocysts often overlap with one another (Long and Joyner, 1984).

E. acervulina oocysts have lengths between 17.7-20.2µm and widths between 13.7-16.3µm. *E. brunetti* oocysts have lengths between 20.7-30.3µm and widths between 18.1-

24.2µm. *E. maxima* oocysts have lengths between 21.5-42.5µm and widths between 16.5-29.8µm. *E. mitis* oocysts have lengths between 11.7-18.7µm and widths between 11.0-18.0µm. *E. necatrix* oocysts have lengths between 13.2-22.7µm and widths between 11.3-18.3µm. *E. praecox* oocysts have lengths between 19.8-24.7µm and widths between 15.7-19.8µm. *E. tenella* oocysts have lengths between 19.5-26.0µm and widths between 16.5-22.8µm (Cervantes et al., 2020).

If further confirmation is needed to identify specific species, polymerase chain reaction (PCR) is a useful tool. For best results with PCR, DNA should be extracted from purified oocysts as feces is a PCR inhibitor (Schnitzler et al., 1999).

Quantitative real-time PCR (qPCR) allows for efficient identification of *Eimeria* species. This form of PCR uses fluorescence-based method of enzymatic amplification that can detect and measure minute quantities of DNA from tissues, feces, and purified oocysts. Nolan et al. (2015) used qPCR to quantify DNA of *E. tenella* in tissue samples using primers from several genes. Vrba et al. (2010) developed a qPCR assay using SCAR markers to identify seven species of *Eimeria* efficiently that was built off a previous study by Blake et al. (2008).

Multiplex PCR that combines all primers for each *Eimeria* species into a single tube is useful for differentiation (You, 2014). Fernandez et al. (2003b) developed a multiplex PCR assay that would allow the identification of seven *Eimeria* species DNA by using primers with amplicons of different sizes. These primers were based from sequence-characterized amplified region (SCAR) markers (Fernandez et al., 2003a). This method allows for the simultaneous diagnosis of several species of *Eimeria*.

Next-generation sequencing (NGS) with the 18s rDNA gene is another molecular technique that can be used to identify all *Eimeria* species from a flock or individual bird (Vermeulen et al., 2016; Hinsu et al., 2018; Hauck et al., 2019a).

Single nucleotide polymorphism (SNP)-based haplotyping has been used for assessing the genetic diversity of *Eimeria* as it is cost effective and quick to use. Gene loci such as the internal transcribed spacer (ITS) or mitochondrial cytochrome oxidase subunit 1 (COX1) have been used widely for speciating *Eimeria* isolates (Schwarz et al., 2009; Clark et al., 2017). Schwarz et al. (2009) identified multiple variants of *E. maxima* and *E. mitis* in their study. Additionally, MALDI-TOF mass spectrometry has been used recently as well to identify SNPs in *Eimeria* proteins such as elongation factor 2 (EF-2), 14-3-3 protein, ubiquitin-conjugating enzyme domain-containing protein (UCE), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and transhydrogenase (Liu et al., 2017). This method has also been used to type *E. tenella* (Liu et al., 2009).

Treatment

In general, poultry flocks are not treated for coccidiosis. There is a greater emphasis on prevention and control. If needed, flocks will be treated with chemicals and only as a last resort are flocks treated with antibiotics (Cervantes et al., 2020).

Sulphonamides may be used as a treatment and are highly effective against *E. brunetti*, *E. maxima*, and *E. acervulina*. However, this drug can lead to intoxications if used as therapy for coccidiosis outbreaks (Peek and Landman, 2011). Toltrazuril is another drug that can be used to treat coccidiosis as it acts against the intracellular stages of all *Eimeria* species from 1 to 168 hours post infection (Greif, 2000).

Prevention

Prevention is key for the control of coccidiosis in commercial broilers and laying hens either through the use of anticoccidials and vaccination.

Ionophores and chemical compounds are typically used for control of coccidiosis and are mainly given through the feed (Peek and Landman, 2011). There are three different categories of anticoccidials: synthetic compounds, polyether antibiotics or ionophores, and mixed products – a combination of ionophore and synthetic compound or a mixture of synthetic compounds (Peek and Landman, 2011). Anticoccidials have different modes of action depending on what category they fall under and can be more effective against one species of *Eimeria* than others. Some anticoccidials can kill the parasites completely (coccidocidal) while others arrest there development – these are known as coccidiostats (Morehouse and Baron, 1970). Some examples of coccidiostats are novastat and quinolones (Wang, 1976). Withdrawal period on coccidiostat drugs is important to be aware of as well as leaky anticoccidials, as once the drug has been withdrawn parasites may continue their development and contaminate the environment with oocysts (Reid et al., 1969; Cervantes et al., 2020). With leaky anticoccidials, some of the parasites may not stop replicating and will still pass into the environment allowing for natural immunity to be built (Chapman, 1999).

Drugs that are currently approved for use in the United States include the ionophores; monensin, narasin, salinomycin, semduramicin, lasalocid and the chemicals; diclazuril, nicarbazin, amprolium, decoquinate, clopidol, sulfadimethoxine/ormetroprim, and sulfaquinoxaline (Cervantes et al., 2020).

Worldwide use of intensive anticoccidials has led to resistance of these drugs, as any long-term exposure will lead to loss of sensitivity (Peek and Landman, 2011). In order to prevent

drug resistance, rotation and shuttle programs are used. Cross-resistance to anticoccidials tends to occur with *Eimeria* spp., so drugs with distinct modes of actions should be used in anticoccidial programs (Jeffers, 1974a; b). For example, a study conducted by Abbas et al. (2008) found cross resistance of *E. tenella* to salinomycin and mauduramicin, as well as varying sensitivity to maduramicin and clopidol to three field isolates of *E. tenella*. Bedrník et al. (1989) found in their study that field isolates of *E. tenella* were not controlled by the monovalent polyether drugs monensin, narasin, and salinomycin, but were controlled effectively by the monovalent monoglycoside polyether maduramicin and the bivalent polyether lasalocid.

Coccidiosis vaccines are also available to build immunity and protect flocks from outbreaks. Vaccines contain live sporulated oocysts and are administered at an early age. Many of these vaccines contain attenuated coccidia that have been selected for a short life cycle (Vermeulen et al., 2001; Cervantes et al., 2020). Vaccines contain 3 or more species of *Eimeria* – generally *E. acervulina*, *E. maxima*, and *E. tenella* for commercial broilers (Chapman et al., 2002).

Vaccination of day old chicks can be administered in a variety of ways: intra-ocular, hatchery spray, edible gel, and spray on feed (Williams, 2002). With intra-ocular administration, the vaccine is sprayed into the eye of the chicken and the oocysts will pass down the nasolachrymal duct and reach the intestines through the buccal cavity. Experimental studies have shown that this method when applied can achieve uniform dosage and birds reared on litter develop immunity to coccidia (Chapman and Cherry, 1997). Hatchery spray administration sprays the vaccine over trays of chicks who then receive there dosage through oral and ocular routes (Bafundo and Jeffers, 1990). Edible gel administration is often done with Immunocox® combined in a green edible gel that is placed in chick trays at the hatchery or in feed trays in the

poultry house right after placement (Danforth, 1998; Chapman, 2000). The last method is the spray on feed administration which sprays the vaccine on the first feed the day old chicks receive after placement in the poultry house. However, this method needs to be done precisely to the manufacturer's instructions as the live oocysts in the spray vaccine can die from desiccation (Williams, 2002). As long as the proper dose is administered to the chickens, administration of a vaccine by spray or gel application should be effective at producing immunity (Albanese et al., 2018; Tensa and Jordan, 2019).

In ovo vaccination is another form of vaccine administration and more ideal than other methods (Williams, 2002). This method can allow precise application of the vaccine dose and give the earliest onset of immunity (Evans et al., 2002). In experimental studies, chicks vaccinated by in ovo injection had significantly reduced lesion scores, improved weight gain, and reduced shedding of oocysts compared to non-immunized chicks (Weber et al., 2004).

2.2 Nematodes

Nematodes are the most important group of helminths in poultry. Species of *Ascaridia*, *Capillaria*, and *Heterakis* are the most common nematodes seen in commercial poultry.

Description & Prevalence

Ascaridia spp.

Nematodes of the genus *Ascaridia* can invade the small intestine and cause light to moderate damage. All species of *Ascaridia* have a direct life cycle and similar morphologies (McDougald, 2020a). Embryonation of *Ascaridia* eggs is vital to the development of the adult worm and their size (Todd et al., 1952). Eggs recovered from the feces of the chicken host are more likely to embryonate than those recovered from the adult worms uteri (Rahimian et al.,

2016). Larvae of *Ascaridia* inhabit the small intestine of poultry, but larvae themselves are not infective to the chicken – only embryonated eggs (Hansen et al., 1956; Luna-Olivares et al., 2012).

Prevalence studies have found that *Ascaridia* spp. are one of the most prevalent of nematodes that infect chickens (Permin et al., 1999; Thapa et al., 2015). *Ascaridia galli* is a re-emerging as cage-free and free-range production systems become more common (Kaufmann et al., 2011; Thapa et al., 2015). *A. galli* can infect chickens as well as turkeys, doves, ducks, and geese. These adult worms can reach up to four inches in length (McDougald, 2020a).

Capillaria spp.

