Methods of Quantification and Characterization of Coccidian Oocysts

by
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Abstract

MARIAM DANIELLE ABUNEMEH: Methods of Quantification and Characterization of Coccidian Oocysts
(Under the direction of Dr. Richard Buchholz)

Coccidiosis is a major economic and health risk in the poultry industry. The oocysts of the causative agent of coccidiosis are excreted in animal feces and must be ingested by a new host for a new infection to begin. These oocysts are microscopic and very similar between species. The ability to quantify and identify the oocysts that are causing the illness is important to controlling this disease. My research first compares methods of quantifying oocysts of domestic turkeys for their ease of use and accuracy. Next, I attempt to identifying novel oocysts from a different turkey species by morphological and molecular approaches. Of the four methods of oocysts isolation and quantification that I compared (Standard Sugar Flotation, Standard Dilution, Hemocytometer, and Howard-Mold counting slide) the Standard Dilution provided the most accuracy relative to the time invested. I attempted morphological identification of oocysts in from the host *Meleagris ocellata* and found that length and width of the oocysts overlapped with those of known coccidian species from domestic turkey. My efforts to obtain molecular descriptions of the oocysts from *M. ocellata*, were not successful, but I report on five means of *DNA* extractions that I attempted.

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Chapter One: General Introduction

Coccidia

Coccidia are single celled, obligate intestinal parasites from the *Apicomplexa* phylum, suborder *Eimeriorina* (Duszynski et al, 2016). Coccidian parasites range over 42 different genera and contain over 2000 species (Duszynski et al, 2016). Coccidian parasites of the apicomplexan phylum frequently cause ill health and severe economic loss in human and animals (Clark et al, 2012). Coccidiosis causes damage to the intestinal tract, which can lead to intestinal tract bleeding (Chapman, 2008). Other symptoms of coccidiosis are malabsorption, inflammation and diarrhea (Chapman, 2008). Coccidiosis is a major economic issue and causes huge financial losses to the poultry industry every year (Vbra and Pakandl, 2014). The estimated cost globally exceeds two billion dollars per year (Fornace, et al., 2013). Coccidia infect most animals, vertebrates and invertebrates, around the world. The genera can be differentiated by the species of their host and the specificity they have to this host. Oocyst morphology and lifecycle differ among genera. Coccidiosis can be used to describe any disease deriving from any coccidian genera, but is most commonly used for infections by *Eimeria* (Clark and Blake, 2012). My study focuses on coccidian of the genus *Eimeria*.

Eimeria

Eimeria consists of over 1800 species, and as many as 98% of the species of this genus may not have been identified yet (Vrba and Pakandl, 2015). It is rare for *Eimeria* coccidia species to have the ability to infect multiple host species, which means they are

host specific. There are very few exceptions to this host specificity. *Eimeria* coccidia infect most mammals, birds and reptiles (Duszynski et al, 2016). *Eimeria* parasites target the intestinal tract of their host; the site of attack depends on the species of the parasite (Vrba and Pakandl, 2014). The morphology of *Eimeria* coccidia is similar for most species: ellipsoidal or circular shaped with a thick cell wall and sporocysts. *Eimeria* have a strict fecal-oral route of transmission (Clark and Blake, 2012).

Lifecycle

According to Duszynski et al, (2016), the *Eimeria* lifecycle can be summarized as follows. *Eimeria* coccidia have both an asexual and a sexual stage. The first part of the cycle is haploid and asexual. The cycle begins when a sporulated oocyst is ingested by a host. A sporulated oocyst contains four sporocysts, which each contain two sporozoites. The oocyst travels to the intestinal tract where it encounters intestinal enzymes, which cause the release of the eight sporozoites. These sporozoites search out specific regions of the intestinal tract for replication. The first stage of replication is the trophozoite during which the parasite is replicating its nucleus and organelles. Next, it enters the schizont stage. In this stage the parasite begins to make copies called merozoites. These merozoites are released by lysing the host cells. Merozoites then develop into gametes to begin the diploid sexual stage of development. The two gametes – micro and macro- fuse to form a zygote, which then develops into the oocyst. The oocysts are released in the feces of the host. After excretion, the oocysts sporulate if they are in a suitable environment. Sporulated oocysts are infectious.

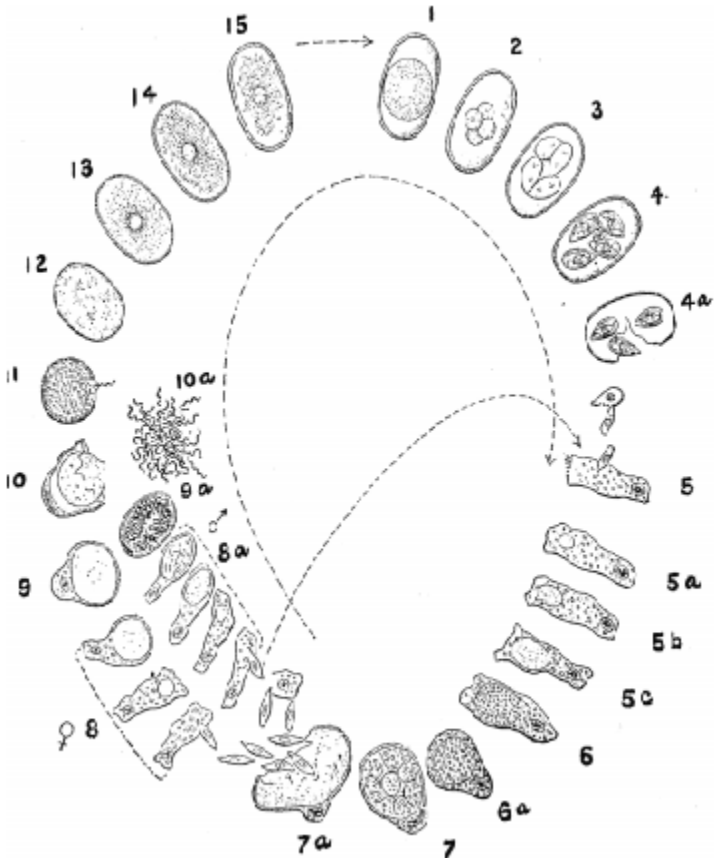

Figure 1: General lifecycle of *Eimeria* (Chapman, 2008). 1 through 4 – sporulation, 4a- oocyst hatches in gut, 5- sporozoite enter cell, 5a through 7- intracellular reproduction, 7a- cell lyse and release of merozoites, 9-9a- gamete formation, 10- micro- and macro- gamete fusion, 11- zygote, 12 through 15- oocysts formation, 15- excretion

