USING NANOPORE NGS TO SEQUENCE AND IDENTIFY CHICKEN EIMERIA IN MIXED POPULATIONS

by

BENJAMIN WILLIAM JACKWOOD

(Under the Direction of Brian Jordan)

ABSTRACT

Eimeria are a type of parasitic protozoan that can cause coccidiosis, an enteric disease, in various animals. In commercial chickens, several species of *Eimeria* can cause devastating outbreaks of coccidiosis, resulting in significant financial losses. To detect and differentiate *Eimeria* species, molecular methods such as polymerase chain reactions (PCR) and next-generation sequencing (NGS) technology can be used. However, a routine and straightforward protocol and bioinformatic platform for using NGS to differentiate *Eimeria* species has not been developed yet. To address this issue, our laboratory aimed to evaluate the ability of nanopore NGS to sequence PCR amplicons of *Eimeria* gene regions and create a protocol to sequence, identify, and distinguish *Eimeria* species in mixed populations. We generated amplicons of Internal Transcribed Spacer-1 (ITS1), Ribosomal 18S DNA (18S), and Cytochrome Oxidase 1 (CO1) genes using pan-species primers and sequenced them with nanopore NGS. Our first study found that nanopore NGS can sequence *Eimeria* amplicons in coccidiosis vaccines with >97% contig sequence identity across replicates. Additionally, the high amplicon sequencing depth allowed us to create detailed phylogenetic trees with existing reference sequences, identifying all species present. In our second study, we replicated these results with field samples. Nanopore NGS was

able to sequence amplicons from oocysts isolated on broiler farms using various coccidiosis control programs. Our protocol was used again to create detailed phylogenetic trees, including field samples, coccidiosis vaccines, and existing references, showing the relatedness between contigs. Finally, we developed a bioinformatic platform to supplement our sequence assembly methods. This platform automates the computational process by subjecting all raw nanopore NGS reads to the Basic Local Alignment Search Tool (BLASTn) and parsing through those results. In conclusion, our work demonstrated the suitability of nanopore NGS to sequence amplicons of identifying gene regions in *Eimeria* and investigate *Eimeria* species present in broiler chicken houses under various coccidiosis control programs. The resulting data produced detailed *Eimeria* gene assemblies and phylogenetic trees, showing the good applicability of nanopore NGS technology for sequencing and discerning mixed species populations from vaccines and field samples.

INDEX WORDS: *Eimeria*; Coccidiosis; Poultry; Broilers; Nanopore NGS; Identification

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by

BENJAMIN WILLIAM JACKWOOD

B.S. Biology, The University of North Georgia, 2015

M.S. Pharmaceutical and Biomedical Science, The University of Georgia, 2017

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BENJAMIN WILLIAM JACKWOOD

Major Professor:	Brian J. Jordan
Committee:	Rami A. Dalloul
	Ramesh K. Selvaraj
	Eric Shepherd

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
May 2023

DEDICATION

I want to dedicate this dissertation to my wife, Meredith. Thank you for your love, encouragement, and belief in me. I would also like to dedicate this work to my father, Mark. I could not have achieved this work without the love and guidance of my wonderful wife and family. I am fortunate to have you all as my support system and I am so thankful for it.

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Thank you.

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CHAPTER 1

INTRODUCTION

Coccidiosis is an enteric poultry disease of universal importance caused by infection from protozoan parasites of the genus *Eimeria*. *Eimeria* are single-celled organisms that flourish under commercial poultry conditions due to their short, direct life cycles. Species known to infect chickens include *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella*. Each species causes host-specific disease, but severity of clinical symptoms ranges from mild to severe. All species appear ubiquitous and may occur in combinations of up to at least six species present on any farm (1). Chickens ingesting food or water containing sporulated oocysts from the environment leads to development of the disease coccidiosis. Each species favorably infects different regions of the intestinal tract and can result in lesions, thickening of the intestinal wall, hemorrhages, and necrosis (2). Diagnosis by examining these characteristics requires time, resources, and the sacrifice of animals for necropsy. Coccidiosis is the most economically significant disease affecting poultry producers worldwide. It costs the poultry industry an estimated $14.4 billion annually due to losses in feed conversion ratios and reduced weight gain (3). Coccidiosis is a known predisposing factor for both necrotic enteritis (NE) caused by *Clostridium perfringens* infection and salmonellosis (4). Leakage of plasma proteins into the gut lumen and increased intestinal mucus from coccidiosis provide a favorable environment for *C. perfringens* (5). Lesions resulting from the subclinical form of NE cause reduced nutrient absorption and feed conversion, costing producers $0.0625 per bird, with worldwide losses due to necrotic enteritis estimated at $6 billion in 2015 (4).

Traditionally, identifying *Eimeria* species infecting poultry has been based on oocysts' morphological and biological characteristics. Some species are identified easily by the location and presence of gross lesions in the intestinal tract or by the size of the oocyst. Still, others are not as easily identifiable by oocyst size, for many species overlap. In an attempt to address this problem, molecular identification approaches such as PCR (Polymerase Chain Reactions) or qPCR (quantitative Polymerase Chain Reactions) assays capable of differentiating *Eimeria* species have existed for more than 20 years. Molecular analysis has the advantage that small populations of some species can be detected, even when gross lesions are not apparent (6). Sequences of genomic DNA originating in *Eimeria* species are used to design oligonucleotide primers, allowing selective gene amplification by PCR. Three genome regions, Internal Transcribed Spacer 1 (ITS1), Ribosomal DNA 18s (18s), and Mitochondrial Cytochrome C Oxidase 1 (CO1), have been used to differentiate organisms and develop taxonomic relationships (7).

Additionally, molecular assays that are a variation of the above described methods have been described and combine all primers for each species in a single tube (8). While several PCR assays have been related to identifying certain *Eimeria* species, very few studies have focused on the applicability of these techniques for identifying Eimeria species in commercially raised poultry throughout the world (9-11). Developing a standardized protocol or newer sequencing technologies supporting high throughput sampling for *Eimeria* will enhance the value of such data while promoting PCR application.

Nanopore sequencing is a unique, rapidly expanding, and accessible technology that enables real-time analysis of varying-length DNA or RNA fragments. Nanopore NGS involves monitoring changes in ion flow as nucleic acids pass through protein nanopores. Dissimilar nucleic acids block ion flow differently, and the change in current is measured to provide sequence

information. One company that pioneered this technology is Oxford Nanopore Technologies (ONT). Based in the United Kingdom, ONT was founded in 2005 as a spin-out from the University of Oxford by Hagan Bayley, Gordon Sanghera, and Spike Willcocks. The company's main products include portable protein nanopore sequencing USB devices such as the MinIon Mk1B. Publications suggested a DNA sequence read rate of 90 nucleotides per second per nanopore with an error rate of 30% during the early phase of its release around 2014 (12). New protein pores with different apertures eventually lowered the sequencing error rate. With the version R9 pore release in 2016 and the latest R10 pore release, raw error rates have been reduced to 2-10% for various types of DNA sequencing (13). These pores work with both 1D and 2D chemistry. Sequencing one strand of the duplex DNA signifies 1D chemistry, and sequencing both the template and complimentary strand illustrates 2D chemistry.

This research aims to assess the ability of nanopore NGS to produce more complex genome assemblies of *Eimeria* parasites affecting poultry. Data from this work may allow for the design of a profiling assay to identify poultry *Eimeria* species in mixed populations. This research hypothesizes that there is enough inter-species variation between vaccine *Eimeria* strains and pathogenic field *Eimeria* to develop a molecular profile for each type. Therefore, a sample sent in for diagnostics may be identified quickly by nanopore NGS as belonging to a coccidiosis vaccine. By conducting this research with nanopore NGS, we can also help evaluate its potential for use in future *Eimeria* sequencing or identification. We propose to test this hypothesis with three specific aims outlined on the next page.