Capillaria spp. are thin, thread-like nematodes that may be found throughout the digestive system depending on the species. There are multiple species of *Capillaria* that infect chickens such as *Capillaria annulata*, *Capillaria contorta*, *Capillaria obsignata*, *Capillaria caudinflata*, *Capillaria bursata*, and *Capillaria anatis* (McDougald, 2020a). *Capillaria* sp. have a direct life cycle and the eggs can survive up to 1 year in the environment (Levine, 1936). Once ingested by the host, embryonated eggs hatch in the small intestine and reach maturity by 19 days (Wehr, 1939).

C. obsignata is one of the most important worms in commercial chickens as it is prevalent in different poultry production systems (Permin et al., 1999; Kaufmann et al., 2011). It is typically found in the mucosa of the small intestine and may cause morbidity and mortality in severe infections (McDougald, 2020a).

Heterakis spp.

Worms of the genus *Heterakis* are short, approximately 2 cm in length, thin and primarily found in the ceca of birds (McDougald, 2020a). *Heterakis gallinarum* can infect chickens,

turkey, duck, goose, grouse, guinea fowl, partridge, pheasant, and quail. It is the most important nematode in poultry due to its role in the transmission of *Histomonas meleagridis* that causes histomoniasis (Hess and McDougald, 2020). *H. gallinarum* has a direct life cycles and earthworms play a role in transmitting its infective eggs (Lund et al., 1966). *H. gallinarum* is more prevalent during rainy seasons as the population of earthworms increases during this time (Mungube et al., 2008).

It is one of the most frequently diagnosed nematodes and is re-emerging due to the shift to cage-free and free-range production systems (Lund et al., 1970; Permin et al., 1999; Maurer et al., 2009).

Clinical Signs

Ascaridia spp.

Clinical signs seen in chickens infected with *A. galli* are a drop in egg production in relation to decreased weight gain and feed intake (Ackert and Herrick, 1928; Kilpinen et al., 2005).

It has been observed that heavy infection with *A. galli* can have an effect on host behavior and also result in increased cannibalism (Ikeme, 1971; Gauly et al., 2007).

Capillaria spp.

Infection with *Capillaria* can result in production losses in breeders as well as significant growth depression and mortality (Ruff, 1999). It may also cause diarrhea and hemorrhagic enteritis (Wakelin, 1965).

Heterakis spp.

There are no specific clinical signs associated with *H. gallinarum*.

Lesions

Ascaridia spp.

In heavy infections, *A. galli* can cause decreased weight gain and high worm burdens can also lead to intestinal blockages (Reid and Carmon, 1958).

Capillaria spp.

C. obsignata can induce catarrhal exudate and the thickening of the intestinal wall (McDougald, 2020a).

Heterakis spp.

In severe infections inflammation and thickening of the cecal walls can be seen. Additionally, *H. gallinarum* may cause cecal and hepatic granulomas in chickens (Riddell and Gajadhar, 1988).

Diagnosis

Diagnostic of nematodes in poultry can be done at necropsy by seeing the worms in the intestinal tract as well as using a flotation method to view the worm eggs in feces under a microscope.

Capillaria spp. has a distinct egg morphology from *Ascaridia* spp. and *Heterakis* spp. *A.galli* and *H. gallinarum* are very similar in egg morphology with the later being slightly smaller in size (McDougald, 2020a).

Further, PCR can be used to detect nematode DNA from feces and earthworms (Bazh, 2013; Tamaru et al., 2015; Cupo and Beckstead, 2019).

Treatment

Flubendazole and tetramisole are drugs that are highly effective in eliminating infection with nematodes (Bruynooghe et al., 1968; Squires et al., 2012).

In experimental trials, tetramisole (also known as levamisole) was found to eliminate 99-100% of mature and immature parasites with no drop in egg production, fertility, and hatchability (Bruynooghe et al., 1968).

In vitro studies have found that garlic oil has anthelmintic properties against *A. galli* and *H. gallinarum* (Singh and Nagaich, 2000). Diatomaceous earth is another product that can be used to treat parasites. A study conducted by Bennett et al. (2011) found that diatomaceous earth significantly decreased nematode fecal egg counts and worm burdens in laying hens.

Prevention

The most effective way to prevent infection with nematodes is through good management practices and biosecurity. Additionally, controlling earthworms in dirt-floor pens can help limit infection with nematodes such as *H. gallinarum* (Ruff, 1999).

2.3 Blastocystis spp.

Description & Prevalence

One parasite of uncertain clinical relevance to veterinary and human medicine is *Blastocystis* (Tan et al., 2010). The parasite has been isolated from a wide spectrum of hosts including pigs, rodents, poultry, and reptiles which suggests a low host specificity (Boreham and Stenzel, 1993; Souppart et al., 2009). Many parasite prevalence studies show that *Blastocystis* is often the most common parasite found. Lee and Stenzel (1999) found that the parasite was prevalent in 95% of the commerical chickens they investigated for their study.

It is not fully understood whether *Blastocystis* is a pathogen, commensal organisms, or an opportunistic organism, and there are many conflicting studies about whether the parasite causes disease or not – previously published reviews outline clinical aspects observed (Zierdt, 1991;

Stenzel and Boreham, 1996; Tan et al., 2002). In recent years however, more research through epidemiological, in vitro, and animal studies have shown there is a strong possibility of *Blastocystis* pathogenic potential (Tan, 2008).

Clinical Signs

Human Disease

In humans, *Blastocystis* infections may results in clinical symptoms such as diarrhea, abdominal cramps, and nausea (Tan, 2004).

Diagnosis

Diagnose of *Blastocystis* infection is done by PCR (Grabensteiner and Hess, 2006). However, due to irregular shedding that makes *Blastocystis* difficult to identify, multiple stool samples should be examined (Suresh et al., 2009).

Blastocystis may also be diagnosed via microscopy.

Treatment

Generally, due to the controversy over the pathogenesis of *Blastocystis*, treatment is not prescribed for infection. If a drug were to be prescribed, metronidazole would be the drug of choice to eliminate the parasite (Stensvold et al., 2010).

Prevention

The most effective way to prevent *Blastocystis* is through good biosecurity and hygiene standards. In the survey conducted by Lee and Stenzel (1999), one farm that did not have *Blastocystis* present had very high hygiene standards where the floors and equipment at the farm were regularly washed to remove feces.

2.4 Cryptosporidium spp.

Description & Prevalence

There are several species of *Cryptosporidium* that are known zoonotic agents with the most common being *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Cryptosporidium* spp. that have been isolated from mammals have been shown to have little to no host specificity (Lindsay et al., 1986). *C. parvum* is not commonly seen in poultry (McDougald, 2020b). *Cryptosporidium meleagridis*, an avian pathogen, has been shown to be increasingly important as a human pathogen as is makes up 10-20% of human cryptosporidiosis cases in Peru and Thailand (Xiao et al., 2001; Gatei et al., 2002). *Cryptosporidium* is known as an opportunistic parasite of humans – especially young children and immunocompromised individuals. Spherical oocysts are the infective stage of the parasite.

Unlike other coccidian parasites, *Cryptosporidium* oocysts do not contain sporocysts, but do contain four sporozoites. These sporozoites, when ingested or inhaled by the host, will excyst into the intestine and invade the epithelial cells of the respiratory system or the intestine (Roberts and Janovy Jr., 2000a).

Clinical Signs

Poultry Disease

In chickens, infection with *C. baileyi* can cause respiratory disease with signs of sneezing and coughing (McDougald, 2020b).

Human Disease

In human cases of cryptosporidiosis, patients with AIDS can exhibit profuse watery diarrhea that lasts for several months with frequency ranges of 6 to 25 times per day. Individuals

that are more immunocompetent have much less severe symptoms and the infection is often self-limiting with either no symptoms or abdominal cramps (Roberts and Janovy Jr., 2000a).

Lesions

Intestinal cryptosporidiosis in chickens caused by *Cryptosporidium baileyi* may result in histological lesions, but is unlikely to result in gross lesions (McDougald, 2020b). Lesions caused by other *Cryptosporidium* spp. have not been described.

Diagnosis

Poultry Disease

Active infections of *Cryptosporidium* can be diagnosed by microscopy to identify oocysts (McDougald, 2020b). This can be done for both respiratory and intestinal infections.

Human Disease

If *Cryptosporidium* oocysts are still intact, one way to diagnose is to identify the oocysts seen in feces. Oocysts begin to shed as early as five days post infection. Diagnosis should be done by using modified acid-fast staining. Other methods that can be used are direct and indirect immunofluorescence microscopy and ELISA (Roberts and Janovy Jr., 2000a). These two methods cannot speciate but can determine if the oocysts are infectious. PCR is the only method that can speciate *Cryptosporidium* and help determine the origin (Widmer, 1998; Tzipori and Ward, 2002). These methods can be used for cryptosporidiosis cases in both humans and poultry.

Treatment

Poultry Disease

There are currently no treatments available for cryptosporidiosis in chickens.

Human Disease

While most cases of cryptosporidiosis are self-limiting, it has been shown that nitazoxanide can be an effective drug against cryptosporidial diarrhea (Doumbo et al., 1997).

Prevention

For poultry, there are currently no available anti-cryptosporidials or vaccines and control can be difficult as oocysts of *Cryptosporidium* are highly resistant in the environment and to most common disinfectants (McDougald, 2020b).

2.5 Histomonas meleagridis

Description & Prevalence

Histomonas meleagridis is a flagellate parasite and member of the family *Trichomonadidae,* that infects chickens and other gallinaceous birds. The parasite causes the disease commonly known as blackhead or histomoniasis.