Turkey coccidia

Coccidiosis may be the most common parasitic disease in turkeys, *Meleagris gallopavo* (Chapman, 2008). It is extremely destructive to the poultry industry and is a major cause of death in young turkeys (Chapman, 2008). There are seven species of *Eimeria* coccidia known to infect turkeys: *E. meleagrimitis, E. dispersa, E. adenoeides, E. gallopavonis, E. meleagridis, E. innocua, E. subrotunda* (Vrba and Pakandl, 2014). These species differ by their size, shape, and the segment of the intestine they infect (Table 1). The overlap in oocysts' size ranges can make it hard to differentiate turkey

coccidian species by morphology alone. In Galliformes, of which *Meleagris gallopavo* is a part, there is a similar ancestry in their *Eimeria* coccidia (Miska and Jenkins, 2010). Some turkey coccidia infect multiple host species. One of these known exceptions is *E. dispersa*, which has been found in various avian hosts, though this has only brought into question the species validity (Chapman, 2008). Also, *E. meleagridis KR* can reproduce in *Perdix perdix* (Grey Partridge) and *E. innocua* can cross-transmit to *Colinus virginianus* (Bobwhite Quails) and *Perdix perdix* (Grey Partridge) (Vrba, 2015). The ocellated turkeys, or *Meleagris ocellata*, is closely related to *M. gallopavo*, but has not been as heavily researched. Therefore, their intestinal parasites are understudied. It is not known if coccidians of the ocellated turkey are the same as those in the North American wild turkey or domestic turkeys.

Species	Size (μm)	Shape	Location
E.meleagrimitis	18.0x15.3 [1] 19.2x16.3 [1] 20.3x16.4 [2] 26.1x21.0 [2]	Subspherical [1]	First and second generation: anterior part of small intestine (upper jejunum and duodenum) and throughout the intestine including rectum and caeca [1]
E. dispersa	25.1x19.7 [1] 26.1x21.0 [2]	Broadly ovoidal [1]	Duodenum and upper intestine, spreads to lower intestine but not caeca
E. adenoeides	25.6x16.3 [1] 25.6x16.6 [2]	Ellipsoidal [1]	First generation: neck of caeca and the terminal inch of small intestine Second generation and sexual: throughout caeca, lower intestine, and rectum [1]
E. gallopavonis	26.3x16.9 [2] 26.6x16.4 [1] 27.1x17.2 [1] 29.5x19.5 [2]	Ellipsoidal [1]	Schizonts: Posterior ileum, caeca, and rectum Sexual: Posterior ileum, caeca, and rectum and small intestine [1]
E. meleagridis	22.5x16.3 [1] 22.9x16.6 [2] 23.8x17.4 [1] 24.4x18.1 [1] 27.1x17.2 [2]	Broadly ovoidal [1]	First-generation: caeca, small intestine either side of yolk sac divertidculum, small intestine upper and mid-ileum and mid-jejunum Later generations and gametes: caeca, rectum, and lower ileum [1]
E. innocua	21.2x18.5 [1] 22.4x20.9 [1] 23.9x20.9 [2]	Spherical [1]	Duodenum, jejunum, and upper ileum [1]
E. subrotunda	21.8x19.8 [1]	Nearly spherical [1]	Duodenum, jejunum, and upper ileum [1]

Table 1: Morphology dimensions of the seven species of *Eimeria* coccidia that infect *eleagris gallopavo*

[1] Duszynski et al, 2016
[2] Vrba and Pakandl, 2014

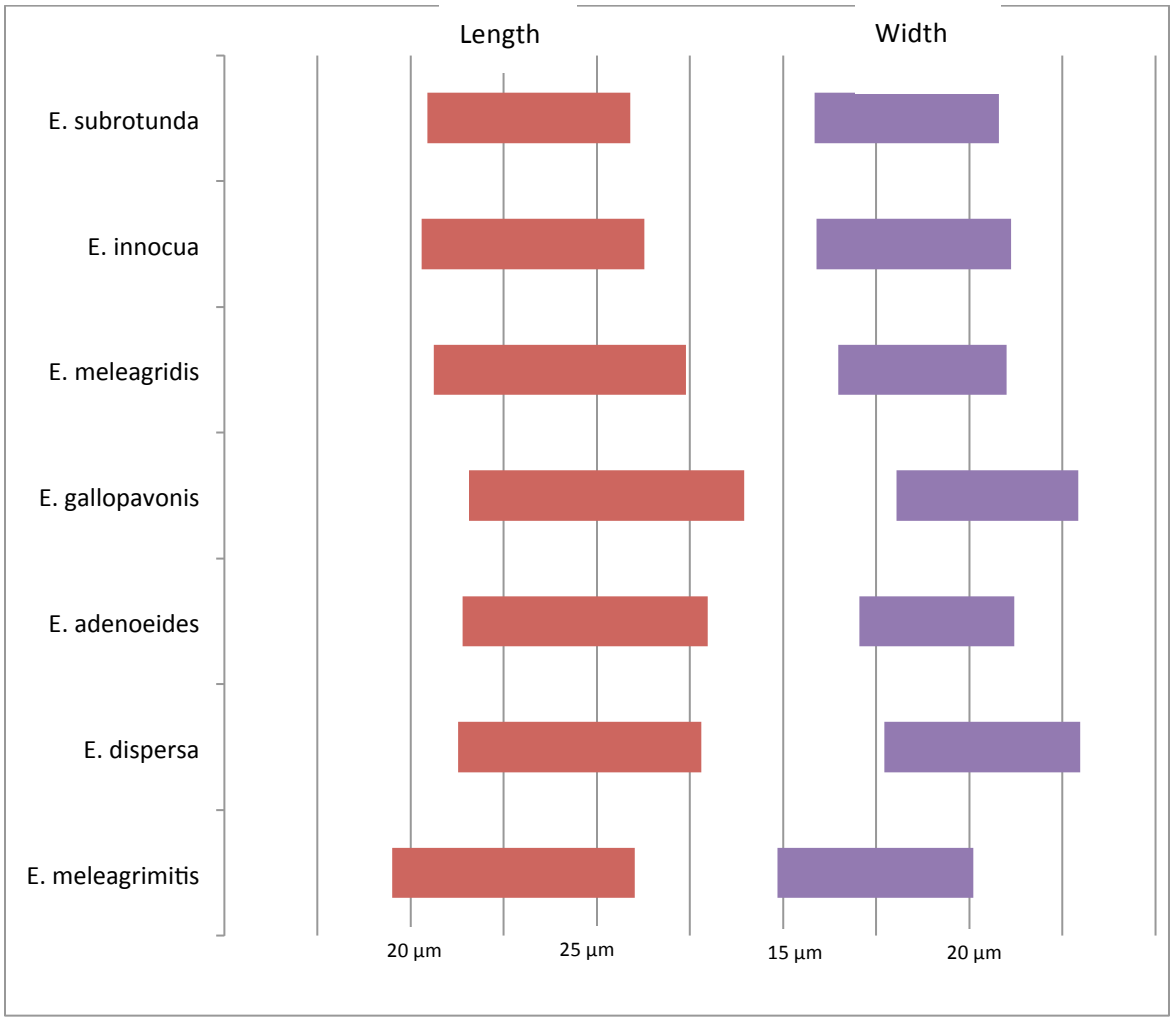

Figure 2: Ranges of oocyst species length and width in turkey. From Vrba and Pakandl (2014) and Duszynski et al (2016). Units on x-axis are approximations.