Specific Aim 1. Assessing the ability of Nanopore NGS to Produce High-Quality Sequences of ITS1, CO1, and 18s Genes from Eimeria in Multi-species Coccidiosis Vaccines

Oocysts are found wherever chickens are raised, but no permanent method to eradicate coccidiosis makes disease control essential. The robust nature of poultry *Eimeria* seems to prevent their elimination by quarantine, disinfection, or sanitation. Coccidiosis can be prevented by using anticoccidials such as ionophores and certain chemicals; however, increased lesions from drug-resistant parasites during the last few weeks of a broiler flock can result in a loss of $0.015 per pound (14). Anticoccidial treatments are effective in protecting against disease outbreaks, but the development of drug resistance, external pressures on the industry, and regulatory changes have producers turning towards vaccination (15, 16). Unlike anticoccidials, coccidiosis vaccines do not cause drug resistance. Coccidiosis vaccines contain sporulated oocysts of varying formulations and concentrations, which are given at low doses to initiate an immunologic response (17, 18). The most common method for coccidiosis vaccination is spraying day-old chicks with a liquid vaccine solution using a spray cabinet. In recent years, the use of a gel diluent for coccidiosis vaccines has become more widespread. This delivery method delivers gel beads containing oocysts in the feed and is shown to be protective (19). Coccidiosis gel vaccines may also be applied to day-old chicks at the hatchery with a spray cabinet via a gel applicator bar.

To evaluate the potential of nanopore NGS technology to identify Eimeria in mixed population samples correctly, *Eimeria* target genes 18S, ITS1, and CO1 were sequenced in triplicate or until high-quality gene sequences of *Eimeria* species with economic importance in commercial poultry were obtained.

Four multispecies coccidiosis vaccines were used for sequencing. The vaccines subjected to sequencing were Coccivac B52 (Merck Animal Health), Advent (Huvepharma), Immucox 3

(Ceva), and Inovocox (Zoetis/Huvepharma). These vaccines contain three or four species of *Eimeria* which are thought to be the most important for broiler chickens. Additionally, Coccivac B52 is labeled 52, denoting five total strains of *Eimeria*. Two of the 5 are *E. maxima,* with 1 being a precocious *E. maxima* strain. Coccidiosis vaccines provide relatively clean samples for DNA isolation and sequencing preparation and clearly label known species. A previously published pan-species PCR reaction for each targeted gene region was performed to obtain sequencing starting material for each vaccine. Amplicons were then purified and sequenced by nanopore NGS technology.

In addition to producing high-quality sequences of commercially important *Eimeria* species, we also tested the reproducibility of nanopore NGS by sequencing each identifying gene region in triplicate or until adequate sequence coverage and depth were compiled. Vaccine sequences were compared between their replicates and to each other, and then consensus sequences were combined end-to-end for each species in each vaccine to form vaccine profiles. These vaccine profiles could be compared to future diagnostic samples and serve as molecular barcodes, which may help differentiate species. Additionally, probed for species not listed on vaccine labels to test the specificity of the sequencing reactions.

Specific Aim 2. Producing High-Quality Sequences of ITS1, CO1, and 18s from Eimeria Field Samples Using Nanopore NGS.

Oocysts of vaccine origin persisting in the environment after use is a significant drawback of using live vaccination as a primary method to control coccidiosis outbreaks. Vaccinating birds typically protects them from future infection. However, the subjective nature of identifying

Eimeria makes it challenging to discern if a sample from the field containing oocysts is a true coccidiosis challenge or simply a remnant from a previous vaccine.

In addition to determining strain origin, sequencing field Eimeria may provide insights into the designation of species themselves. Field samples offer the possibility of identifying species not present in coccidiosis vaccines. Several other species, including *E. hagani* and *E. mivati*, are mentioned in the literature, but their existence as separate *Eimeria* species is still debated. Ten species of Eimeria have been labeled from chickens, with three recently recognized through clustered Operational Taxonomic Units (OTUs) derived from DNA sequences (9). The literature suggests that these cryptic *Eimeria* OTUs possess sufficient genetic and biological diversity to be considered new and distinct species. The names *Eimeria lata*, *Eimeria nagambie*, and *Eimeria zaria* are suggested based on their appearance and origins in the first isolates (9). With the increased use of live coccidiosis vaccination, which relies on species-specific immunity, comes a need to distinguish between all *Eimeria* species affecting poultry properly.

After displaying that high-quality *Eimeria* vaccine sequences from nanopore NGS technology are reproducible, *Eimeria* field samples were obtained from vaccinated and non-vaccinated commercial poultry farms for sequencing. Collecting samples from farms using coccidiosis vaccines where the oocysts have passed through live chickens will allow us to measure generational variance, if any. In contrast, samples obtained from commercial chicken houses with no history of coccidiosis vaccine usage will provide a template for genetic variation of potentially pathogenic *Eimeria* species and sequences of any species not present in coccidiosis vaccines.

Field samples were treated similarly to vaccine samples for this project. DNA was isolated from oocysts, amplified by the initial step PCR using pan-species primers, and analyzed by the nanopore Mk1B sequencer. The only difference in the sample preparation of field samples was the

separation of oocysts from litter and feces. DNA sequences collected from birds treated with known coccidiosis vaccines were compared to DNA from non-vaccinated farms. With these data, we can assess any changes in the three identifying gene regions mentioned after at least one generation of parasite replication.

Specific Aim 3. Phylogenic Analysis of Sequences and Validation of Gene Assemblies

The phylogenetic analysis includes investigating the evolutionary development of *Eimeria* species by comparing their identifying gene regions. Genomic differences between species may allow for the development of a rapid, high throughput molecular screening assay for differentiating poultry *Eimeria* species in the future. The first step involves bioinformatic software tools that will be used to construct gene assemblies for vaccine and field oocysts. Geneious 9.1.8 (Biomatters Ltd) is a world-leading bioinformatics software for sequence analysis, and Bowtie 2 (20) is a fast and memory-efficient tool for aligning sequence reads to long reference sequences. A consensus sequence from each gene assembly was developed using these tools and data from the nanopore NGS platform. Consensus sequences were compared to previously known GenBank references to confirm percent identities. Next, phylogenetic trees containing vaccine and field sample sequences and previously published chicken *Eimeria* GenBank references were produced. In addition to investigating phylogenetic relationships, this project developed a Standard Operating Procedure (SOP) detailing the workflow necessary to identify *Eimeria* using nanopore NGS data. The workflow includes developing and organizing a series of executable shell scripts on the Sapelo2 computing cluster to form a bioinformatics pipeline to characterize sequence reads further. Data from sequencing vaccine and field origin oocysts will be used as a reference tool for future diagnostic samples in a species-identifying assay.

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CHAPTER 2

LITERATURE REVIEW

2.1 COCCIDIOSIS: INTRODUCTION

Eimeria life cycle

Eimeria that infect chickens flourish under commercial conditions due to their short, direct life cycles. Depending on the species, the life cycle process takes 4-6 days, but reproductive potential from a single ingested parasite is relatively consistent (6, 20). When a susceptible host ingests a sporulated oocyst, the oocyst wall is crushed in the ventriculus resulting in the excystation of sporozoites from the sporocyst. Sporozoites may also emerge from the sporocysts with the help of bile salts in the small intestine (6, 21). Sporozoites are the infective form of coccidian parasites and move by gliding motility. Sporozoites enter cells in the intestine's mucosa and begin the cell cycle leading to reproduction (22). After recognizing target epithelial cells, organelles in the apical complex achieve host cell penetration (23, 24). Inside the host cell, a parasitophorous vacuole forms around the sporozoite, where the sporozoite will begin merogony (25).

There are three crucial *Eimeria* cell cycle stages; merogony, gamogony, and sporogony. Merogony consists of at least two generations of asexual parasite replication and sometimes as many as four (6, 26, 27). Each round of asexual replication results in more host cell damage. Depending on the parasite, merogony can result in 2-100,000 merozoites formed from a single sporozoite (6). Mature merozoites rupture the host cell to penetrate new epithelial cells and begin further generations of merogony. The number of asexual replication cycles will depend on the

parasitic species. If there are as many as four generations of merogony, this will likely be the stage that causes the most pathogenic lesions (28-30). In some species, like *E. tenella*, maximum tissue damage may occur when second-generation schizonts, immature merozoites, rupture cell walls to become second-generation merozoites (6). After the final round of asexual replication, merozoites enter epithelial cells for development into gametes.