Trophozoites of *H. meleagridis* on their own cannot persist in the environment for long. However, the flagellate parasite is known to be transmitted through *H. gallinarum* eggs and when encased in an egg, can remain viable for years under good conditions (Graybill and Smith, 1920; McDougald, 1998). If the *H. gallinarum* eggs are eaten by the appropriate bird, the eggs will then hatch in the intestines and the worms will pass into the cecum where *H. meleagridis* can begin its pathogenesis.

Infection with *H. meleagridis* trophozoites can occur through intracloacal routes – also referred to as cloacal drinking – or the fecal-oral route. Unlike turkeys where transmission of histomoniasis occurs rapidly from bird to bird, in chickens the route of transmission is more dynamic and different. Hu et al. (2006), found that infected chickens did not spread *H. meleagridis* to uninfected birds within the same floor pen or battery cage under experimental infection

conditions. However, another study found that there was some transmission from bird to bird, but still much less compared to the rapid transmission in turkeys (Hess et al., 2006).

Clinical Signs

Infected birds will show clinical signs of droopiness, ruffled feathers, and hanging wings. Typically, yellow diarrhea is also seen. In laying type chickens, there may also be a drop in egg production. Unlike turkeys, histomoniasis in chickens may or may not present any clinical signs (Hess and McDougald, 2020).

Lesions

The lesions caused by histomoniasis are seen in the cecum and liver. The ceca become filled with cheesy plugs that can adhere to cecal walls. Liver lesions are rounded and will be white or green in areas of necrosis (Hess and McDougald, 2020).

In an experimental infection where chickens were challenged with *H. meleagridis* via the oral route, severe lesions were seen in the ceca (Windisch and Hess, 2010). Another experimental infection challenged chickens with *H. meleagridis* intracloacally and found that these birds had severe cecal lesions, but few liver lesions (Hu et al., 2006).

Diagnosis

Most experienced poultry field workers can diagnose histmoniasis in turkeys based on the distinct gross lesions of necrotic nodules on the liver, but since chickens are more likely to only have lesions in the ceca it can be confused with other diseases like coccidiosis (Hess and McDougald, 2020).

Molecular biological tools have greatly enhanced the diagnostics of *H. meleagridis*. Techniques such as PCR allow for the detection of the parasite's DNA in various organs, dust, and in feces (Huber et al., 2005; Hafez et al., 2005; Grabensteiner and Hess, 2006; Sulejmanovic

et al., 2019). Other molecular techniques such as *in situ* hybridization and immunohistochemistry have been used to detect *H. meleagridis* in tissues (Liebhart et al., 2006; Singh et al., 2008).

Treatment

There are no effective drugs against histomoniasis available in the United States (Clark and Kimminau, 2017). Previously drugs such as nitrofurans and nitromidazoles have been highly effective but their use is now banned due to residue and carcinogenic concerns (McDougald, 2005).

Prevention

The best method of control for blackhead disease is to have proper biosecurity practices and an intensive worming program as the *H. gallinarum* worm larvae release histomonads in the process of molting to their adult stage in chickens rather than at their death (Fine, 1975; Ruff, 1999; Hu et al., 2006).

Additionally, in free-range chicken flocks pasture rotation can also be very successful in the prevention of infection with *H. meleagridis* (Roberts and Janovy Jr., 2000b).

While a commercial vaccine is not available for histomoniasis, it has been seen experimentally that vaccination with *in vitro* attenuated *H. meleagridis* can help prevent a drop in egg production in laying hens and protection against the parasite (Liebhart et al., 2011, 2013).

2.6 Tetratrichomonas gallinarum

Description & Prevalence

Tetratrichomonas gallinarum, formerly known as *Trichomonas gallinarum* (Honigberg, 1963), is a very common intestinal flagellate parasite of fowl and gallinaceous birds, both domesticated and wild (McDowell, 1953). It had been previously thought that *T. gallinarum*

caused fatal enterohepatitis, but the true source of the infection was *H. meleagridis* another member of the *Trichomonadidae* (Tyzzer, 1919).

In order for the parasite to be transmitted, it is likely that a bird needs to have direct exposure to the parasite in the feces. Typically, *T. gallinarum* can survive in feces for 24 – 48 hours at room temperature (McLaughlin, 1957). In experimental infections of *T. gallinarum* in specific pathogen free (SPF) chickens via incoluations through the rectum or when consumer in water resulted in an infection (Friedhoff et al., 1991).

T. gallinarum from different avian species are capable of infecting chickens and possibly humans (Hegner, 1929; Friedhoff et al., 1991; Cepicka et al., 2005; Amin et al., 2014). However, *T. gallinarum* includes cryptic species and is thought to be a species complex shown through the existence of molecular polymorphisms (Cepicka et al., 2005).

Clinical Signs

Clinical signs of disease caused by *T. gallinarum* included emaciation, ruffled feathers, diarrhea, and dilation of the ceca (Friedhoff et al., 1991).

Lesions

T. gallinarum infection may result in lesions of the liver that are similar to the lesions caused by an infection with *H. meleagridis* as well as in the cecum (Kemp and Reid, 1965; Hauck et al., 2019b).

In some cases, there have been outbreaks of *T. gallinarum* that have produced granuloma disease that left granulomas in ceca and livers of laying hens in both free range and conventional production systems (Landman et al., 2016, 2019). The study by Landman et al. (2016) further confirmed that *T. gallinarum* was the source of the granulomas by satisfying Koch's Postulates

and doing an experimental infection with the original parasite from the outbreak. This infection was in the absence of *H. meleagridis*.

A study that looked at the presence of *T. gallinarum* and *H. meleagridis* DNA is poultry with suspected cases of histomoniasis from 2004-2008 found *T. gallinarum* DNA in 5.3% of flocks infected with *H. meleagridis* and 27.4% of flocks that were uninfected (Hauck et al., 2010).

Diagnosis

Due to being closely related to *H. meleagridis*, diagnosis of an infection with *T. gallinarum* can be done using a sensitive PCR assay (Grabensteiner and Hess, 2006).

Treatment

Similar to *H. meleagridis*, due to the in availability of drugs there are no legal treatment options for *T. gallinarum* as nitroimidazoles would be affective (Amin et al., 2014). These drugs can be used on birds not used for food production.

Prevention

The best way to prevent infections with *T. gallinarum* are through good biosecurity practices. There are no special prevention methods.

2.7 Multi-locus Sequence Typing

Multi-locus sequence typing (MLST) is a nucleotide sequence typing system that reflects the population and evolutionary biology of bacterial pathogens, but MLST schemes have also been developed for some eukaryotic organisms, specifically protozoa – *Babesia bovis*, *Babesia bigemina,* and *Cryptosporidium hominis* (Gatei et al., 2007; Guillemi et al., 2013).

In an MLST scheme, sequences of 450-500 base pairs (bp) in length for several housekeeping genes are given different allele numbers depending on if they different at a single site or many sites (Maiden et al., 1998). This is because any single point mutation or recombination can result in a new allele. Additionally, it is recommended that a nested strategy be utilized when designing primers for the MLST system to obtain higher quality sequenced data (Urwin and Maiden, 2003).

Majority of bacterial species have enough variation within house-keeping genes to provide many alleles per locus, which allows for many distinct allelic profiles to be distinguished from the chosen house-keeping genes. It is advantageous to use MLST because sequence data is generally unambiguous, and the allelic profiles of isolates can be compared to a large database easily (Chan et al., 2001). Due to its simple technique this typing system can be done in almost any lab that can perform PCR, MLST can be a cost-effective approach to monitoring pathogenic organisms (Maiden et al., 1998; Chan et al., 2001).

MLST typing systems can be useful tools for population and epidemiological studies as it has the capability of examining the evolution of a pathogen and can allow for global monitoring of a specific pathogen (Urwin and Maiden, 2003).

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Chapter 3. Prevalence of select intestinal parasites in Alabama backyard chicken flocks

Abstract

Keeping chickens as backyard pets has become increasingly popular in the United States in recent years. However, biosecurity is generally low in backyard flocks. As a consequence, they can serve as reservoirs for various pathogens that pose a risk for commercial poultry or human health. Eighty-four fecal samples from 64 backyard chicken flocks throughout the state of Alabama were collected in the summer of 2017 and 2018. Coccidia oocysts were seen in 64.1% of flocks with oocyst counts in most samples below 10,000 oocysts per gram. Eggs of *Ascaridia* spp. or *Heterakis gallinarum* were seen in 20.3% of the flocks, and eggs of *Capillaria* spp. in 26.6% of the flocks. Egg counts were low, rarely exceeding 1,000 eggs per gram. DNA extracted directly from fecal samples was investigated by PCR for other relevant parasites. The results showed that 4.7% of flocks were positive for *Histomonas meleagridis*, 18.8% of flocks for *Tetratrichomonas gallinarum*, 18.8% of flocks for *Cryptosporidium* spp. and 87.5% of flocks for *Blastocystis* spp..The results will help to provide information that can be used to design outreach programs to improve health and wellbeing of birds in backyard flocks.

Introduction

In recent years, there seems to be a trend in people wanting to own chickens and having their own backyard flocks in the United States (Pollock et al., 2012; Whitehead and Roberts, 2014; Elkhoraibi et al., 2014; Crespo and Senties-Cue, 2015; Tobin et al., 2015; Cadmus et al., 2019). "Backyard flock" is a term that generally refers to a privately-owned flock of chickens that are kept at a residence. Some states, like California, have a more specific definition where a backyard flock is a flock with less than 1,000 chickens not for commercial use (Cadmus et al., 2019). In

most cases though, these backyard flocks are much smaller as some cities have ordinances that prohibit having more than a certain number of birds.