Molecular Identification of Coccidia

Molecular identification of coccidia is important because of the overlapping morphological similarities between *Eimeria* species (Kumar et al, 2014). Gene sequencing for *Eimeria* began in 2002 with the Houghton strain of *Eimeria tenella;* the resources for sequencing prior to 2002 were impractical for *Eimeria* (Blake, 2015). Now there are completely sequenced coccidia and important genes from specific species, as well (Kumar et al, 2014). Matching newly isolated and sequenced oocysts to these previously sequenced coccidian species is how to determine the species of oocysts. Genomes differ between all living things but each species conserves some genes. These conserved genes are different for each species and are used to identify coccida species.

DNA Isolation

DNA isolation was first done by Friedrich Miescher in 1869; since that time the process has advanced and became more accurate (Tan et al., 2009). The basic procedure for DNA extraction is lysing or breaking of the cell to release the DNA from the cell (Rice, 2015). In coccidians, this is difficult because the oocyst is surrounded by a tough outer wall. This can be accomplished by vortexing with beads (glass, metal, etc.) (Cha, 2014) or freeze-thaw cycles (Fritzler, 2011). Next, it is necessary to degrade the cellular proteins to prevent contamination of the DNA isolate. This usually leaves a salt residue on the DNA, so the next step washes the DNA with alcohol to remove the salt (Rice, 2015). If DNA has been extracted from cells, then a gel electrophoresis will reveal the presence of DNA stained with ethidium bromide.

Hypothesis and Objectives

The overall objective of my research was to test the success of various methods for quantifying the oocysts of the turkey and identifying them to species. Accurate

quantification is important for determining the severity of infection of the host and for testing efficacy of anti-coccidial drugs (Hodgson, 1970). The identification of coccidia species is necessary because species vary in the severity of their harm to their host. Also new parasite species may become threats to the US poultry industry as tropical deforestation, international travel and climate change create novel encounters of hosts and parasites. In the next two chapters of my thesis I first compare the results of four alternative methods of quantifying oocysts. Counting oocysts is time consuming and laborious. My objective is to identify the most consistent and efficient method of counting a host's parasite burden. In the subsequent chapter, I use a traditional morphological approach to testing the identities of oocysts found in the Neotropical ocellated turkey. The parasite community of this close relative of the North American wild turkey has never been identified to species. My objective was to link my morphological description of oocysts to nucleotide sequences for the same oocysts (after Dolnik et al 2009). Unfortunately this part of my project was unsuccessful. The various methods I attempted to isolate coccidian DNA did not result in successful extraction nor amplification by PCR. As a result I report only the comparative methodological issues that I encountered with the approaches that I attempted.

Chapter 2: Comparison of Techniques for Quantifying Oocyst Number

Introduction:

Methods to accurately count organisms, whether the organisms are humans attending a rally on the National Mall in Washington, DC (Goodier, 2011) or bacterial spores on a microscope slide (Cook and Lund, 1962) must be accurate, provide repeatable results, and meet the practical requirements of the researcher. Accuracy is a measure of how closely a counting method approximates the true count (Rago, 2011). Repeatability refers to the reliability of repeated measurements using the same methodology (Rago, 2011). Practical constraints on counting methods include the time needed to conduct counts, the availability of the equipment or supplies necessary for that method of counting, and the financial and opportunity costs of the method (Dryden et al., 2005). Proper counting of coccidian oocysts is crucial for studies of variation in individual host susceptibility to infection, and the efficacy of anti-coccidial drugs and vaccines (Hodgson, 1970). The methods for counting coccidian oocysts have undergone a complex evolution (Haug et al, 2005). Oocysts are shed in the host's feces and thus must be differentiated from the fecal debris for counting. The first challenge in counting oocysts is to either stain them so that they are visible or separate them from the rest of the fecal matter. Because oocysts are extremely abundant during an outbreak of coccidiosis on a poultry farm (e.g. many hundreds of thousands per gram of feces; Price and Barta, 2010), and the oocyst wall is impervious to most stains (Jenkins et al., 1997), veterinarians typically have not bothered to stain samples. Instead they "float" oocysts in

a concentrated solution so that the different components of the feces separate out in a gradient according to their specific gravities (Dryden et al, 2005). The most common flotation solutions use inexpensive reagents to achieve a specific gravity of 1.18-1.20, depending on the coccidian species and stage of development (Dryden et al., 2005), and include 33% copper sulfate, saturated sodium chloride, and 70% sucrose. In Dr. Buchholz' lab, we use a standard quantification technique used in most parasitology labs (Dryden et al., 2005). The standard approach mixes a known quantity of fresh or preserved feces (1g or 1ml, respectively) with the sucrose flotation solution in a conical centrifuge tube. Centrifugation allows the oocysts to float to the top quickly where they adhere to a glass coverslip capping the tube. It is assumed that a sample of oocysts proportional to the number actually in the feces are transferred to a microscope slide with the coverslip when it is plucked vertically off the centrifuge tube. At one extreme, when oocysts are rare in a fecal sample, for example during latent infections or during certain times of day (Martinez-Bakker and Helm, 2015), the few and translucent oocysts might be easily overlooked by the observer. At the other extreme, when oocysts are super-abundant, the density of oocysts on the coverslip may be so great that they obscure one another and cannot be counted accurately. Even when oocysts do not overlap, it is time consuming and exhausting to count many thousands on each slide.