During gamogony, either macro or microgametocytes can be formed. This phase results in sexual reproduction where small, motile, male-line microgametes seek out larger, female-line macrogametes to form a zygote. The fertilized egg will then mature into an unsporulated oocyst and exit through the feces. Oocysts released from the intestinal mucosa are shed in the feces for several days after patency is reached (6, 31).

The final stage, sporogony, occurs outside the host and is the process by which the parasite becomes infective. Immature, unsporulated oocysts undergo sporulation to form 4 sporocysts, each containing 2 sporozoites, at which point it is mature and infective to a new susceptible host (30, 32, 33). Once in the environment, sporulation begins immediately but requires warmth (25-30°C), moisture, and oxygen. Depending on the species of *Eimeria*, an unsporulated oocyst can become sporulated and infectious in as little as 12-30 hours (34).

Incidence and distribution

Eimeria are ubiquitous in the environment, with surveys in South and North America showing chicken *Eimeria* parasites present in almost all broiler farms (35-37). High positive flocks are also found in Europe (38-40). Depending on location, the most prevalent species in broiler houses in the United States appear to be *E. acervulina* and *E. maxima*, followed by *E. praecox* and *E. tenella* (6). *E. acervulina* is the most frequently encountered species in commercial poultry in

North and South America (8, 41, 42). Concurrent infection with two or more species of *Eimeria* is common (35, 39, 41, 43, 44). However, strict host specificity eliminates wild birds as sources of infection unless as mechanical vectors, the most common means of spreading coccidiosis disease. Examples of mechanical coccidiosis vectors include humans walking between farms and flies. For poultry, all 60 billion birds produced worldwide are at risk of exposure (45).

Reports on coccidiosis disease incidence worldwide indicate that the most significant contribution to the development of the disease in poultry appears to be stocking density. Rearing chickens in houses with littered floors provide more opportunity for infection. In a conventional broiler house rearing system, using recycled litter already seeded with *Eimeria* species allows immunity to develop more quickly than when using fresh litter (46). However, high stocking density and litter moisture, mainly from recycled litter, may provide an environment that promotes rapid sporulation of oocysts and high numbers of oocysts in the litter, leading to an increased incidence of clinical disease (47, 48). Oocysts in the poultry litter or droppings are usually most numerous between 3 and 5 weeks of age and typically decline afterward, though anecdotal evidence exists for late-breaking coccidiosis after transfer to a new house for broiler breeders and layer birds around 19 to 26 weeks of age (49, 50).

Unlike bacterial and viral diseases, coccidia infections are self-limiting and depend primarily on the number of sporulated oocysts ingested and on the immune state of the bird. Coccidia infections in free-ranging birds are rarely a significant problem because birds harbor asymptomatic infections that rarely cause mortality. *Eimeria* have been found in the free-living ancestors of domestic chickens from Asia, the wild Red Junglefowl (Gallus gallus) and Ceylon Junglefowl (Gallus lafayetii), but reports of disease in these hosts are lacking (51, 52). Renal coccidiosis may become problematic when susceptible wild avian species are kept in captivity

(53). However, *Eimeria* seem to plague the commercial chicken industry more than wild avian species. A possible explanation for this tendency may be explained by the fact that *Eimeria* need time to become sporulated before becoming infectious, allowing free-range wild avian species to move away from oocysts shed in their excrement. Commercial chickens often do not have large spaces to roam and are usually confined to houses where large numbers of oocysts aggregate. Maintaining clean housing or raising birds on wire prevents the buildup of infective oocysts and can decrease the risk of coccidiosis (54).

Disease epidemiology in commercial poultry

In the late 1800s and early 1900s, families typically kept small flocks of chickens for their meat and egg production and allowed them to roam freely. However, as poultry farming evolved into large-scale operations, disease control became increasingly important. Coccidiosis was particularly problematic. In 1929, Tyzzer identified the cause as a parasite called Eimeria avium, which was found in almost all poultry farms (55). Although coccidiosis was prevalent in many flocks, the move towards closed housing and higher stocking densities worsened the problem as chickens ingested more oocysts, increasing the severity of the disease. Johnson, another pioneer in coccidiosis research, demonstrated that E. avium consisted of multiple species of Eimeria that did not cross-protect, and immunity could be achieved by inoculating chickens with small numbers of oocysts (56). As methods for diagnosing, treating, and preventing diseases improved, the poultry industry expanded significantly. Today, understanding the molecular epidemiology of coccidiosis is critical in protecting the over 9 billion eggs and 802 million broilers produced annually in the United States alone (57).

Diagnosis of coccidiosis

Coccidiosis is primarily associated with enteric disease and can be subdivided into hemorrhagic or malabsorptive pathologies (58). Most infections are mild or subclinical and can be diagnosed in birds euthanized for immediate necropsy. If oocysts are found by microscopic examination of intestines, it indicates the presence of infection but is not a diagnosis for clinical coccidiosis (59). Despite anticoccidials, mild lesions occur in the intestines of 3-6 week old birds in most flocks (6). A diagnosis is justified when gross lesions are severe. The severity of disease based on gross lesions is graded on a scale of 0-4, as described by Johnson and Reid, where 0 is normal, and 4 is the most severe (60). While this technique was initially designed for scoring the severity of pure infections in research, many parasitologists and veterinarians have adopted it to gauge the severity of natural conditions in fieldwork (61). Four sections of the intestine are scored: the duodenum, midgut, lower small intestine, and ceca. Lesions in birds that have been culled should not be used for diagnosis. Instead, a necropsy should be performed on representative birds from the flock.

Multiple factors, including parasite and host genotype, the size and age of oocyst dose, poultry management system, consequential level of oocyst sporulation, and previous exposure history, can influence the severity of infection. It may also be indicated by counting the number of oocysts per gram of feces (OPG) (62, 63). However, the pattern of oocyst excretion is highly variable, and OPGs are used more as a tool to determine the situation of coccidiosis in chickens on a farm (31). OPGs may be used for rapid and relatively truthful disease ratings in laboratory infections still, this technique has apparent complications where birds are infected with more than one species of *Eimeria,* as replication time varies among species (64).

The biological characteristics of *Eimeria* are adequate for identification and diagnosis by experienced diagnosticians. After diagnosis, the species of coccidia identified is essential as some species are more pathogenic than others. Likewise, histopathological methods are satisfactory for routine examination of coccidia-infected tissues. Sections stained with H&E or other common histologic stains will demonstrate developing stages, and a microscopic lesion scoring method has been implemented (65). However, molecular diagnosis by PCR is used when further confirmation is needed, like in surveys.

Classification and taxonomic relationships

Coccidia are members of the phylum Apicomplexa, characterized by an apical complex in the sporozoites. While many species of *Eimeria* have been described from chickens, only seven are universally recognized (8, 9, 37, 38, 41, 43, 44, 66-69). Biological characteristics helpful in identifying species include the location and appearance of gross lesions in the intestine, oocyst size and shape, endogenous tissue stages, area of parasites within tissues, prepatent time, and immunogenicity compared to reference strains. More recently, sequences of DNA unique to each species have been combined with traditional methods of identification using biological characteristics (11, 67, 68, 70). Additionally, using electrophoretic patterns of metabolic enzymes as an identification technique holds value as a tool for species identification (71). Species can usually be adequately diagnosed using a comprehensive list of differential characteristics from Norton and Joyner (72). If further confirmation is required, cross-immunity and biochemical studies are needed.

Molecular diagnosis of coccidiosis

DNA sequences unique to each *Eimeria* species can be used to design oligonucleotide primers, thus allowing selective identification by PCR. Multiplex PCR techniques have been described that combine all primers for each *Eimeria* species in one tube (8, 70, 73-78). Pan-species primers are specific and sensitive, amplifying genes in samples containing as few as 20 oocysts in individual and multiplex PCR (79). For any molecular identification method to be successful, the DNA extraction technique is crucial. The starting material may be intestinal tissue, fecal droppings, or litter samples. An oocyst rupturing step is needed, often by bead-beating or grinding, in a buffer containing ethylenediaminetetraacetic acid (EDTA) to prevent DNA degradation (68, 80). DNA can then be obtained by ethanol precipitation or commercial extraction kits. PCR products may be checked by simple agarose gel electrophoresis, where a target band of the predicted size may identify the *Eimeria* species in a sample, or loop-mediated isothermal amplification (LAMP) (81, 82). The main goal of any *Eimeria* DNA extraction method is to obtain high-quality DNA for downstream applications. Newer downstream applications include qualitative real-time PCR and NGS (68, 83-85).