The most common reasons for backyard flock ownership are to keep the chickens as pets, a learning tool for children or as a source of eggs (Elkhoraibi et al., 2014). Regardless of their reasons for owning chickens, many of these backyard flock owners tend to lack knowledge of proper biosecurity measures, e.g. wearing designated clothes/shoes, not allowing guests to interact with the chickens. They are not aware of the risks associated with exposing their backyard flocks to wild birds and rodents (Karabozhilova et al., 2012; Elkhoraibi et al., 2014). This may be an issue as backyard flocks can become a reservoir for pathogens.

A study conducted by Derksen et al. (2017) investigated antibodies present in California backyard chicken flocks in different proximities to commercial broiler flocks; 77.5% of the investigated flocks had antibodies against Newcastle Disease. Birds being close to a commercial poultry facility were more likely to have antibodies against Newcastle Disease. This study's findings suggest that these pathogens can be transmitted from commercial flocks to backyard flocks and vice versa (Derksen et al., 2017). In addition, zoonotic avian diseases such as salmonellosis are well-documented and recognized as issues in regard to backyard chicken flocks. According to the CDC, as many as 67% of 511 reported illnesses in 2019 being associated with direct contact with poultry (CDC, 2019).

Currently, limited information is available about parasites found in backyard flocks. In birds submitted from backyard flocks to eight veterinary diagnostic laboratories, internal parasites were regarded as the primary cause of mortality in 2.6% of the birds. However, parasitic infections were the most common secondary finding, being seen in 25.5% of the birds (Cadmus et al., 2019). While the cited study looked specifically at diseased birds and flocks, the aim of the present study was to

determine the population of relevant parasitic organisms found in backyard poultry flocks without ongoing disease.

Materials and Methods

Sample collection. Eighty-four fecal samples from 64 different, non-commercial backyard chicken flocks with less than 50 chickens throughout the state of Alabama were included in the study. The flocks were selected opportunistically, and the samples were collected and submitted by the owners. Forty-seven samples from 41 flocks were submitted in the summer of 2017 and 37 samples from 23 flocks in the summer of 2018. Two of the fecal samples were from turkeys kept on the same premises with chickens that were also sampled. Each sample was stored at 4°C upon arrival for microscopy and at -20°C for DNA extraction. Four owners submitted samples of their flocks in both 2017 and 2018; however, in the present study they are considered different flocks.

Oocysts and nematode egg detection. Each backyard fecal sample was mixed thoroughly, and 1 g was suspended in 29 mL saturated NaCl solution. Debris was filtered out through a sieve. A McMaster chamber was filled with the fecal mixture and placed on a microscope where *Eimeria* spp. oocysts and nematode eggs were counted. Total number of oocysts and eggs in the chambers were multiplied by 100 to get the oocysts per gram (opg) and eggs per gram (epg). *Ascaridia* spp. and *Heterakis gallinarum* eggs were not differentiated due to similar egg morphology.

Oocyst purification and qPCR to detect *Eimeria*. Oocysts were purified and concentrated from 4 g feces of 47 samples with *Eimeria* oocysts as described by Hafeez et al. (2015). Three positive samples were not further processed due to lack of material. DNA was extracted from the

purified oocysts using the QIAGEN QiaAmp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol, and *Eimeria* DNA was quantified by qPCR with 45 cycles detecting *Eimeria* 5S rDNA as described (Blake et al., 2006; Hauck et al., 2019). Correlation between parasite load in opg and detected DNA load expressed as the number of the cycles of the qPCR minus the quantification cycle (Cq) was assessed by calculating Spearman's rho using R 3.6.0 (R Core Team, 2019).

Stool DNA Extraction and PCR for other parasites. DNA was extracted from one fecal sample per flock using the QIAGEN QIAamp Stool Mini Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). *Histomonas meleagridis*, *Tetratrichomonas gallinarum*, *Blastocystis* spp., and *Cryptosporidium* spp. and were detected by PCR using established protocols. Primers and references are listed in Table 1.

Results

***Eimeria* and nematode prevalence**. *Eimeria* were detected in 41 flocks (64.1%) and 50 samples (59.5%). Median parasite load was 800 opg; however, several samples had greater than 10,000 opg. In the two samples from turkeys, no coccidia were observed. *Ascaridia* spp. or *H. gallinarum* eggs were detected in 13 flocks (20.3%) and 16 samples (19.0%), while *Capillaria* spp. were present in 17 flocks (26.6%) and 22 samples (26.2%). Median epg for all nematodes was less than 500 (Table 2, Fig. 1). In one of the two samples from turkeys, 200 epg *Ascaridia* spp. or *H. gallinarum* eggs were seen. Of the four flocks that submitted samples in both 2017 and 2018, one flock had a change in status for coccidia from negative to positive and two had changes in *Ascaridia/Heterakis* egg status from negative to positive. There were no changes in prevalence of *Capillaria* spp. eggs from year 2017 and 2018.

Quantification of *Eimeria* oocysts by qPCR. The 5S rDNA qPCR failed to detect *Eimeria* DNA in four samples with 100 opg (two samples), 4,000 opg, and 30,800 opg. A spearman's rho of 0.31 showed only a weak correlation between the parasite load seen in the feces and the DNA load detected by qPCR (Fig. 2).

Prevalence of other parasites. Of the 64 DNA samples, one from each flock, tested by PCR, 4.7% were positive for *H. meleagridis*, 18.8% for *T. gallinarum*, 18.8% for *Cryptosporidium* spp., and 87.5% for *Blastocystis* spp. (Table 3). Of the four flocks whose owners submitted samples in both 2017 and 2018, only one flock had a change in status for both *T. gallinarum* and *Blastocystis* spp. with the flock being positive in 2017 but negative in 2018. There were no changes in any of the other species of parasites in the flocks that submitted samples in both years.

Discussion

Backyard flocks may be a concern to public health and the commercial industry as they could potentially be a reservoir for pathogens. This is due to the fact that many of these flocks have poor biosecurity and have frequent access to the outdoors, which allows them to come in contact with wild birds and other animals, such as rodents, that can transmit disease (Whitehead and Roberts, 2014; Pohjola et al., 2016). In the present study, the prevalence of avian pathogens as well as zoonotic parasites in backyard flocks was investigated. Poultry pathogens included *Eimeria* spp., *Ascardia* spp., *H. gallinarum*, *Capillaria* spp., *H. meleagridis*, and *T. gallinarum*.

Eimeria spp. are considered ubiquitous in chicken flocks. However, in this study, their oocysts were only detected in 59.5% of the samples and 64.1% of the flocks, and oocyst counts in most samples were low. This reflected the equilibrium between infection and immune answer present

in older chickens, as well as the lower stocking density in backyard flocks, which decreases the infection pressure.

Four samples in which coccidia oocysts had been seen tested negative by qPCR. One likely reason for the discrepancy is a lack of sensitivity of the qPCR: Two of the samples in question contained only 100 opg. In consequence, each PCR reaction contained DNA of less than two oocysts, not taking into account inevitable losses during purification of the oocysts and extraction of the DNA. On the other hand, two samples with considerably higher oocyst counts were tested negative as well. The most likely reason is that the observed oocysts were not *Eimeria* infecting chickens but other hosts; the primers of this qPCR were designed based on sequences of *Eimeria* infecting chickens (Blake et al., 2006) and might not amplify other *Eimeria* spp.. In fact, *Eimeria* from other hosts such as squirrels and mice were detected in some of the samples when amplified by pan-*Eimeria* PCR primers (Results published by Hauck et al., 2019); the samples in question here were not processed further after the negative qPCR result.

There was only a weak correlation between the parasite load seen in the feces and the DNA load detected by qPCR. The reasons probably include the presence of *Eimeria* from other hosts in addition to varying losses of oocysts during the purification of the oocysts and age of the sample. Testing samples from commercial poultry by the same methods showed a better correlation and no sample in which *Eimeria* oocysts had been seen tested negative by qPCR (results not shown).

The prevalence of *A. galli* and *Capillaria* spp. in the present study was lower than in organic layers in Europe, where flock prevalence of the two parasites as determined by investigation of fecal samples was between 49.3% and 100% (Thapa et al., 2015). However, the mean of 576 epg was similar to the results presented here (Thapa et al., 2015).

In European commercial pullet and layer flocks, antibodies against *H. meleagridis* were detected in up to 37.3% of the tested birds and 89.3% of the tested flocks (Grafl et al., 2011; van der Heijden and Landman, 2011). In contrast, in the present study, the prevalence as detected by PCR was extremely low. This compares to findings by Cadmus et al. (2019) who diagnosed histomoniasis based on lesions only in very few chickens. No nematode eggs were detected in the samples that tested positive for *H. meleagridis*. However, unfortunately these samples were very dry, which decreased the likelihood to detect nematode eggs.

In commercial poultry in Germany, *T. gallinarum* DNA was detected in 17.7% of flocks in which lesions resembling histomonosis were observed, which is similar to the flock prevalence found here (Hauck et al., 2010). To our knowledge, there are no previous studies investigating its prevalence in not-diseased commercial or backyard poultry. In the present study, a single flock had concurrent infect with both *T. gallinarum* and *H. meleagridis*.