The objective of this chapter of my thesis is to evaluate alternative methods for oocyst quantification in the hopes of finding one that is more practical and efficient while remaining accurate. I compare the standard sugar flotation to three other approaches: a) standard dilution, b) hemocytometer, and c) Howard mold slide. As the name suggests, the standard dilution simply dilutes the floated oocysts by a known amount so that a sub-

sample is counted more quickly. A hemocytometer is a slide with a grid pattern etched into it. The grid pattern and sample well is comprised so that the total area in the blocks has a known volume (Grigoryev, 2014). Originally designed for counting blood cells, now it is used to count microbes as well (Grigoryev, 2014). The Howard mold counting slide was invented to find the presence of mold spores in food, specifically tomato products (Anonymous, 2010). Its key feature is the raised edges on the left and right of the stage, which ensure a volume of 0.1 mL under the coverslip. It has been adapted to count other microscopic organisms, but it is thought to be inaccurate when the study organism occurs at low densities.

Methods:

Four counting techniques were compared for their ease of use and consistency of result. For all methods, preserved fecal sample 1A Black/Red 2014 was used. Sample 1A Black/Red 2014 was from a domestic turkey that had been fed feces from wild turkeys. For the first two counts of the Standard Sugar Flotation, an Olympus BX40 microscope was used. Because Dr. Buchholz's graduate student need to use the Olympus BX40, a Reichert-Jung Series 150 microscope was used to count oocysts from the rest of the Standard Sugar Flotation method samples and all those from the three other methods. Oocysts counts are reported as oocysts per one gram. To achieve these units, results from the Standard Sugar Flotation and the Hemocytometer were converted from oocyst per milliliter using the conversion where 0.905 ml of feces equals one gram.

Standard Sugar Flotation:

1 mL of fecal solution was added to a 15 mL conical tube, which was then filled with 70% sucrose solution until the liquid formed a convexity at the top of the tube. The

tubes were placed in the IEC HN-SII centrifuge and coverslips placed on top. These were centrifuged at 2000 rpm (706.5 g) for 12 minutes. The coverslips were lifted off vertically and placed on individual microscope slides for counting. The slides were allowed to sit for a couple of minutes to allow the oocysts to float up again after disturbance. All oocysts under the coverslip were counted and viewed under 100x magnification. These steps were repeated for each of the ten replicate flotations using the standard sugar flotation.

Dilution:

Two grams of the fecal solution were diluted with 60 mL of the 70% sucrose solution. This solution was mixed vigorously, and 16 µL was placed on a clean microscope slide and a coverslip placed on top. 16 µL was used because it was the volume that best allowed for minimal bubbles and leakage from under the coverslip. Slides rested for a couple of minutes and then were viewed under 100x magnification. All oocysts under the coverslip were counted. Conversions were used to attain the units of oocyst per gram of feces. These steps were repeated until ten replicates were achieved.

Hemocytometer:

1 mL of fecal solution was added to a 1.5 mL microcentrifuge tube; 0.1 ml of that was added to another microcentrifuge tube and was diluted with 0.9 mL of 70% sucrose solution. The diluted solution in the microcentrifuge tube was vortexed. The hemocytometer used was a Bulldog Bio 4-Chip Disposable Hemocytometer. 6 µL of the dilution was added under the permanent coverslip of the hemocytometer. The slide was allowed to rest for a couple of minutes and then viewed under 100x magnification. The oocysts inside the four 4x4 squares of the grid pattern were the only oocysts counted and

were averaged together. The equation A*10*D was used to calculate the number of oocyst in one milliliter. Where A is the average from the four 4x4 squares on the hemocytometer, 10 is the inverse of the volume in one 4x4 square which had units of inverse microliters, and D is the dilution of the sample, which had no units. The resulting units are oocyst per microliters, so simple conversions were used to convert the units to oocyst per milliliters. These steps were repeated until ten replicates were completed.

Howard Mold:

Two grams of the fecal solution were diluted with 60 mL of the 70% sucrose solution. This was mixed vigorously and a small amount transferred to the stage of the slide. The coverslip was placed on top carefully to ensure no bubbles formed and that the volume was 0.1 mL. The slides rested for a couple of minutes before being viewed under 100x magnification. Oocysts in 30 view fields were counted, then the counting was repeated for another thirty view fields. The two totals were averaged. The average was used in an equation (A*13778)/2, where A is the count average and 13778 is a multiplication factor found by dividing the total volume of the dilution by the volume in 30 view fields, to calculate the number of oocyst per gram. This process was repeated until replicate counts were achieved.

Results

The numbers of oocysts per gram were varied for each method (Figure1, Appendix 1). The most accurate and consistent method of quantifying oocyst was the Standard Sugar Flotation ($\bar{x}$: 34,582 ± 3,025, CV: 8.7%). The CV was significantly lower for the Standard Sugar Flotation. The averages, standard deviations, and coefficient of variation of the dilution methods are as followed: Standard Dilution ($\bar{x}$: 40,891 ± 6,607, CV: 16.2%), Hemocytometer ($\bar{x}$: 186,022 ± 64,863, CV: 34.9%), and Howard Mold ($\bar{x}$:

235,604 ± 41,973, CV: 17.8%). A t-test was performed to compare the Standard Sugar Flotation and the Standard Dilution. The difference between the two is significant but small (t = -2.75, n = 10, p = 0.007).