2.2 COCCIDIOSIS CONTROL AND VACCINES

Control and prevention

A significant advantage for the poultry industry was the discovery in 1939 by P. P. Levine that chemotherapeutics could be used as anticoccidials (86, 87). Antibiotic treatment was effective initially at curbing coccidiosis-related production losses. Eventually, drug use led to parasite resistance, demanding an alternative method for control (88). Ionophore use began as an alternative to chemotherapeutics, as anticoccidial activity was produced without developing resistance (89). Polyether ionophores became the drugs of choice for coccidiosis prevention in 1972 and remain extensively used today (6). Additionally, the knowledge that repeated exposure to low levels of oocysts can stimulate immunity is currently exploited in coccidiosis vaccines, which may be used alone to protect against the disease or as part of a hybrid program incorporating ionophores (16, 90, 91). Older methods for coccidiosis control often suggest directions for sanitation and disinfection to prevent outbreaks. Besides oocysts being extremely resistant to common disinfectants, a significant reason why eradication is no longer considered valid is that an oocyst-free environment for floor-maintained birds could prevent the early establishment of immunity causing later outbreaks (43, 44, 92, 93).

Anticoccidials

The concept of preventive medication emerged with the realization that most damage is already done when signs of coccidiosis are widespread in a flock. The chicken industry is most

concerned with the pathological changes that a given *Eimeria* species might induce in chickens while producing meat or eggs, and the FDA first approved most anticoccidial molecules in the second half of the 20th century. Among the first such compounds were sulfonamides (94). However, ionophores and chemically synthesized anticoccidials eventually became the standard coccidiosis control method (95). Chemical compounds vary in the type of action exercised on the parasite. Some of these mechanisms are highly detailed, while others have yet to be discovered. Some drugs kill the parasite, while others halt parasite development (95).

Anticoccidial control relies primarily on routine chemoprophylaxis using ionophores and chemical drugs, but resistance develops rapidly and is now widespread (96, 97). Anticoccidials may be efficacious against more than one *Eimeria* species, but few are equally productive against all. FDA-approved compounds are used in animal feed, and most have good selective toxicity. They provide toxicity for the parasite but remain nontoxic to vertebrates. Still, anticoccidials have many downsides, such as overdose from formulation errors, an increase in the development of drug resistance, and the growing demand for broiler chickens raised without antibiotics or drugs. With overdoses, most ionophores depress weight gain under laboratory conditions, and a withdrawal period is often needed to allow compensatory growth to compensate for the loss gain (98).

Programs for the use of anticoccidial drugs

Several types of anticoccidial programs are practiced depending on bird species. The objective of broilers is to produce maximum feed and growth efficiency with minimal disease. In long-lived birds like layers and breeders, the aim is to protect against early acute infections and to provide long-lasting immunity. The program choice often depends on the year's season or current

exposure factors, and it is considered sound management to make periodic changes in anticoccidial drug use. Most producers in the U.S. consider changes in the spring and fall. In the U.S., the administration of live coccidiosis vaccines had worked well, especially when roxarsone, an arsenical compound, was available (99-101). The U.S. FDA (Food and Drug Administration) classifies roxarsone as an antibiotic growth promoter with some anticoccidial activity against *E. tenella* (102). A voluntary halt in sales of roxarsone by the manufacturer over concerns about converting organic arsenic to inorganic arsenic, a carcinogen, caused vaccine manufacturers and broiler producers to develop bio-shuttle programs. Bio-shuttle programs add an anticoccidial drug at low concentrations to the feed and are carried out over a specified period to reduce oocyst shedding. Again, different anticoccidials are often interchanged during the Winter or Summer months (103). Following the halt in sales of roxarsone, bio-shuttle programs generally added an anticoccidial to the grower feed starting at 16-18 days of age to remedy the performance issues. Unfortunately, non-vaccinated birds reared using anticoccidial drugs remain susceptible to infection after drug withdrawal before processing or egg production (104, 105).

Drug resistance

A significant limitation to the effectiveness of anticoccidials is coccidia's ability to develop tolerance after exposure to medication (106, 107). Anticoccidial control relies primarily on routine chemoprophylaxis using ionophore or chemical drugs, but resistance develops rapidly and is now widespread. Surveys reveal drug-resistant coccidia in the United States, South America, and Europe (16, 35-37, 42, 96, 108). Long-term exposure to any drug will produce a loss in sensitivity and, eventually, resistance. Two or more drugs in combination help reduce or delay the development of oocyst resistance and improve weight performance in birds (109, 110). Delayed

resistance resulting in selecting resistant strains when compounds are used simultaneously is much less than if those compounds are used alone.

Drug resistance is a genetic phenomenon, and only selection pressure or genetic drift will force the return of sensitivity in a population. Many drugs appear to select resistance in coccidia immediately. Resistance can develop quickly to drugs such as decoquinate, where coccidia are selected for cytochromes that do not bind readily to the drug. In turn, coccidia can become drug-resistant after only a few months (111). It has taken many years for coccidia to become resistant to other medications, like polyether ionophores, which have a more complicated mechanism of action involving the active transport of alkaline metal cations across cell membranes (112).

Despite the efficacy of drug combinations, the primary defense against drug resistance is the use of less intense programs combined with a program rotation (113). This alone will not prevent drug resistance development however, and it has recently become common practice to incorporate live coccidiosis vaccines to replace drug-resistant wildtypes. This approach has demonstrated effects on drug sensitivity where it is utilized (114-117). The poultry industry has increasingly learned to take advantage of developed immunity from live vaccines, where a small handful of studies have shown vaccine strains remain sensitive to anticoccidials (118, 119).

Coccidiosis vaccines

Poultry coccidia induce a strong immunity, and vaccination has been investigated as an alternative to drugs for controlling disease (120-122). In the U.S., controlled exposure of fully virulent *Eimeria* strains are administered in the hatchery at a low dose, and immunity starts building after two weeks. Broilers are commonly administered a multispecies vaccine containing *E. tenella, E. acervulina, and E. maxima*. Upon replication in intestinal cells, these strains stimulate

the immune response, mainly the T-cell response, providing a robust immunity against later exposure. Newly hatched birds are not fully susceptible to coccidial infection because of their high maternal antibodies and the inefficient excystation of sporozoites because of lower total chymotrypsin activity and bile salts in the intestinal tract (123, 124). Without proper administration in the hatchery and management of temperature, humidity, and stocking density on the farm, these fully virulent strains may cause disease in portions of the flock. In addition, some poultry producers use anticoccidial drugs at the peak protozoan replication to reduce vaccine-induced gut lesions for an improved feed conversion rate (125).

In contrast, the EU uses attenuated strains termed precocious, where oocysts with shorter prepatent times are artificially selected for after multiple passages. Precocious strains omit several replication cycles in the asexual phase, leading to less intestinal damage (126). Precocious *Eimeria* exhibit reduced pathogenicity but retain their immunogenicity (123, 127, 128). One exception is an *E. tenella* line selected for growth in embryonic chicks (129, 130).

Several published reviews deal with vaccines to prevent coccidiosis in poultry (17, 128, 131). Chickens are given live sporulated oocysts from natural infection or attenuated vaccines and often develop immunity or may even establish substantial immunity while receiving anticoccidial drugs (132-134). Vaccine strain pathogenicity is attenuated mainly by dose size and means of administration, but attenuated strains are less pathogenetic by having significantly lower lesion scores (132, 135). Additionally, all coccidiosis vaccines contain strains of *Eimeria* species susceptible to most anticoccidial drugs currently on the market (114, 115).