Zoonotic parasites that were investigated included *Cryptosproidium* spp. and *Blastocystis* spp. There are several species of *Cryptosporidium* that are known zoonotic agents. *Cryptosporidium meleagridis*, an avian pathogen, has been shown to be increasingly important as a human pathogen as is makes up 10-20% of human cryptosporidiosis cases in Peru and Thailand (Xiao and Feng, 2008). Due to the low host specificity of *C. meleagridis* and other *Cryptosporidium* spp., it is important for backyard flock owners to be aware of this and improve biosecurity as they could potentially become ill.

Blastocystis spp. are very common in chickens and seems to have a low host specificity. *Blastocystis* infections may results in clinical symptoms such as diarrhea, abdominal cramps, and nausea. However, is unclear if *Blastocystis* spp. infecting chickens can cause disease in humans (Tan, 2004; Souppart et al., 2009). In this study, there was a high prevalence of *Blastocystis* spp.

with 87.5% of backyard flocks being infected. This is unsurprising as another study found a *Blastocystis* prevalence of 95% in commercial chickens (Lee and Stenzel, 1999).

Overall, prevalence of the investigated parasites in backyard flocks was lower than expected as chickens with access to the outdoors generally have higher rates of parasites (Permin et al., 1999; Kaufmann et al., 2011). However, this prevalence may be underestimated due to sample quality, shedding patterns of the parasites, and possible treatments administered to flocks. Only one flock was free of the parasites investigated and 11 flocks were only infected with a single parasite (Appendix). 52 flocks had concurrent infection with two or more of the slected parasites the flocks were examined for.

Some samples were received dried out, which made detection of *Eimeria* oocysts and nematode eggs with the McMaster method difficult. Other samples contained more dirt and debris than fecal matter. In addition, the cool chain during transport to the laboratory was not maintained for all samples.

Shedding patterns of the parasites may have an effect on the results. *Eimeria* are shed in variable amounts based on days post infection (MyungJo, 2014). A study that looked into diurnal fluctuations of nematode egg excretion found that egg shedding was higher during the day, early morning to noon, than in the afternoon and night (Wongrak et al., 2015), and *H. meleagridis* is shed only intermittently from chronically infected chickens (Hess et al., 2006). Conesquently, the low prevalence of parasites, is likely underestimated.

However, the low parasite loads did not indicate a risk that these backyard flocks pose as a parasite reservoir to commercial poultry. In addition, backyard coccidia populations differed from those in commercial flocks, which does not indicate an exchange of coccidia between commercial and backyard chickens (Hauck et al., 2019). However, the cited study also found sequences of

Eimeria spp. of other hosts in these backyard flocks indicating that biosecurity is lacking in many backyard flocks and more care needs to be taken to keep the risk of parasite spread from backyard flocks into commercial chickens low. In addition, the results of this study will help to provide information to the owners of backyard chicken flocks that can be used to design timely and appropriate extension outreach programming based on the types of internal parasites to improve health and wellbeing of birds in backyard flocks.

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Acknowledgment

This project was supported by the Alabama Agricultural Experiment Station and the Hatch program of the National Institute of Food and Agriculture, U.S. Department of Agriculture and the Animal Health and Disease Research (AHDR) program, College of Veterinary Medicine, Auburn University.

Table 1. Nucleotide sequences and references of the primers used to detect the parasites in fecal samples of backyard chicken flocks.

Target species	Target gene	Primer sequence		Amplicon size in base pairs	Reference
		Forward (5'-3')	Reverse (5'-3')		
Eimeria spp.	5S rRNA	TCATCACCCAAAGGGATT Probe: [6-FAM] CGC CGC TTA ACT TCG GAG TTC AGA TGG GAT [BHQ-1][1]	TTCATACTGCGTCTAATGCAC	~110	Blake et al., 2006
Blastocystis spp.	18S rRNA	TAACCGTAGTAATTCTAGGGC	AACGTTAATATACGCTATTGG	459	Grabensteiner and Hess, 2006
Cryptosporidium spp. (outer)	18S rRNA	TTCTAGAGCTAATACATGCG	CCCTAATCCTTCGAAACAGGA	1325	Grabensteiner and Hess, 2006
Cryptosporidium spp. (nested)	18S rRNA	GGAAGGGTTGTATTTATTAGATAAAG	AAGGAGTAAGGAACAACCTCCA	830	Helmy et al., 2017
Histomonas meleagridis	18S rRNA	CCGTGATGTCCTTTAGATGC	GATCTTTTCAAATTAGCTTTAAATTATTC	603	Hauck and Hafez, 2012
Tetratrichomonas gallinarum	18S rRNA	GCAATTGTTTCTCCAGAAGTG	GATGGCTCTCTTTGAGCTTG	526	Grabensteiner and Hess, 2006

[1][6-FAM] is fluorophore attached at 5’ end [BHQ-1] is the quencher molecule that attaches at the 3’ end.

Table 2. Prevalence of *Eimeria* spp. oocysts and nematodes eggs in fecal samples of backyard chicken flocks and median oocysts (OPG) and eggs per gram (EPG).

Species	Positive samples (n=84)	Positive flocks (n=64)	Median opg/epg
Eimeria spp.	50 (59.5%)	41 (64.1%)	800
Ascaridia galli/Heterakis gallinarum	16 (19%)	13 (20.3%)	350
Capillaria spp.	22 (26.2%)	17 (26.6%)	200

Table 3. Prevalence of the parasites investigated in backyard chicken flocks

	Eimeria spp.	*Ascaridia galli*/ *Heterakis gallinarum*	*Capillaria* spp.	*Histomonas meleagridis*	*Tetratrichimonas gallinarum*	*Cryptosporidium* spp.	*Blastocystis* spp.
Positive (n=64)	41 (64.1%)	13 (20.3%)	17 (26.6%)	3 (4.7%)	12 (18.8%)	12 (18.8%)	56 (87.5%)

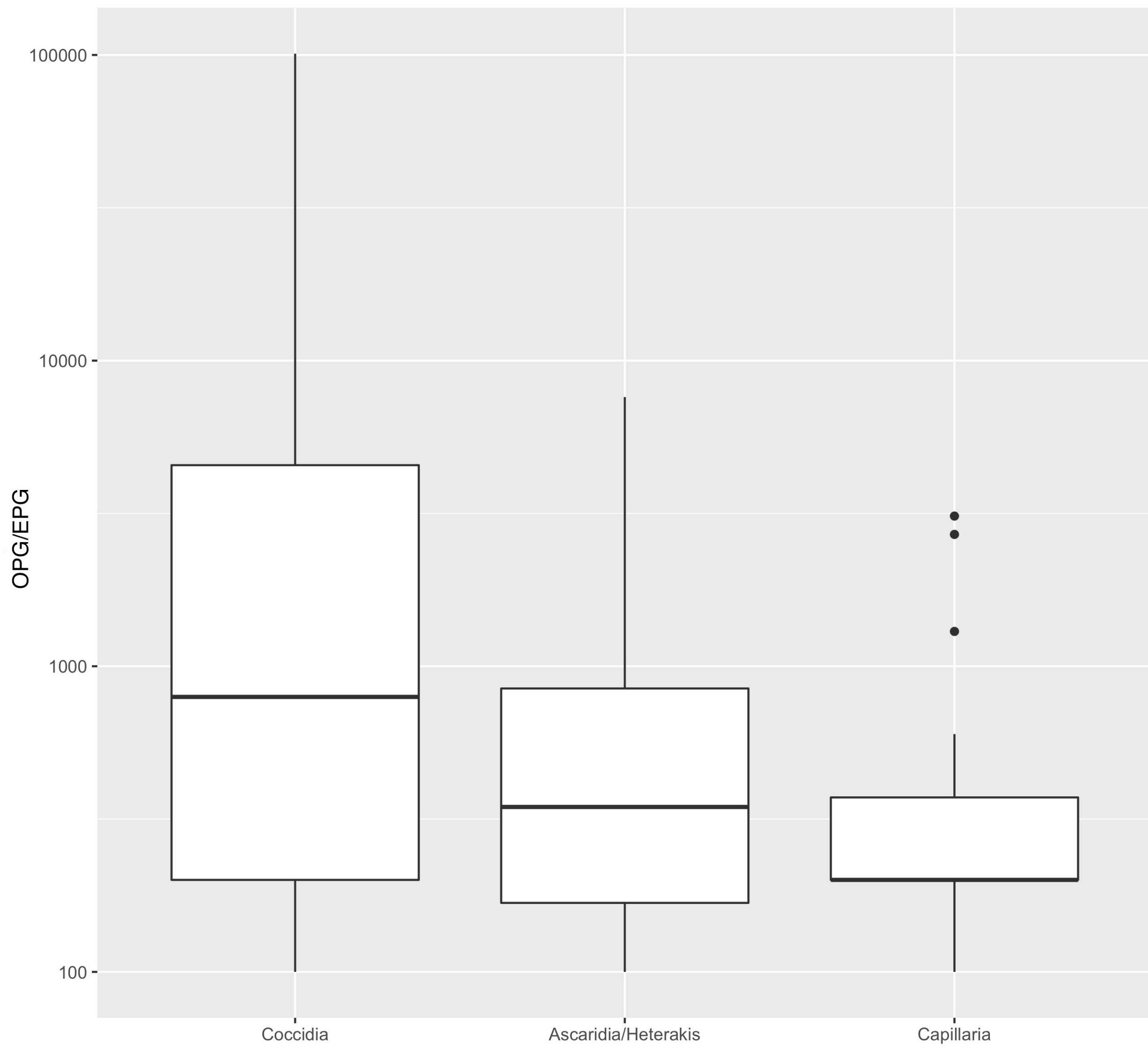

Figure 1. Coccidia oocysts per gram feces (OPG) and Ascaricia galli / Heterakis gallinarum eggs per gram feces (EPG) detected in fecal samples of backyard chicken flocks shown on log scale.