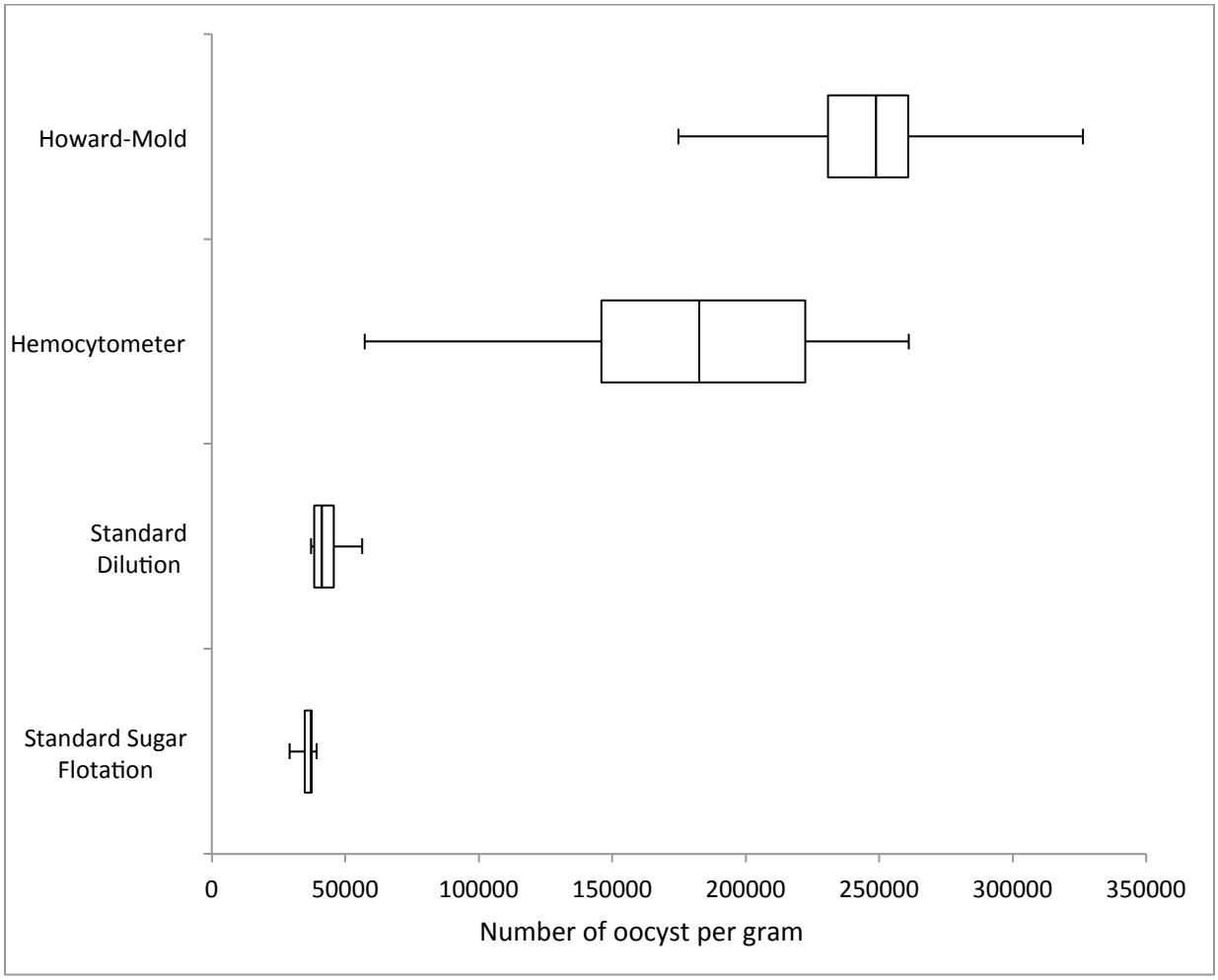

Figure 3: Box plots showing results of four methods for counting oocysts. The box is the standard deviation and the line inside the boxes is the mean. The lines are the 95% confidence interval, or 95% of all counts will fall into this range.

Discussion

My results correspond with those of Haug et al (2005), who concluded that the methods with the most volume of fecal samples, or least diluted, are the most accurate and consistent. The Standard Sugar Flotation was less diluted than the Howard-Mold and Standard Dilution methods, and was the most repeatable. The low CV of the Standard Sugar Flotation method suggests it is the most repeatable method for oocyst quantification. Ease of use of this method though is low. The Standard Sugar Flotation is very time consuming at this oocyst density; it took approximately 6 hours to count one slide. The difference between the Diluted Sugar Flotation and the Standard Sugar Flotation was significant ($t = -2.75$, $n = 10$, $p = 0.007$). In contrast the Diluted Sugar Flotation approach had good repeatability (CV:16.2%) and required only approximately 1 hour to count each slide. Although the mean count by this method was 10% higher than the standard, the time saving still recommends this approach as the most efficient for most purposes. The Hemocytometer and Howard-Mold method did not give accurate or repeatable results probably because the number of oocyst per slide was too low. Grigoryev (2014) found that at least 100 oocysts per slide was required for accurate estimates. My counts per slide (Hemocytometer: 3-12 oocysts per slide, Howard-Mold: 23-46 oocysts per 30 view fields) were well below this cutoff value. For both methods this problem can be solved by lowering the dilution of the samples. For the Hemocytometer, this problem can be fixed by lowering the dilution of the sample from 10x to 2x. The method for the Howard-Mold involved adding 60 mL of 70% sucrose solution to a fecal solution, halving this amount could solve the problems with the method. For both the Hemocytometer and Howard-Mold methods the ease of use was

high. Both methods were not time consuming; taking only a few minutes to dilute samples and count the number of oocysts. The convenience of these methods does not negate the inconsistency in the results. For this reason, the Hemocytometer and Howard-Mold methods should not be used for quantifying oocysts in fecal samples with low oocysts density. For all methods, inconsistencies in counts can be explained by oocyst distribution in the samples. The samples were vortexed so that the oocysts could distribute evenly throughout the samples. It is possible that the oocysts did not distribute evenly and an incorrect amount- too many or too few- was transferred to the counting apparatuses. Once the oocysts were on the counting apparatuses they were allowed to sit for a couple of minutes, so the oocysts could resettle at the top for counting, but in the time it takes to count oocysts, especially for the Sugar Flotation, more oocysts can float up. This would distort the counts because areas that have already been counted would suddenly have oocysts that could not be counted.

Chapter Three: Species Identification of Oocyst Through Morphological and Molecular Approaches

Introduction

Parasitological research in the early 20th century focused on identifying coccidian oocyst species (Haug et al, 2005). In *Eimeria* the traditional way to determine species was by morphology, host specificity, pathology, and geographical occurence; however, molecular methods are now seen as essential to accurately determine species (Yang et al, 2015). The move to molecular means of species determination occurred because of the similarities between oocysts morphology among species (Yang et al, 2015). The first *Eimeria* coccidian species to be sequenced was in 2002 (Blake, 2015). Since then research into identifying *Eimeria* species by sequencing extracted DNA has become prevalent. An important aspect of molecular identification of coccidian species is PCR because it can amplify the small amount of DNA retrieved from extraction so that its nucleotides can be sequenced. PCR has been pivotal to sequencing and species determination in *Eimeria*; 14 species have been determined from different PCR methods (Tewari and Maharana, 2011).

Eimeria species have very strict host specificity with very few exceptions (Vrba and Pakandl, 2015). Vrba and Pakandl (2015) found that three species of turkey coccidian can transmit to bird species in different genera. No one has investigated generic specificity of turkey coccidia. This means it is quite possible that the ocellated turkey of

Central America has some cross-species coccidian. This possibility of cross-species transmission could cause economic troubles in the United States of America if global warming causes habitat migration northward. Perez-Rodriguez and Hera (2014) predicted that avian blood parasites are going to redistribute northward with warming climates. As climates warm and resources move to more sustainable environments, tropical birds will either move up in elevation or travel further north- depending on the elevational changes in the surroundings (Serkercioglu et al., 2012). The area that will be most affected by these shifts is the South and Central American mountains and Central American biodiversity hotspots (Serkercioglu et al., 2012). The ocellated turkey- the focus of my research- is both a tropical and Central American bird. This predicted shift in tropical habitats could bring the coccidia that infect *M. ocellata* to areas with *Meleagris gallopavo*-wild and domesticated, or spread North American coccidians southward. If these coccidians have the ability to cross-transmit like some turkey coccidians can, then it would be important to recognize the new species, so that medicines and vaccines can be developed to prevent any ill effects to both turkeys and our economy. Identification of *Eimeria* species is important to diagnosis and control (Gadelhaq et al., 2015). The objectives of this chapter are to determine *M. ocellata's* oocyst species through morphological means and test five methods of extracting DNA from *M. gallopavo* to determine the best method of extraction for *M. ocellata*.