A major hurdle associated with live vaccines is the large-scale dosing of birds in commercial applications. Environmental cycling of vaccine strains is required to establish protective immunity. Variable exposure across large populations of birds may result in

asynchronous immunity in flocks, causing reduced performance and increased susceptibility to disease. Vaccine coverage is essential, as chicks not receiving oocysts on the day of hatch will later be exposed to oocysts in the litter. Until recently, the most common method for vaccination against coccidia in the U.S. was via liquid spray in a hatchery cabinet. To be vaccinated, chicks must ingest a low dose of live oocysts suspended in spray droplets. The spray pattern, the liquid droplet size, and the count of oocysts per droplet can influence coverage (136). When using a liquid spray as a diluent, there is a concern that oocysts sink to the bottom of the vessel containing the vaccine, and agitation of the solution needs to take place frequently to ensure that all birds are given the proper vaccine dose.

Considerable research on poultry vaccines has produced several new live coccidiosis vaccines. A few products have even been developed to immunize turkey poults against turkey coccidia, although these products are only available in some countries (137). On the hatch day, broiler chicks may be mass vaccinated using a spray cabinet after they are sorted and counted into baskets. The chick baskets move down a conveyor belt and pass through a spray cabinet, triggering the vaccine application from one or more bore nozzles or a gel application bar. The vaccine is evenly distributed to the chicks, and exposure may occur through oral ingestion (138). Spray cabinet vaccination is standard for coccidiosis, with the dose volume, vaccine diluent, and vaccine application method varying per the vaccine type (139-142). Once chickens have been placed on the farm, field vaccination can also occur. Chicks are sprayed with vaccines once placed on the floor or ingested via edible gel or feed.

Potential issues with spray application have led to gel vaccine suspensions for coccidia. Gel diluents are novel in the U.S., though they have been used in other countries. There are multiple manufacturers of gel diluents and multiple viscosities of individual gel products. Some

gels are slightly more viscous than water, but they create more stable droplets on the chicks when applied. Other gels are highly viscous and create very defined and gelatinous drops on chicks. The less-dense gel suspensions can be used by traditional spray or a gel applicator bar; highly viscous gels can only be applied through an application bar. The concept behind gel diluents is that oocysts remain evenly suspended within the gel instead of sinking through the liquid. Using more stable "beads" of vaccine will increase the available vaccine for ingestion and improve the amount of time the vaccine is on the chicks. One product was mixed into gels and placed into the chick boxes for the chicks to eat (143).

Another approach to coccidiosis control is using coccidian proteins to confer maternal protection. One product based on this approach is CoxAbic. Antigens, developed from a monoclonal protein produced in the gametocyte of *E. maxima*, are grown in bacterial cells possessing the gene that encodes the protein. CoxAbic is given to hens in 2 doses to confer maternal protection during the first three weeks of brooding (144). Antigens isolated from *Eimeria maxima* gametocytes were also found to be highly conserved for different *Eimeria* species (145, 146). Despite the complexity of generating live coccidiosis vaccines, studies are still being conducted to optimize this strategy by producing a vaccine with a long shelf life that addresses antigenic variation yet remains relatively inexpensive to manufacture (147-149).

Vaccine immune response

In chickens, the primary immune response to *Eimeria* infection is mediated by T-cells (150-152). Although both humoral and cell-mediated responses are activated during infection, immunity development relies on the cell-mediated response (153). Immunoglobulins IgA and IgM are predominantly secreted during coccidia infection but their ability to limit infection is limited

(120, 122). This was demonstrated in birds, where reduced T-cell proliferation increased *Eimeria* susceptibility, despite enhanced IgA and IgG coccidia-specific responses (154). Anti-*Eimeria* IgM, IgY, and IgA antibodies are produced but ineffective in eliminating the parasite (120, 121). Conversely, bursectomized chickens can resist reinfection with *Eimeria* (120).

The sporozoite stage plays a critical role in invasion and is likely the most responsive target for triggering the host immune response. In immune birds, sporozoites undergo very limited development or fail to penetrate cells in the intestinal tract (155-157). During early infection, exposure to sporozoites causes inflammation, lymphocyte infiltration, and increased mucous production in the gut. The Th1 immune response dominates, with the secretion of IFN-γ, IL-2, and TNF-β that activate macrophages, NK cells, and CD8+ cytotoxic T lymphocytes (154). Natural killer (NK) cells and macrophages participate in sporozoite destruction, whereas CD8+ T-cells are suspected of playing a role in sporozoite transport to the epithelial cells since sporozoites are found inside of the CD8+ T-cells immediately following infection and later in the epithelial cells (120, 158). CD4+ T-cells recognize peptides associated with MHC class II signaling during primary infection. CD8+ T-cells are stimulated during reinfection and stop parasite proliferation by identifying infected epithelial cells through MHC class I signaling and destroying them (159). Protective immunity does not prevent initial sporozoite invasion of epithelial cells but contains sporozoite replication (160, 161). Moreover, protective immunity is species-specific and differs in immunogenicity between species, with *E. maxima* being the most immunogenic (151). Other species, like *E. mitis*, are not very immunogenic. Third-generation recombinant coccidia vaccines with adjuvants such as IL-2 and IgY increase the immune response to vaccination (162-164).

2.3 *EIMERIA*: IDENTIFICATION OF SPECIES

Introduction

Traditionally, the identification of chicken *Eimeria* species has been based on the morphological characteristics of oocysts, parasite biology, clinical signs of the affected animals, and macroscopic lesions assessed during necropsy (165). A microscopic identification method means observing oocysts under an objective lens and then deciding based on physical characteristics such as oocyst size and shape. Digital image analysis can be helpful in this type of identification by measuring curvature characterization, size and symmetry, and internal structure quantification (166). Measuring 20-30 oocysts of the predominant population can give a good indication, but morphological characteristics alone are not helpful as diagnostic characteristics (6). Biological characteristics must also be considered to have confidence in the diagnosis. Biological characteristics useful in the identification of species include the location and appearance of lesions in the gut. Each species favorably infects epithelial cells in particular regions of the intestinal tract and can result in lesions of varying severity, including thickening of the intestinal wall, hemorrhages, and necrosis (2). *E. tenella*, for example, is well-known among diagnosticians because of the easily recognizable cecal lesions, often with prominent blood (167).

Unfortunately, morphological characteristics and biological characteristics often overlap, hindering accurate diagnosis. Identifying species through pathogenicity and the severity of lesions is subjective, as severity is also proportional to the number of sporulated oocysts ingested. Additionally, assessing macroscopic lesions during necropsy requires time and the sacrifice of

animals. Mixed infections also pose a problem for the precise discrimination of species using morphological methods. Thus, it has been suggested that these methods should not be used in isolation to differentiate *Eimeria* species (165).

Molecular identification

Molecular approaches such as PCR or qPCR assays capable of identifying and differentiating *Eimeria* species have been available for over 20 years (168, 169). Despite recognition as the gold standard for detecting many pathogens, PCR has yet to replace traditional coccidial diagnostics altogether. The double oocyst cell wall's resistance to mechanical disruption limits template DNA access. However, protocols for DNA isolation and sporozoite excystation without mechanical disruption have been described (80, 170). While several PCR assays have been described to identify specific *Eimeria* species, only some studies have focused on the applicability of these techniques for identifying *Eimeria* species in commercially raised poultry worldwide (9-11, 79). The development of a standardized protocol or new sequencing technologies supporting high throughput sampling for *Eimeria* will enhance the value of such data while promoting PCR application.

Known sequences of DNA originating in *Eimeria* species are used to design oligonucleotide primers that allow selective PCR amplification. Molecular assays have been described that combine all primers for each species in a single tube (8). Technologies, including real-time quantitative PCR, eliminate the need for gel electrophoresis. The DNA isolation or extraction method is crucial in both PCR and qPCR. An oocyst rupturing step by bead-beating or grinding is often used but not needed. Treatment to prevent genome degradation and promote

ethanol DNA precipitation often follows. Commercial DNA extraction kits containing mini-columns that selectively bind DNA are also available.