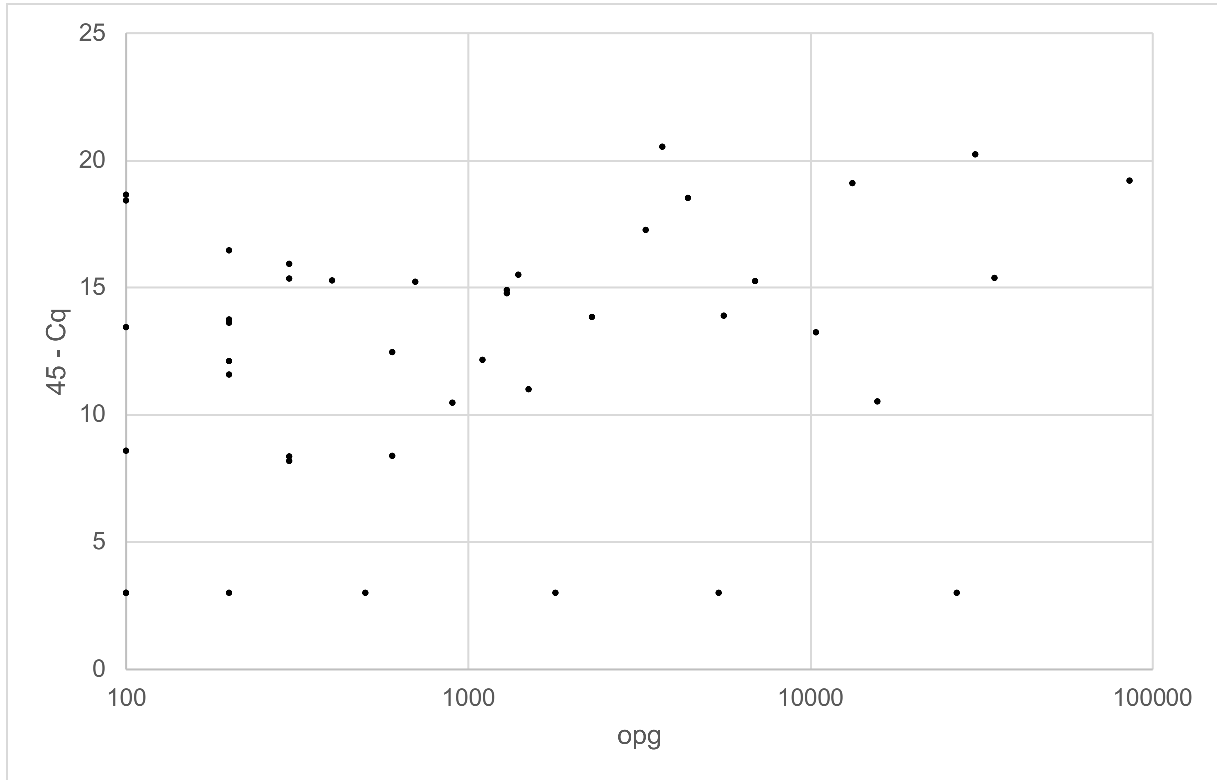

Figure 2. Scatterplot correlating the number of coccidia oocysts per gram feces (opg) and the detected DNA load expressed as the number of the cycles of the qPCR minus the quantification cycle Cq.

Chapter 4. Multi locus sequence typing of *Eimeria maxima* from Alabama & Tennessee, USA

Abstract

Eimeria maxima is a parasite of chickens that can cause coccidiosis and predispose its host to secondary infections. Typing *E. maxima* is important as some strains have been shown to not provide cross protection to one another and some genotypes have been linked to low performance on broilers. E. *maxima* isolates have been typed by sequencing the internal transcribed spacer (ITS) region or sequencing the partial cytochrome oxidase I (COI) gene. Multi-locus sequence typing (MLST) is a nucleotide sequence typing system that reflects the population and evolutionary biology of bacterial pathogens, but MLST schemes have also been developed for some eukaryotes. The aim of the present investigation was to develop a MLST scheme for *E. maxima* and to compare strains from different farms.

Nineteen fecal and litter samples were collected from commercial broiler flocks in Alabama and Tennessee. Oocysts were isolated from the feces and tested by PCR for *E. maxima*. If found positive for *E. maxima*, six gene loci that had been identified based on MLST schemes of several different parasites were amplified and sequenced. Sequences were compared to the reference genome in GenBank.

There were more than one allele variants in all six genes. In four of the genes, insertions and deletions were detected. Mutations were present in coding or non-coding sequences in all genes. MLST was able to distinguish two commercially available vaccines and showed that at least 14 of the field isolates were not similar to these two vaccines.

The results show that MLST is a potential tool for epidemiologic investigation of *E. maxima*. The information gained from epidemiological investigations using the method will be useful in the development and evaluation of vaccine programs.

Introduction

Coccidiosis is one of the most economically important diseases in commercial broiler production (USAHA Committee, 2019). It is caused by infection with the parasite *Eimeria*, which has several species that infects the gastrointestinal tract of chickens. *Eimeria maxima* is one of the most common species of *Eimeria* in commercial broilers that can predispose the host to secondary infections with *Clostridium perfringens*, the causative agent of Necrotic Enteritis (NE) (Jang et al., 2013).

Typing of *E. maxima* is important because some strains do not produce cross-protection for others. Some genotypes have been linked to low performance in broilers. Typing can be done through animal studies or by using molecular techniques.

Martin et al. (1997) used two lab strains of *E. maxima* and three field isolates of *E. maxima* all from North America. Day-old chicks were inoculated to immunize with the strains and challenged at day 10. It was found that the two lab strains and one of the field strains produced cross-protection for each other which the other two field strains only produced protection for their homologous strain.

Single nucleotide polymorphism (SNP)-based haplotyping has been used for assessing the genetic diversity of *Eimeria* as it is cost effective and quick to use (Blake et al., 2015). Gene loci such as the internal transcribed spacer (ITS) or mitochondrial cytochrome oxidase subunit 1 (COX1) have been used widely for speciating *Eimeria* isolates (Clark et al., 2017). Schwarz et al. (2009) analyzed sequence of *Eimeria* at three loci: 18S rRNA, ITS, and cytochrome oxidase I (COI) genes. In the study they identified multiple variants of *E. maxima* and *Eimeria mitis,* with two of the *E. maxima* strains being associated to low performing farms.

MALDI-TOF mass spectrometry has been used recently as well to identify proteins and has been used to type *Eimeria tenella* (Liu et al., 2009, 2017). Additionally, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is another genotyping tool that has be utilized to detect SNPs for *E. tenella* but found limited variation within the UK and Ireland (Pegg et al., 2016).

Clark et al. (2016) sequenced *Eimeria* spp. from 21 countries with the ITS gene. That study found that there was widespread variation of *E. tenella* throughout the world but that certain species variants – OTUs X, Y, and Z – were only identified in the southern hemisphere of the world.

Multi-locus sequence typing (MLST) schemes are tools that utilize nucleotide sequencing to characterize isolates of pathogens – mostly bacterial, but some eukaryotic organisms have been typed with this typing scheme. Sequences of 450-500 base pairs (bp) in length of several housekeeping genes are used to characterize the pathogen (Urwin and Maiden, 2003).

In an MLST scheme, sequences are given different allele numbers depending on if they different at a single site or many sites. This is because any single point mutation or recombination can result in a new allele. Majority of bacterial species have enough variation within house-keeping genes to provide many alleles per locus, which allows for many distinct allelic profiles to be distinguished from the chosen house-keeping genes. It is advantageous to use MLST because sequence data is generally unambiguous, and the allelic profiles of isolates can be compared to a large database easily.

While most MLST schemes are used to type bacterial species, there have been some eukaryotic organisms, specifically protozoa, that have been typed – *Babesia bovis*, *Babesia bigemina,* and *Cryptosporidium hominis* (Gatei et al., 2007; Guillemi et al., 2013).

The objective of this study was to develop a MLST scheme for *Eimeria maxima* to compare strains from different farms and production systems.

Matherials & Methods

Sample collection. Nineteen fecal and litter samples were collected from commercial broiler houses. Samples 47c-19 and 48a-19 come from the same farm, but different houses months apart. Samples were stored at room temperature and processed within 24 hours. Additionally, three backyard flock fecal samples and three commercial vaccines containing *E. maxima* were included in the study. OTU X DNA was kindly provided by Dr. Damer Blake.

Oocysts detection. For each sample, the sample was mixed thoroughly, and 1 g was suspended in 29 mL saturated NaCl solution. The solution was combined, and debris was filtered out through a sieve. A McMaster chamber was filled with the fecal mixture and placed on a microscope where *Eimeria* spp. oocysts were counted. Total number of oocysts and eggs in the chambers were multiplied by 100 to get the oocysts per gram (opg).

Oocyst purification and qPCR to detect *Eimeria* maxima. Oocysts were purified and concentrated from 4 g feces from field samples in which *Eimeria* oocysts had been detected as described by Hafeez et al. (2015). Commercial vaccine samples A, B, and C were not further purified. DNA was extracted from the purified oocysts and vaccines using the QiaAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and *Eimeria* DNA was quantified by a qPCR with 45 cycles detecting *Eimeria* 5S rDNA as described (Blake et al., 2006). All samples used and DNA from OTU-X were checked for *E. maxima* using genus

specific primers (Vrba et al., 2010). Samples not positive for *E. maxima* were not included in the present study, with the exception of vaccine C. Population of *Eimeria* species in 15 samples identified using next-generation amplicon sequencing methods previously described by Hauck et al. (2019).