Methods of Morphological Identification

Isolating Oocyst

0.5 mL of the fecal sample was put in a 15 milliliter conical tube and filled with a 70% sucrose solution. The tube was placed in the IEC HN-SII centrifuge and more 70% sucrose solution was added until a convexity was formed. A coverslip was placed on top

carefully to ensure no large bubbles form under it. The sample was then centrifuged for 12 minutes at 2000 rpms (706.5 g). Coverslips were removed from the top of the tube vertically and placed on a clean microscope slide. The slide was left to sit for a couple of minutes before microscopy.

Microscopy

The slide was first examined under 100x magnifications to ascertain if there were oocysts on the slide. Once an oocyst was found on the slide, the magnification was increased to 400x magnification. The oocyst was centered and focused before adding immersion oil and moving on to the 1000x magnification or oil immersion lens. At 1000x magnification, the oocyst's dimensions, length and width, were measured with the ruler micrometer and morphological features, such as sporulated or unsporulated, were noted. After the first use of the slide, the coverslip was removed and the samples rinsed with water into labelled plastic cups. The samples were set aside to be measured another day. When ready to count again, a glass Pasteur pipet was used to make a single, small drop on a new, clean microscope slide and covered with a coverslip. The process leading up to oil immersion was repeated until the entire sample was rechecked for oocysts and all visible oocysts were measured.

Results of Morphological Identification

Samples 46, 1, 28, 42, 27, and 21, collected from wild *M. ocellata,* were examined for this project. Only oocysts from samples 46, 42, and 27 were measured. From these three samples, 17 oocysts were measured. The measurements, morphological features, and my predictions about species identity were recorded for the 17 oocysts (Table 2). The prediction of species identity are based on documented morphological variation of the oocysts from *M. gallopavo*.

		Shape	Size (µm)	Sporulated?	Putative Species
Sample 46	1	Circular	24.8 x 23.3	Unsporulated	*E. subrotunda or E. innocua*
	2	Oval	25.3 x 20.2	Unsporulated	*E. dispersa or E. meleagrimitis*
	3	Oval	25.3 x 20.2	Unsporulated	*E. dispersa or E. meleagrimitis*
	4	Oval	26.8 x 20.7	Sporulated	*E. dispersa or E. meleagrimitis*
	5	Oval	22.8 x 20.2	Unsporulated	*E. meleagrimitis*
	6	Oval	24.3 x 21.3	Unsporulated	*E. meleagrimitis*
	7	Oval	25.3 x 17.7	Sporulated	*E. gallopavonis or E. adenoeides*
Sample 42	8	Oval	17.7 x 13.7	Sporulated	*E. meleagrimitis*
	9	Oval	22.8 x 17.7	Sporulated	*E. meleagrimitis*
	10	Oval	17.7 x 13.7	Sporulated	*E. meleagrimitis*
Sample 27	11	Oval	20.2 x 18.2	Sporulated	*E. meleagrimitis*
	12	Oval	20.2 x 18.8	Sporulated	*E. meleagrimitis*
	13	Oval	22.8 x 15.2	Sporulated	*E. meleagridis*
	14	Circular	17.7 x17.7	Sporulated	*E. subrotunda or E. innocua*
	15	Oval	25.3 x 18.7	Sporulated	*E. adenoeides*
	16	Oval	22.8 x 15.2	Sporulated	*E. meleagridis*
	17	Oval	22.8 x 15.2	Sporulated	*E. meleagridis*
		Average:	23.1 x 18.4		*E. meleagridis*

Table 2: Dimensions and morphological features of oocysts from *Meleagris ocellata*

Discussion Morphological Identification

Oocysts were determined to be *Eimeria* because they had the 4 sporocysts, a thin cell wall, and an ellipsoid shape. Based on morphological features alone, it would seem that I did not find a new species of *Eimeria* coccidian. All the oocysts that were isolated were similar morphologically to oocysts previously found in *Meleagris gallopavo* (Table 1). The morphological descriptions do show a strong possibility that there is more than one type of *Eimeria* coccidian species in *Meleagris ocellata.* However, the pressure on the coverslip by the microscope objective and the hypertonic environment can both distort the oocysts (Vrba and Pakandl, 2014).

Morphological descriptions are not enough to determine if I found a new species or if there are multiple species, because of the very similar morphology for *Eimeria* coccidia, especially in *M. gallopavo* (Chapman, 2008). DNA extraction and nucleotide sequencing would be the next step to determining the novelty of these coccidians. Indeed, Vrba and Pakandl (2014) found that turkey coccidian species determined by morphological features did not align with the species determined by DNA sequence.

Methods of Molecular Identification

Microscopy

Isolation of the oocyst was the same as in the morphological procedure. The slide was scanned in 100x magnification to ascertain the presence of oocyst. If oocysts were present, the slide was rinsed into a 1.5 mL microcentrifuge tube. Samples 1-D through 6-D came from fecal sample Orange/Purple 6/18/14, samples 1-E through 6-E came from fecal sample Orange/Purple 2014, and sample 1-F through 3-F came from fecal sample 1A Black/Red 2014. Then, five washes in reverse osmosis (RO) water were performed to remove any contaminates that might interfere with DNA extraction. For the five washes,

the microcentrifuge tube was filled with RO water and centrifuged in the Qualitron Microcentrifuge DW-41. This model runs at 6400 rpms or 2000 g. Samples 1-6 D and 1-6 E were centrifuged for 5 minutes and samples 1-3 F were centrifuged for 10 minutes. The time of centrifugation was increased in an attempt to recover more oocysts. Finally, the sample was resuspended in 0.1 mL of RO water. Fifteen microliters was taken from the sample and placed on a clean microscope slide with coverslip. The number of oocysts under the coverslip was counted, and proportions were used to estimate the number of oocyst in one milliliter.

DNA Extraction

Five methods of DNA extraction from the oocysts were attempted. Fecal samples from *Meleagris gallopavo* were used for these testing purposes. Table 6 has a summary of all methods attempted and the results of each trial.