<u>*Sanger sequencing*</u>

Sanger sequencing is a DNA sequencing method that relies on chain-terminating dideoxynucleotides (ddNTPS) which selectively incorporate during *in vitro* DNA replication. After being first developed by Frederick Sanger and his colleagues in 1977, it became the most widely used sequencing method for over 4 decades (171, 172). Despite its wide use, the Sanger method faces several challenges, including poor quality in the first 15-40 bases of the sequence due to primer binding and deteriorating quality of sequencing traces after 700-900 bases (173). The technique can only sequence short DNA fragments (300-1000 nucleotides), making it challenging to resolve large DNA fragments that differ in length by only one nucleotide. Additionally, complete genome sequencing through Sanger sequencing is expensive, with the human genome costing approximately $500/Mb compared to less than $0.50/Mb for Next generation sequencing (NGS) platforms in 2014 (174). Moreover, Sanger sequencing can only sequence a single DNA fragment at a time, meaning it has low sensitivity for minority populations which may be missed.

<u>*Next generation sequencing*</u>

Since 2007, the cost of next-generation sequencing (NGS) has decreased dramatically (175). NGS is also called second-generation sequencing and is such an expanding field of science that if a textbook were published now, it would be out of date in a year. NGS is a high-throughput approach to DNA sequencing using massively parallel processing, which results in sequencing

millions of fragments simultaneously per run. NGS technologies emerged between 1994 and 1998 and have been commercially available since 2005 (176). One company, Solexa, now part of Illumina, was founded by Shankar Balasubramanian and David Klenerman in 1998. Solexa developed a sequencing method based on reversible dye-terminator technology with engineered polymerases (177). Illumina acquired Solexa in early 2007 (178). The Illumina approach attaches single template DNA molecules to a solid surface before polymerase-based amplification. The inclusion of base-specific fluorescently labeled reversible terminator bases translate into sequence detection. Illumina can produce massive numbers of sequence reads per run, ranging from one million to several billion. Low read length and sometimes individual base accuracy are the downsides for Illumina technology. Pacific Biosciences (PacBio) offers a similar technology that focuses on long-read raw sequence data with error rates of 13-15% (179, 180).

Nanopore NGS

Nanopore sequencing is a unique, accessible technology that enables direct, real-time analysis of varying-length DNA or RNA fragments. Nanopore NGS involves monitoring changes in electrical current as nucleic acids pass through protein nanopores. The concept is that nucleic acids induce different ionic currents when traversing through a pore (181, 182). In 1996, Deamer, Branton, and colleagues reported on DNA translocation through α-hemolysin, a toxic pore-forming protein secreted by *Staphylococcus aureus* (183). One company that pioneered this technology to become accessible worldwide was Oxford Nanopore Technologies Limited (ONT). Based in the United Kingdom, ONT was founded in 2005 as a spin-out from the University of Oxford by Hagan Bayley, Gordon Sanghera, and Spike Willcocks. The company's main products include portable protein nanopore sequencing USB devices such as the MinIon Mk1B.

Publications suggest a DNA sequence read rate of 90 nucleotides per second per nanopore with an error rate of 30% during the early phase of its release around 2014 (184, 185). Later, new protein pores with different apertures lowered the sequencing error rate. With the version R9 pore release in 2016 and the latest R10 pore release, raw error rates have been reduced to < 1% for various types of DNA sequencing (186-189). These pores work with both 1D and 2D chemistry. Sequencing one strand of the duplex DNA indicates 1D chemistry, and sequencing the template; complimentary strands mean 2D chemistry.

2.4 MOLECULAR IDENTIFICATION OF *EIMERIA*

Introduction

For two decades, molecular phylogenies depended on data from a few genes, typically generated using PCR amplification and Sanger sequencing (190). The first years of molecular phylogenetics were dominated by studies using a small set of universal orthologous genes, including the small and large subunit ribosomal RNAs and the mitochondrial genome (191, 192). The widespread use of rRNAs stemmed from the ease of PCR amplification using universal primers, clear orthologs amongst conserved genes, and an extensive database of sequences. Now, the development of NGS has resulted in large datasets of multiple genes, and the ease and low cost of genome or transcriptome sequencing have meant that data for creating phylogenetic trees are increasingly available.

Before 2003, genomic *Eimeria* resources were limited mainly to single Sanger sequencing reads covering the ribosomal DNA clusters and sporozoite or second-generation merozoite expressed sequence tags (ESTs) (193-195). Genome sequencing started for chicken *Eimeria* species around 2003 with the *Eimeria tenella* Houghton strain. Sanger sequencing produced 8.3 fold genome coverage (196). At the time, the genome had almost no functional annotation, but the presence of distinct, ubiquitous trinucleotide sequences had already been identified (197, 198). An abundance of the trinucleotide GCA and its complement TGC repeats are present, ranging from short to medium stretches, typically up to 16 repetitions within all chromosomes, as demonstrated by hybridization experiments and sequence analyses of over 3700 ESTs (168). The biological

significance of these triplet repeats in *Eimeria* has yet to be discovered, but trinucleotide repeats are not exclusive to *Eimeria (199, 200)*.

The genomes of representative *Eimeria* species have yet to be studied systematically. Today, the *E. tenella* Houghton strain genome exists as several long gene assemblies, and its sequences are available from the NIH genetic sequence database under the accession number GCA_905310635.1. It consists of a nuclear genome comprising 14 chromosomes of 1–7 Mb, a mitochondrial genome of 6200 bp, and a 35 kb circular apicoplast genome (201-204). Approximately 8,618 genes are within *E. tenella's* roughly 60 Mbp genome (204). Subsequently, similar genomes for the other 6 universally recognized species of *Eimeria* that infect chickens have also been published with NGS technology generating more than 12-fold coverage of the *E. maxima* Houghton strain genome in 2012 (205).

<u>Molecular detection and identification of *Eimeria*</u>

NGS technologies have supported the development of new approaches to define population structures for various microorganisms, including *Eimeria*. For example, deep sequencing of PCR amplicons has been used widely to determine the presence/absence and occurrence level of bacterial populations (167). For *Eimeria*, three groups have published deep amplicon Illumina NGS studies for whole *Eimeria* populations (206-208). The sequence analysis provides a sensitive assessment of *Eimeria* species occurrence, validated by standard and quantitative species-specific PCR. However, detecting low levels of DNA representing other *Eimeria* species not classically associated with chickens did appear to indicate a background noise level. These results suggest a requirement for additional validation and more specific primers.

One study targeted *Eimeria* 18S rDNA (18S) and mitochondrial cytochrome c oxidase subunit 1 (CO1) genes but followed a nested PCR approach using inner and outer primers to increase specificity and sensitivity (208). Applying the nested 18S and CO1 NGS assays to live coccidiosis vaccines and field samples collected from backyard and commercial chickens showed a high sensitivity for the 18S rDNA, confirmed by quantitative TaqMan PCR. However, as for the publication by Hinsu et al., a series of additional sequences associated with non-chicken hosts were also detected, including ferrets, rodents, and rock partridge. Furthermore, novel sequences lacking a matching annotated reference sequence were also detected. Further validation is required to confirm that these sequences do not simply represent environmental contamination or DNA from other sources. Still, the studies demonstrate the value of NGS amplicon sequencing for *Eimeria* populations but highlight the requirement for optimal primer design and quality control.

Molecular-based assays for detecting and identifying *Eimeria* have been described that target various gene regions. ITS regions 1 and 2, along with ribosomal RNA subunit 18S, have successfully differentiated *Eimeria* species before (70, 73, 75-78, 209). Species-specific PCR primers targeting the mitochondrial CO1 locus were generated to identify the most common *Eimeria* species infecting turkeys (210). Sequence-characterized amplified regions (SCAR) have also been determined through random amplified polymorphic DNA (211, 212). 18S ribosomal RNA (rRNA) is a part of the rRNA molecule in cells that forms the ribosome organelle. The gene region coding for 18S rRNA is referred to as 18S. The length of the 18S gene region varies from species to species, but *Eimeria* gene references in GenBank list approximately 1,750 base pairs. The 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and is an important marker for PCR in environmental biodiversity screening (7). As a part of the ribosomal functional core, the 18S gene is exposed to similar selective forces in all living beings. Their

repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in the smallest organisms like poultry *Eimeria* (213). In general, rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for universal or pan-species primers (140).