Sequencing of selected genes. Six genes were determined to be suitable for the MLST scheme. These genes include: dnaJ domain containing protein (*DNAJ*), 70-kDa heat shock protein (*HSP*), prolyl endopeptidase (*PEP*), regulator of chromosome condensation domain containing protein (*RCC*), serine carboxypeptidase (*SCP*), and vacuolar proton-translocating ATPase subunit (*VPT*). Nested PCR primer sets with two forward and two reverse primers were designed for each of the 6 genes using Primer3Plus (Untergasser et al., 2012) with standard settings. All primers were checked *in silico* against other *Eimeria* species (Table 4). These primer sets were designed to include coding and non-coding regions. Samples that were positive for *E. maxima* were run with the outer primer set through the PCR program twice. Reaction was set up using Q5 High-Fidelity 2X Master Mix (New England BioLabs, Ipswich, MA) according to manufacturer's instructions. Cycling conditions consisted of an initial denaturation at 94°C for 3 min and 40 cycles of 30 sec at 65°C and 1 min at 72°C, with a final extension step at 72°C for 10 min. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were sequenced forward and reverse using the inner primers set by MGH CCIB DNA Core (Cambridge, MA).

Sequence analysis. Sequences of high enough quality (forward and reverse) were imported into GeneStudio v.2.2 (GeneStudio Incorporation, Suwanne, GA) to generate a consensus

sequence. The consensus sequences were then imported into MEGA and analyzed for any SNPs, insertions, or deletions. If insertions or deletions occurred that was considered an allele variant to the reference and was assigned a different number (i.e. 1, 2, or 3). If SNPs occurred these were identified using a letter (i.e. a,b,...).

Results

Oocysts detection. Of the 3 backyard samples used for the scheme there was a mean opg of 133.3. Of the nineteen commercial broiler samples used for the scheme there was a mean opg of 68,110.5. All 22 samples and 2 vaccines were positive by qPCR for *E. maxima* (Table 5).

Species detection. Fifteen of the commercial samples were sequenced using next-generation amplicon sequencing with the 18S rRNA and COI genes (Figure 3). Samples were positive for *Eimeria acervulina* (87%), *E. maxima* (100%), *E. mitis* or *E. mavati* (47%), *Eimeria necatrix* or *E. tenella* (33%), and *Eimeria praecox* (53%).

Sequence analysis. In all genes with the exception of pep, there was at least one allele variant (Figure 4). Two isolates from different companies were consistent with vaccine B in at least 4 of the genes. One isolate from a company was consistent with vaccine A in 3 of the gene. There was variation between each farm, with no farm having identical strains. In the backyard strains, there was a third allele variant present in the *HSP* and *RCC* genes that was not consistent with the vaccine strains or commercial broiler flock strains. Vaccine C did not amplify with the primers used in this study.

Twenty-two amplicons were not obtained, and 40 amplicons had consensus sequences of poor quality – meaning one sequence was good and its opposite was poor in quality.

In the genes *DNAJ* and *HSP*, these allele variants were seen in the non-coding regions of the gene. In the *DNAJ* locus there was a 9 bp insertion. With the *HSP* gene there were two allele variants, with one having a 10 bp insertion and the other a 3 bp insertion. In the genes *RCC* and *SCP*, these allele variants were seen in the coding regions of the gene. With the *RCC* gene, there were two allele variants with one having a 3 bp insertion and the other having a 3 bp deletion. In the *SCP* locus there was a 3 bp insertion. All point mutations seen in these loci that occurred in the coding regions of the gene resulted in silent mutations (Table 6).

Discussion

The development of an MLST scheme for *E. maxima* was done in the present study. Initially the study began with 19 potential genes. The genes were identified from MLST schemes already developed for other protozoa such as *Babesisa* (*DNAJ* and *RCC*) and *Cryptosporidium* (*HSP*) as well as genes with differential expression in *E. tenella* (*PEP*, *SCP*, and *VPT*) (del Cacho et al., 1998; del Cacho et al., 2005; Guillemi et al., 2013; Feng et al., 2014; Matsubayashi et al., 2016). These genes were then screened using two different coccidiosis vaccines and if any variation a field sample was used to screen. The six genes chosen in this study had variation from the published genome in the first screen with the vaccine strains (results not shown).

The amplicons of the chosen genes range from 276 bp to 604 bp. Typically in MLST schemes, the desired amplicon size is between 450 and 500 bp (Maiden et al., 1998). In our scheme we were constrained when designing primers as we did not want the primers to amplify DNA of other *Eimeria* spp. Having a longer amplicon between 450 and 500 bp would allow for more variation to be seen, while having a shorter amplicon would have greater sensitivity.

The present study found that there were differences in several loci between vaccine strains, commercial strains, and strains found in backyard flocks. Strains that were found in

backyard flocks were not similar to strains of *E. maxima* observed in vaccines. There were some consistencies between strains found in commercial flocks and the vaccine strains as these flocks were likely vaccinated for coccidiosis. While there were limited amounts of variability, there was enough to distinguish field strains from vaccine strains. Flocks sampled in 2019 were not vaccinated for coccidiosis and were on a rotational program to reduce the number of oocysts shed into the environment.

Samples where no amplicon sequence was not obtained could be due to sensitivity issues with the PCR as there is limited amounts of DNA available in the fecal samples or mutations that prevent the primers from binding. Whereas sequences of poor quality could be due to cross-reactivity with other species of *Eimeria* present in the sample.

The information gathered from this study can provide a tool that can be used in any lab that does PCR for epidemiology studies. This tool could be used to improve vaccination programs as it would be possible to tell what strain of *E. maxima* is causing issue within a flock. However, many more samples from different types of poultry flocks need to be analyzed using this tool to fully demonstrate its efficacy.

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Table 4. Primers and probes used in the study.

Gene	Type	Forward	Reverse	Amplicon Length (bp)	Accession Number	Reference
5s		TCATCACCCAAAGGGATT	TTCATACTGCGTCTAATGCAC	~110		Blake et al., 2006
		Probe: [6-FAM] CGC CGC TTA ACT TCG GAG TTC AGA TGG GAT [BHQ-1]				
E. maxima		TCGTTGCATTCGACAGATTC	TAGCGACTGCTCAAGGGTTT	138		Vrba et al., 2010
		Probe: [6-FAM] ATT GTC CAG CCA AGG TTC CCT TCG [BHQ-1]				
DNA	outer	CGTTTTGCCGTTGCTCATGA	ATCACCAGATGCACCACCTG	667	EMWEY_00047020	This study
	inner	AATTTGCTGTTGCCCCTCCT	TAGCAGCACCAGGGGAAGTA	598		This study
HSP	outer	ACACCGTTTTGTATGGGGCT	AACCGTGCAAAACAGCCATC	796	EMWEY_00031510	This study
	inner	AAGAAACTCCACCTGAGCCG	AAAGACACCGCTGCCATGTA	353		This study
PEP	outer	CAGTGGCTGTTGTTGACGTG	TTCCGGGTCTTCAAGCCATC	828	EMWEY_00002070	This study
	inner	TGCCACACCTATGTTCACCC	GAGTTGATTTGGGCGCCATC	276		This study
RCC	outer	AGACGCTGAACCTCCAACTG	TGATGCTGCTCGAACTCCTG	603	EMWEY_00042910	This study
	inner	GGTATCTGAGCTGAGTGGCC	ATAAGGAAGTGTGGGGCAGC	396		This study
SCP	outer	TAATAGTGGAGACGCAGCGC	AGCAGCAAGTCCGTCAACTT	1028	EMWEY_00045680	This study
	inner	AAGGGGCAGAGAGTGAATGC	TTACTCCCTCTGAGCCTCCC	387		This study
VPT	outer	ATATTCGTCACCCTCGTGGC	AAACAAACGCATGCACACCA	945	EMWEY_00015190;	This study

	inner	AAAGCAACTGCAGCAACGAG	CCACGCTGCTGACAATGATG	604	This study

[1][6-FAM] is fluorophore attached at 5’ end [BHQ-1] is the quencher molecule that attaches at the 3’ end.

Table 5. OPG and Cq for *E. maxima* qPCR for all samples used in MLST scheme.

Sample	Type	opg	Cq
22-17	backyard	200	37.87
22a-18	backyard	100	38.97
23a-18	backyard	100	37.71
2-18	broiler	23,600	38.19
5-18	broiler	39,200	42
6-18	broiler	57,600	36.48
7-18	broiler	254,000	29.67
8-18	broiler	23,600	35.63
9-18	broiler	23,400	37.98
11-18	broiler	56,200	35.17
13-18	broiler	6,300	38.09
14-18	broiler	1,700	35.12
15-18	broiler	195,000	34.7
20-18	broiler	7,900	36.2
49a-17	broiler	144,000	42
49b-17	broiler	108,000	35.2
49d-17	broiler	179,000	39.38
49f-17	broiler	152,000	42
44b-19	broiler	4,350	33.68
45b-19	broiler	3,100	34.09
47c-19	broiler	2,950	36.55
48a-19	broiler	12,200	39.35
OUT-X	-	-	36.81

Table 6. All allele variants and point mutations in the selected gene loci for MLST relative to the reference sequences.