Method 1

For method 1, BiOstic Bacteremia DNA Isolation Kit (MO BIO Laboratories, Inc., Catalog No.: 12240-S) was used with no modifications. The number of oocysts in the sample is unknown because the sample was made before counting methodology was begun.

Method 2

This method was taken from Fritzler et al (2011). The kit used was the QIAMP DNA Stool Mini Kit (QIAGEN, Catalog No.: 51504). Two modifications were added to the kit instructions - a bleach wash and 10 freeze-thaw cycles. Samples were washed in 10% bleach. The wash was performed by first re-suspending the oocyst in 1 mL of 10% bleach followed by incubation in ice for 5 minutes. Next, the oocysts were re-pelleted

with an Eppendorf Centrifuge 5415 C for 2 minutes at 11,000 rpm (10,000 g) and the supernatant drained off. The bleach was washed off the oocyst by re-suspending in reverse osmosis water 6 times. The six washes were centrifuged in the Qualitron Microcentrifuge DW-41 for 5 minutes. While the oocysts were suspended in the lysis buffer from the kit, 10 freeze-thaw cycles in liquid nitrogen were performed. The sample used contained 522 oocysts and came from Orange/Purple 2014.

Method 3 and 4

Both method 3 and 4 came from sample Orange/Purple 6/18/14. Sample for Method 3 contained 1221 oocysts and the amount of oocysts is unknown for Method 4. PowerMicrobiome RNA Isolation kit (MO BIO Laboratories Inc., Lot No.: PMR15J6-S) was used for these two methods. The kits DNase steps and reagents were skipped because DNA, not RNA, was being isolated. For Method 3, the washed and reverse osmosis water suspended sample was used with no modifications to the kit. For method 4, 5 mL of Orange/Purple 6/18/14 fecal sample was spun down in a 15 mL conical vial in the IEC HN-SII centrifuge for 12 min at 2000 rpm (706.5 g) to separate the feces from potassium dichromate. The potassium dichromate supernatant was drained off and the pelleted feces rinsed with reverse osmosis water into microcentrifuge tubes. These tubes where then washed with reverse osmosis water in the Qualitron Microcentrifuge DW-41 for 5 minutes. This was repeated until 0.227 g of feces was obtained. There were no modifications to the kit for Method 4.

Method 5

Three samples- one with 85µL of RO water (1 F) and the other two with water decanted off - in microcentrifuge tubes were used in this method. A ball bearing is added

to each tube and placed in vortex adaptor on dry ice for 30 minutes. Then, the adaptor was placed on the QIAGEN TissueLyser LT and shaken at 50 Hz for 2 minutes. 180 µL of ASL buffer and 20 µL of Proteinase K were added to the three samples and the tubes were left to incubate at 56°C overnight. The QIAamp DNA Mini Kit (QIAGEN, Catalog No.: 51306) was used, and instruction followed with no modifications. The diluted sample had 175 oocysts. The two decanted samples had 1944 and 1502 oocysts. All samples were taken from fecal sample 1A Black/Red 2014.

PCR

To amplify DNA, PCR was used. The procedure used for PCR was copied from Dolnik et. al (2009). The outside primers and procedure were only used. PCR was performed in an Eppendorf Mastercycler gradient. The PCR solution was 12.5µL of the GoTaq® Green Master Mix, 1µL of primer COX tenella R2, 1µL primer COX tenella F2, 5µL of DNA sample, and 5.5µL of nucleotide free water. For the negative control all was the same expect there was 10.5µL of nucleotide free water and no DNA sample. The procedure for PCR was to preheat the hot plate to 95°C and the lid to 105°C, start with a denaturing step at 94°C for 3 min, then 12 touchdown cycles with a denaturing step at 94°C for 30 seconds, annealing step at 57°C (-0.7°C for each cycle) for 30 seconds, elongating step at 72°C for 30 seconds. After the touchdown cycling is complete, eight more cycles were run of denaturing at 94°C for 30 seconds, annealing at 48°C for 30 seconds, elongation at 72°C for 30 seconds. Lastly, there is an elongation step at 72°C for 10 min.

Gel Electrophoresis

Gel electrophoresis was used to check if DNA was isolated and PCR was successful. DNA isolation and PCR checks were run on 1.5% gels (10x7x1.5cm) for 60

minutes at 100 volts. The first gel was not run as described above. The composition of the gel was the same, but it was run for 50 minutes at 98 volts, then 20 more minutes at 110 volts. For DNA isolation confirmation, 5µL of the ladder and all 6.5µL of a mixture of 4.0µL of DNA isolation sample and 2.5µL of blue dye were loaded into separate wells. For PCR confirmation, 5µL of the ladder and 6.5µL of the PCR product were loaded into separate wells. The electrophoresis methods used to check the PCR results were the same as those used to assess DNA isolation.

	Method 1	Method 2	Method 3	Method 4	Method 5
Source of Method	none	*Fritzler, 2011*	none	None	None
Manufacturer of the kit	BiOstic Bacteremia DNA Isolation Kit	QIAMP DNA Stool Mini Kit	PowerMicrobiome RNA Isolation Kit	PowerMicrobiome RNA Isolation Kit	QIAamp DNA Mini Kit
Preparation	Instructions from kit followed	Samples were 10% washed with bleach	Instructions from kit followed	Instructions from kit followed	Instruction from kit followed
Method of oocyst disruption	Mechanical: with quartz in lyse buffer	10 Freeze/thaw cycles in liquid nitrogen while in lyse buffer	Mechanical: with glass beads in lyse buffer	Mechanical: with glass beads in lyse buffer	Mechanical, ball bearings shaken in tissue vortex and frozen in dry ice
Number of oocysts	N/A	522	1221	Unknown: 0.227g fecal sample was used	1: 175 2: 1944 3: 1502

Table 3: Attempted approach to DNA isolation from coccidian oocyst in *Meleagris gallopavo* feces

Results of Molecular Identification

None of the methods used to isolate DNA were successful. Figures 6-9 show the results of the gel electrophoresis for DNA isolation. For all methods, the size standard appears on the gel, but there is no evidence of coccidian DNA. PCR was attempted to amplify any possible DNA in methods 2, 3, and 4. Figures 5 and 6 show the results of the gel electrophoresis for the PCRs. No DNA bands were present on any of the PCR gels.