Genetic diversity within *Eimeria* species has commonly been studied by looking at the ITS 1 and 2 sequences (214). ITS is a non-coding spacer DNA between 18S and 5.8S rRNA genes. The length of the ITS1 region varies from species to species, but *Eimeria* gene references in GenBank list an approximate size of 500 base pairs. Another ITS region labeled ITS2 originated as an insertion that interrupted the ancestral 23S rRNA gene (215, 216). ITS1 and ITS2 contain short repetitive elements, which match motifs found in the promotor. These act to concentrate RNA Polymerase I molecules in the region so they may be shuttled more quickly to the promoter. Like 18S, sequence comparisons of ITS regions are widely used in taxonomy and molecular phylogeny because of several favorable properties. Still, ITS1 has a high degree of variation between closely related species. This can be explained by the relatively low evolutionary pressure on non-coding spacer sequences.

CO1 is the main subunit of the last enzyme in the respiratory electron transport chain of cells located in the membrane. It is one of the most widely used genetic markers for resolving phylogenetic relationships because its mutation rate is often fast enough to distinguish closely related species, and its sequence is highly conserved among species. Studies have indicated that the CO1 gene is efficacious for resolving the phylogenetic relationships among closely related species (217-220).

Contested species of Eimeria

Accurate identification of *Eimeria* species is essential for diagnosing disease and managing subclinical infection, developing and applying effective control strategies, and conducting biological and epidemiological studies (221, 222). As mentioned, seven well-recognized *Eimeria* species are known only to infect chickens. Other potential species, including *E. hagani* and *E. mivati*, are mentioned in the literature, but their existence as separate *Eimeria* species is questioned (218, 223-225). *E. hagani* is described as a new species only once by P.P. Levine in 1938, who classified it on the grounds of having its own specified immunity. Still, no one has been able to isolate *E. hagani* since (226).

The name mivati refers to a Sanskrit word meaning to move or change and was chosen because location changes occur during endogenous development in the host, and second-generation merozoites were noted to move from the duodenal loop to complete replication in the lower small intestine (227). In 1973, Long suggested that *E. mivati* be regarded as a variant of *E. acervulina* after re-examining the life cycle of both species. He found that both species had four generations of schizogony within the first four days of infection (228). Furthermore, both species were capable of disease of the ceca and the chorioallantoic membrane of chicken embryos. However, only *E. mivati* could carry out a complete replication cycle to produce oocysts (228, 229). This claim was contested by Ryley, who showed that the two species could not interbreed, so they must be distinct (230). It was later discovered that Long's theory about *E. mivati* being a variant of *E. acervulina* arose because the laboratory cultures of *E. mivati* were contaminated with *E. acervulina*. The culture studied was then purified of *E. acervulina* via a passage in embryonated eggs to produce only *E. mivati* oocysts (231). This monoculture was confirmed through challenge

studies that showed a lack of cross-protection between the two species and a notable difference in pathogenicity produced from infection with either species (72, 224, 232).

Although the distinction between *E. mivati* and *E. acervulina* was made, there were still postulations that *E. mivati* was a variant of *E. mitis*. In 1983, Shirley, Jeffers, and Long compared field strains of "*E. mitis*/*E. mivati*" and a laboratory strain of *E. mivati*. After examining parameters such as oocyst dimensions, absence of gross lesions, and cross-immunity, they concluded that *E. mivati* was not a species (223). There was still doubt regarding *E. mivati* status as a distinct species continuing into the 1990s when a supposed monoculture of *E. mivati* was used in drug and vaccine trials (89, 101, 129, 233-235). However, simultaneously other researchers were claiming to be using *E. mivati/E. mitis* and did not consider the two species' distinct (236-239). One proponent of *E. mivati* as an actual separate species of *Eimeria* was Fitz-Coy, who compared *E. mivati* and *E. mitis* schizonts and merozoites based on size, number, and location in the host cell and found differences between both species (240).

Later, in a field survey of commercial broiler farms in Arkansas and North Carolina, oocysts were sequenced with genus-specific primers to analyze the 18S rRNA, ITS 1 and 2, and CO1 regions of the genome. Both *E. mitis* and *E. mivati*-like genomes were detected based on homology searches. These sequences had enough polymorphisms in the 18S rRNA nuclear genomic locus and the CO1 mitochondrial locus to form distinct clades (209). This phylogenetic relationship was confirmed by other researchers (218, 242). Still, doubt about *E. mivati* as a separate species was again cast upon the debate when single-oocyst-derived strains of *E. mitis* and *mivati* were analyzed by sequencing 18S and CO1 genes in 2011. Vrba et al. identified two types of the 18S gene, which differ significantly in their secondary structure, and were found to be present in each strain in roughly equal ratios (225). The authors conclude that if the strains carrying

only one or the other 18S type exist, then they will likely cross-breed and still represent a single species. However, insufficient sampling may cause the detection of only one or the other 18S type by sequencing, and the biological significance of possessing two 18S gene variants in *E. mitis* has yet to be discovered. Vrba et al. hypothesize that 18S variants might be used in different stages of the parasite's life-cycle (225).

Currently, ten species of *Eimeria* have been labeled from chickens, with three recently recognized through clustered Operational Taxonomic Units (OTUs) derived from DNA sequences (243). Phylogenetic analysis using mitochondrial sequence data suggests that these 3 OTU variants are related to *E. maxima, E. brunetti,* and *E. mitis (203)*. The literature indicates that the OTUs possess sufficient genetic and biological diversity to be considered a new and distinct species (243). The names *E. lata*, *E. nagambie*, and *E. zaria* were suggested based on their appearance and origins in the first isolates. The cryptic *Eimeria* OTUs x, y, and z were detected in North America for the first time in 2019 (208).

The future of Eimeria identification

Eimeria pose a risk to commercial chickens as a cause of coccidiosis, reducing productivity and compromising animal welfare. NGS technologies may provide potential insight into *Eimeria* phylogenies, genome functions, target virulent factors, and future transgenic populations or diagnostic assays for controlling coccidiosis. Such insights will provide a rich resource for analyzing potential therapeutic and vaccine intervention targets. The development of transfection methodologies will promote studies on the function of *Eimeria* genes. Recently, a start has been made by transient transfection technologies with sporozoites of *E. tenella* published in 2021 *(244)*.

Vaccine-origin parasites persisting in the environment post-vaccination is one major drawback of exercising vaccines to control coccidiosis outbreaks. Birds typically become protected from future infection. However, the subjective nature of identifying parasites by morphology or species-specific legions makes it challenging to know if sample field parasites are a true coccidiosis challenge or simply remnants from a previous vaccine. After assessing the ability of nanopore NGS to accurately and repeatably sequence and identify chicken *Eimeria*, we propose its potential use to differentiate *Eimeria* parasites from field and vaccine origin. The nanopore NGS process simplifies the purification and PCR step; no separation of species is required. It will output vaccine sequence information for all sequences present in the sample. Sequence information from the 18S, ITS1, and CO1 gene regions could be used to develop extensive vaccine and field origin *Eimeria* profiles. Data from this work have the potential to differentiate *Eimeria* strains based on sequence homology, which will be a useful diagnostic tool in the future.

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CHAPTER 3

ASSESSING THE ABILITY OF NANOPORE NGS TO SEQUENCE AND IDENTIFY CHICKEN *EIMERIA* IN MULTISPECIES COCCIDIOSIS VACCINES

Ben Jackwood[1] Brian Jordan[2]

[1]Department of Poultry Science, [2]Department of Population Health, University of Georgia, Athens, Ga.