Gene	Insertion/Deletion; Region	Point mutations
DNAJ	9 bp insertion; non-coding	1; non-coding
HSP	10 bp insertion; non-coding 3 bp insertion; non-coding	3; non-coding
PEP		1; coding (silent)
RCC	3 bp deletion; coding 3 bp insertion; coding	1; coding (silent)
SCP	3 bp insertion; coding	
VPT		1; non-coding

Sample	EA		EB		EMax		EMiv	EMit	EMit/EMiv	EN/ET	EN	ET	EP		OTU-X		OTU-Y		OTU-Z	
	18S	COI	18S	COI	18S	COI	18S	18S	COI	18S	COI	COI	18S	COI	18S	COI	18S	COI	18S	COI
7-18	34.3	27.49			53.6	61.13	2.0	2.3												
15-18	48.2	28.16			36.2	61.01	1.0	1.2		7.9										
49a-17	57.1	27.93			32.2	59.77	1.6	1.6		2.2										
49b-17	47.6	26.20			35.9	63.68	1.7	1.5		6.1										
49d-17	78.0	31.33			16.0	55.25				1.6										
49f-17	72.9	24.73			4.2	53.05	4.8	4.8	4.43	1.4			7.0	3.04						
2-18	43.5	7.87			46.8	72.26							4.5	10.98						
5-18	65.8	52.50			9.8	41.71														
6-18					87.2	97.90							2.2							
8-18	46.8	28.52			40.8	60.43								2.18						
9-18	64.8	27.64			23.9	42.34							6.9	16.43						
11-18	51.2	36.16			43.3	54.69														
13-18					82.5	93.96							4.3	3.04						
14-18	7.1	2.98			45.0	84.18	15.9	22.6	4.33					1.93						
20-18	17.2	6.58			40.7	84.23	9.4	8.1					1.9	2.47						

Figure 3. Next-generation amplicon sequencing results for 18S rRNA and COI genes for *Eimeria* spp and OTUs X, Y, and Z. Green indicates a positive result for that *Eimeria* spp.. *E. acervulina* (EA), *E. brunetti* (EB), *E. maxima* (EMax), *E. mitis* (Emit), *E. mivati* (EMiv), *E. necatrix* (EN), *E. tenella* (ET), and *E. praecox* (EP).

Sample	***DNAJ***	***HSP***	***PEP***	***RCC***	***SCP***	***VPT***	
Ref	1a	1a	1a	1a	1a	1a	
A	2a	2a	1b	2a	2a**	1a	
A2	2a	2a	1b			1a	
B	2b	1a	1a		1a	1a	
C							
22-17		1a		3a		1a	
22a-18		3a	1a			1a	
23a-18							
2-18	2b	1a	1b	2a			
5-18	2b					1a	Consistent with B
6-18		1a	1a	2a	1a	1a	Consistent with B
7-18		2b		2a		1a	
8-18	2b		1b		1a	1a	
9-18		1b	1a		1a	1b	
11-18	2b	2b	1a	2a	1a	1a	
13-18		1a	1b	2a	1a	1b	
14-18			1b	2a		1a	Consistent with A
15-18	2a				1a	1a	
20-18	2b	1a	1b		1a	1a	Consistent with B
49a-17	2b	2b	1a	2b	1a	1a	
49b-17				2a			Consistent with A
49d-17	2b	2c	1a		1a		
49f-17	2b		1b			1b	
44b-19	2b	1a	1b	2a		1b	
45b-19			1b			1b	
47c-19		1a	1a	2a		1a	Consistent with B
48a-19	2b	2a	1b			2a	
OTU-X							

Figure 4. Allele variants and point mutations for each gene. Blank space indicates no sequence obtained; grey spaces indicate poor sequence quality. Allele variants were assigned a different number and color group (i.e. 1, 2, or 3). If SNPs occurred these were identified using a letter and a different shade of the color group (i.e. a,b,c).

Appendix

Occurrence of selected parasites in all backyard flocks. For *Histomonas*, *T. gallinarum*, *Cryptosporidium*, and *Blastocystis* only one sample per flock was examined.

Sample Number	*Eimeria*	*Ascaridia*	*Capillaria*	*Histomonas*	*Tetratrichomonas gallinarum*	*Cryptosporidium*	*Blastocystis*
003/2017	pos	neg	neg	neg	pos	neg	pos
004/2017	pos	neg	pos	neg	neg	neg	pos
005/2017	pos	neg	neg	neg	neg	pos	neg
006/2017	neg	neg	neg	neg	neg	neg	pos
007/2017	neg	neg	pos	neg	neg	neg	pos
008/2017	pos	neg	neg	neg	neg	pos	pos
011a/2017	neg	neg	neg	neg	neg	neg	pos
011b/2017	neg	neg	neg				
011c/2017	neg	neg	neg				
012/2017	pos	neg	neg	neg	neg	neg	pos
013/2017	pos	neg	neg	neg	neg	neg	pos
014/2017	pos	neg	neg	neg	neg	neg	pos
015/2017	pos	neg	pos	neg	pos	neg	pos
016a/2017	pos	pos	pos	neg	neg	neg	pos
016b/2017	pos	pos	pos				
016c/2017	neg	neg	neg				
016d/2017	pos	neg	neg				
017/2017	neg	neg	neg	neg	neg	neg	pos
018/2017	pos	neg	pos	neg	pos	neg	pos
019/2017	neg	neg	neg	neg	neg	neg	pos
020/2017	pos	pos	pos	neg	pos	neg	pos
021/2017	neg	neg	neg	neg	neg	neg	pos
022/2017	pos	neg	neg	neg	neg	pos	pos
023/2017	neg	neg	neg	pos	neg	pos	pos
024/2017	pos	pos	pos	neg	pos	neg	pos
025/2017	neg	neg	neg	neg	neg	neg	pos
026/2017	neg	neg	neg	pos	pos	neg	pos
027/2017	pos	neg	neg	neg	neg	neg	pos
028/2017	neg	neg	neg	pos	neg	neg	neg
029/2017	neg	neg	neg	neg	pos	neg	pos
030/2017	neg	neg	neg	neg	pos	neg	pos
031/2017	pos	pos	neg	neg	neg	neg	pos
032/2017	pos	pos	neg	neg	pos	neg	pos

Sample Number	*Eimeria*	*Ascaridia*	*Capillaria*	*Histomonas*	*Tetratrichomonas gallinarum*	*Cryptosporidium*	*Blastocystis*
033a/2017	pos	neg	neg				
033b/2017	pos	neg	neg	neg	neg	neg	pos
034/2017	pos	neg	neg	neg	neg	neg	pos
035/2017	neg	neg	neg	neg	neg	pos	pos
036/2017	pos	pos	neg	neg	neg	neg	pos
037/2017	pos	neg	neg	neg	neg	neg	pos
038/2017	neg	pos	neg	neg	neg	neg	pos
039/2017	pos	neg	neg	neg	neg	neg	pos
040/2017	neg	neg	neg	neg	neg	pos	pos
041/2017	pos	neg	neg	neg	neg	pos	pos
042/2017	pos	neg	neg	neg	pos	neg	pos
043/2017	pos	neg	pos	neg	neg	neg	pos
044/2017	neg	neg	neg	neg	neg	pos	pos
045/2017	neg	neg	neg	neg	neg	pos	pos
021a/2018	neg	pos	neg	neg	neg	neg	pos
021b/2018	pos	pos	neg				
022a/2018	pos	pos	neg	neg	neg	neg	pos
022b/2018	pos	neg	pos				
022c/2018	neg	pos	pos				
023a/2018	pos	neg	pos	neg	neg	neg	neg
023b/2018	neg	neg	neg				
023c/2018	neg	neg	pos				
026a/2018	neg	neg	neg	neg	neg	neg	pos
026b/2018	pos	neg	neg				
026c/2018	neg	neg	neg				
027/2018	pos	neg	pos	neg	neg	neg	pos
028/2018	neg	neg	neg	neg	neg	neg	pos
029/2018	pos	neg	neg	neg	neg	neg	pos
030a/2018	pos	neg	pos	neg	neg	neg	pos
030b/2018	neg	pos	neg				
030c/2018	pos	neg	neg				
031/2018	neg	neg	neg	neg	neg	neg	pos
032/2018	pos	neg	neg	neg	pos	neg	pos
033a/2018	pos	neg	pos	neg	neg	neg	pos
033b/2018	pos	neg	pos				
034/2018	neg	neg	neg	neg	neg	neg	neg

Sample Number	*Eimeria*	*Ascaridia*	*Capillaria*	*Histomonas*	*Tetratrichomonas gallinarum*	*Cryptosporidium*	*Blastocystis*
035/ 2018	neg	neg	neg	neg	neg	neg	pos
036/2018	pos	pos	pos	neg	pos	neg	pos
037/2018	pos	neg	neg	neg	neg	pos	pos
038a/2018	pos	neg	neg	neg	neg	neg	pos
038b/2018	pos	neg	neg				
038c/2018	pos	neg	neg				
039/2018	pos	neg	neg	neg	neg	neg	pos
040/2018	pos	neg	pos	neg	neg	neg	neg
041/2018	neg	pos	neg	neg	neg	neg	pos
042a/2018	pos	neg	neg	neg	neg	neg	neg
042b/2018	pos	pos	pos				
24/2018	pos	neg	neg	neg	neg	neg	neg
25/2018	neg	neg	neg	neg	neg	pos	pos
43a/2018	neg	neg	pos	neg	neg	pos	neg
43b/2018	pos	neg	pos				