Figure 4: Gel Electrophoresis Results for Method 1 of DNA Isolation. Well 1 and 7- Size Standard, Well 4- DNA

Figure 5: Gel Electrophoresis Results for Method 2 of DNA Isolation. Well 2- Size Standard, Well 5- DNA

Figure 6: Gel Electrophoresis Results for Method 3 and 4 of DNA Isolation. Well 1- Size Standard, Well 3- the wash sample, Well 5-the fecal sample

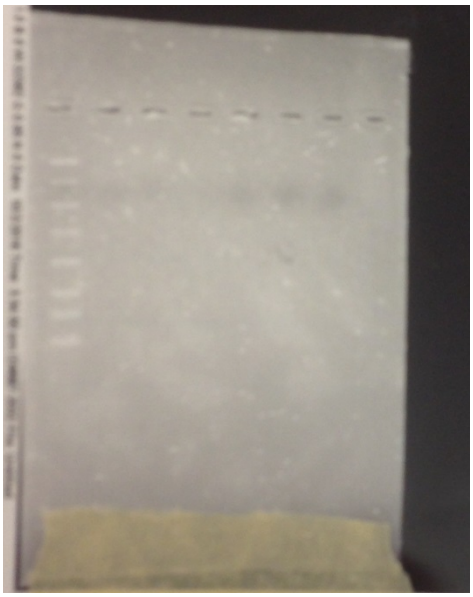

Figure 7: Gel Electrophoresis Results for Method 5. Well 1- Size Standard, Well 2- product 1, Well 3- product 2, Well 4- product 3, Well 5- product 1A, Well 6- product 2A, Well 7- product 3A.

Figure 8: Gel Electrophoresis Results for PCR of Method 2. Well 1- Size Standard, Well 3- PCR product 1, Well 5- PCR product 2, well 7- Negative control

Figure 9: Gel Electrophoresis Results for PCR of Method 3 and 4. Well 1- Size Standard, Well 2- the wash sample PCR product 1, Well 3- the wash sample PCR product 2, Well 4- fecal sample PCR product 1, Well 5- fecal sample PCR product 2, Well 6- Negative control

Discussion of Molecular Identification

Despite the success of previous researchers, my research never isolated DNA from oocysts. Successful DNA isolation would have appeared as bands on the gel. Oocysts have strong cell walls that would inhibit DNA extraction. No DNA extraction kit I used was specifically made to break up these kinds of cell walls. Cell lysing with glass beads is dependent on vortexing speed, time, and size of the beads (Cha et al., 2014). Only methods 3 and 4 used glass beads, the size of the beads was 0.1 mm. The smallest glass beads studied in Cha et al (2014) research was 0.5 mm. This probably means that the glass beads used in the PowerMicrobiome RNA Isolation Kit were too small and probably went too slow to break open the cell wall of the oocysts, so DNA could not be extracted. Table 4 summarizes all the faults and mistakes made for each kit. All kits had some flaws that would make it less likely to extract DNA from the oocysts. Method 2 came from Fritzler et al (2011), and they were able to extract DNA from oocysts. The reason the extraction did not work in my research might be that my oocysts were older; therefore, the oocysts had weaker cell walls. The bleaching step may have allowed 10% bleach to leak into the oocysts destroying the DNA.

If amounts of DNA too small to appear on the gel were extracted, then PCR should have amplified them. The results of no band on the gel suggest that DNA extraction did not happen. Unfortunately although I included a negative control to check for contaminates in the PCR procedure, there was no positive control to prove the PCR could amplify *Eimeria* DNA. The primers used in the PCR were originally made for coccidian of blackcaps (Dolnik et al, 2009). This might lower the chance that the primer and DNA template will anneal. I checked GenBank for the primers and found extreme redundancy, so that the primers were connected to many species. The primer sequence,

when searched for in GenBank, resulted in many organisms matching that sequence. This means that the primer can anneal to many organism's DNA to amplify during PCR. For these reasons, I believe the primers probably did not contribute to the non-results of the PCR.

Table 4: Errors during DNA extraction for each method attempted

	Method 1	Method 2	Method 3	Method 4	Method 5
Errors during Use of Kit	1-Equipment was not appropriate for kit -Incubator wells not large enough for tubes provided in kit -Vortex did not have adaptor so sample was not shaken continuously 2-Samples were in RO water which could have destroyed the oocysts and diluted reagents in the kit	1-Vortex did not have adaptor so sample was not shaken continuously 2-Samples were in RO water which could have destroyed the oocysts and diluted reagents in the kit	1-Samples were in RO water which could have destroyed the oocysts and diluted reagents in the kit 2-Had no vortex adaptor so advisor created one. Do not know if samples were properly shaken in apparatus	1-Kit calls for 0.25g of fecal matter. I could only obtain 0.227g of fecal matter. 2-Had no vortex adaptor so advisor created one. Do not know if samples were properly shaken in apparatus	1-Samples were in RO water which could have destroyed the oocysts and diluted reagents in the kit -Only partially true for 2-F and 3-F because most of the water was decanted off

Conclusion

In conclusion, my research has determined that the most repeatable method of quantifying oocysts is the Standard Sugar Flotation, which supports Haug et al (2005) findings that the least diluted solution is the most consistent. The other three methods had higher CVs than Standard Sugar Flotation, but were more practical in their ease of use. For this reason, I have concluded that the Standard Dilution is the best method to use, because its repeatability is only slightly lower than the Standard Sugar Flotation and its ease of use is exceptionally higher. Additionally, I have not determined a new species through oocyst morphology or nucleotide sequencing. Morphology was too broad as a descriptive mechanism because of species similarities (Chapman, 2008), but the oocysts in *M. ocellata* do have many similarities to those of *M. gallopavo*. This suggests that either they have the same oocyst species or that any new species is very morphologically similar to *M. gallopavo*'s oocyst species. While DNA extractions and PCR would have provided a more specific means of species identification, I could not do these successfully. Further work with better oocysts counts and quality should result in DNA extractions in the future.

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Appendix I:

Oocysts Counts for Each Method of Quantifying Oocysts

Standard Sugar Flotation	
Trial	Number of oocyst per gram
1	27,680
2	32,163
3	37,688
4	35,920
5	35,447
6	35,832
7	35,787
8	34,738
9	32,763
10	37,801
Avg	34581.86
StD	3025.464
Hemocytometer	
Trial	Number of oocyst per gram
1	221,951
2	266,341
3	266,341
4	166,882
5	62,580
6	166,882
7	125,161
8	229,462
9	146,021
10	208,602
Avg	186,022
StD	64,862.94

Howard-Mold	
Trial	Number of oocyst per gram
1	313,449
2	161,891
3	217,003
4	196,336
5	248,004
6	279,004
7	220,448
8	230,781
9	248,004
10	241,115
Avg	235,604
StD	41,973.33
Standard Dilution	
Trial	Number of oocyst per gram
1	35987
2	35987
3	39586
4	44657
5	34832
6	38405
7	39257
8	53922
9	35949
10	50329
Avg	40,891.1
StD	6,606.832