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ABSTRACT

Nanopore next-generation sequencing (NGS) technology offers a novel method for molecular sequencing and identifying *Eimeria* parasites in poultry. *Eimeria* are parasitic protozoans that cause coccidiosis, a gastrointestinal disease, in various animals. Coccidiosis outbreaks caused by several species of *Eimeria* in commercial chickens can result in massive financial losses. Traditionally, species detection has relied on superficial morphological or biological characteristics. However, molecular methods using polymerase chain reactions (PCR) have been developed to detect and differentiate species. In this study, nanopore NGS was evaluated for its ability to sequence amplicons of identifying gene regions and produce high-quality DNA profiles to aid in identifying *Eimeria* strains in commercial coccidiosis vaccines. Three identifying gene regions - Internal Transcribed Spacer-1 (ITS1), Ribosomal 18S DNA (18S), and Cytochrome Oxidase 1 (CO1) - were used to differentiate coccidia species. Pan-species primers were used to generate amplicons from oocysts in four commercial US coccidiosis vaccines, which were sequenced in triplicate using nanopore NGS. The resulting data were used to construct detailed gene assemblies and phylogenetic trees of chicken *Eimeria*. Nanopore NGS identified all species indicated on the vaccine labels, and no other stains were detected. Contigs of the species present displayed high percent identities across replicates. Additionally, vaccine sequences for each identifying gene region used for identification were clustered together in the same clade as references of the same species. High sequence identity among replicates and homology between vaccine sequences and references indicate the usefulness of nanopore sequencing for detecting and differentiating mixed *Eimeria* populations.

Key words: *Eimeria*; coccidiosis vaccination; Coccivac B52 (B52); Advent; Immucox 3; Inovocox; nanopore sequencing; next generation sequencing; Minion mk1b; bioinformatics; sequence assemblies.

INTRODUCTION

Coccidiosis is a worldwide parasitic disease caused by infection with *Eimeria*, which are single-celled parasitic protozoa labeled Apicomplexa. *Eimeria* infect the gut of multiple animal species, with seven well-recognized species known only to infect chickens (1-11). These species include *E. acervulina, E. brunetti, E. maxima, E. mitis (1), E. necatrix, E. praecox, and E. tenella.* Each species causes disease, but clinical symptoms range from mild to severe. All species appear ubiquitous and may occur in combinations of up to six species on any farm (2-4). Ingesting food or water containing sporulated oocysts from the environment leads to the disease coccidiosis, which is often characterized by morbidity, lost weight gain, and sometimes mortality (5). Coccidiosis is the most economically significant disease affecting poultry producers worldwide. It costs the poultry industry an estimated $14 billion annually due to losses in feed conversion ratios and reduced weight gain (6). Enteric tissue damage caused by coccidiosis is also a known predisposing factor for salmonellosis and necrotic enteritis (NE) caused by *Clostridium perfringens* infection (7, 8).

Each species favorably infects the intestinal tract along varying regions and can result in lesions of varying severity, including thickening of the intestinal wall, hemorrhages, and necrosis (9). Examining lesions requires time, resources, and sacrifice of the animals for necropsy. The disease is regularly diagnosed in birds brought to diagnostic laboratories, but most cases are diagnosed in the field (5). Few oocysts are found after birds are removed from a farm because the parasites are killed by ammonia or composting heat in poultry litter (5). Still, poultry *Eimeria's* ubiquitous and resistant nature precludes their elimination or total prevention. No cross-immunity exists between species of poultry *Eimeria,* and later outbreaks may result from different species or even simultaneous infection with two or more species (10-14). Identifying species is crucial for

disease management as infection severity depends on the type and quantity of oocysts present. Knowledge of the species present in a region allows veterinarians and pharmaceutical companies to create tailored protection strategies to prevent losses from disease.

Methods for oocyst identification include microscopic examination of morphology, biological life cycle characteristics, and molecular sequencing. In recent years, the increased use of molecular methods for *Eimeria* detection has allowed for additional characterization of *Eimeria* species. Nanopore NGS is a molecular method that facilitates deep amplicon sequencing by supporting significant parallel sequencing reactions and analyzing all DNA molecules in a sample. Nanopore sequencing is a distinctive, fast-growing, and easily accessible technology that enables rapid analysis of DNA or RNA fragments of different lengths. This technique works by monitoring alterations in ion flow as nucleic acids pass through protein nanopores. Since different nucleic acids obstruct ion flow in different ways, the alteration in electric current is quantified to provide sequence data.

In the current study, detailed genome assemblies of *Eimeria* parasites were produced from nanopore NGS data and combined with conventional identification methods to solidify correct species identification using commercial coccidia vaccines. The repeatability of using nanopore NGS to identify *Eimeria* species in mixed populations was investigated and the resulting sequences were assembled to construct comprehensive phylogenetic trees establishing relatedness and identities for each species present.

MATERIALS AND METHODS

Multispecies coccidiosis vaccines

This study used commercially available multispecies vaccines containing *Eimeria acervulina, Eimeria maxima, Eimeria maxima MFP, Eimeria mivati, and Eimeria tenella.* Vaccines for Advent, Coccivac B52, Immucox 3, and Inovocox were obtained from their commercial retailers[a,b,c,d] and stored at 4°C. A fifth vaccine, Hatchpak III, was initially used but failed to produce any PCR products using the pan-species primers in Table 1.

DNA extraction and PCR amplification

Genomic DNA (gDNA) was purified from oocysts using a series of reagents followed by ethanol precipitation. For this study, oocysts found in 4 US brand coccidiosis vaccines were sequenced by nanopore NGS in triplicate. A new vaccine vial was used for each run, and 12 total vaccine vials were used. From each vial, approximately 20,000 oocysts were pelleted by centrifuge and resuspended in DNAzol[e]. Oocysts were homogenized in 1.4 mm ceramic bead tubes at 6m/s for 40 seconds. The resulting cell lysate was incubated in 50 µl Proteinase K[g] with a 160 µg/ml concentration at 65°C for 15 minutes. The mixture was cooled on ice for 1 minute, followed by additional centrifugation. Finally, gDNA was isolated from the resulting supernatant by ethanol precipitation.

Previously published pan-species primers (Table 3-1) for each identifying gene region were used on the 12 gDNA samples to obtain sequence starting material. Reactions were prepared in triplicate for each gene region to obtain enough PCR product (4 vaccines x 3 replicates per vaccine x 3 replicates per gene region x 3 gene regions = 108 PCR reactions) for sequencing using Platinum PCR SuperMix High Fidelity[f]. All 40 µl reactions included ~100 ngs of gDNA, 25 pmol forward

and reverse pan-specific primers, and an adequate amount of Platinum PCR SuperMix High Fidelity according to the manufacturer[f]. Thermal cycling was done with an initial denaturing step at 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, an annealing temperature specified in Table 1 for 30 seconds, and 72°C for 90 seconds. A final extension step at 72°C for 10 minutes was also performed. The amplification of the PCR product was checked by gel electrophoresis in 2% agarose gels stained with 0.5 g/ml ethidium bromide. The 5 µl PCR product used for gel electrophoresis was not used for nanopore NGS sequencing. If successful bands were produced, the three resulting PCR replicates were pooled together for each gene. Amplicons were cleaned and prepared for sequencing using the GeneJET PCR Purification Kit according to the manufacturer's instructions[h] and eluted into 20 µl of buffer.

Nanopore NGS sequencing

Four vaccines and three identifying gene regions produced 12 sequencing samples in the study. All 12 samples were sequenced using the Oxford Nanopore Technologies (ONT) MinIon Mk1B sequencer[i]. This experiment was repeated three times for a total of 36 sequencing runs. A simple workflow for 1 nanopore NGS run is shown in Figure 3-1. Samples were prepared with a Rapid Sequencing Kit following the manufacturer's instructions[j] and loaded onto an R9 version Spot-ON Flow Cell.[k] Sequence run times varied but were approximately 6 hours long. The software program MinKnow was used to operate the MinIon Mk1B. R9 Flow cells were washed after each use following a protocol associated with the ONT Wash Kit.[l] Nanopore sequencing runs generated output fast5 and fastq files containing fasta sequences of passed reads. Passed reads were generated from a minimum Q-score of 7 (~80% base call accuracy) with the base calling parameter set to fast. The raw sequence data were then imported into the bioinformatic software

program Geneious to create gene assemblies and phylogenetic trees. Sequence reads failing to meet the minimum Q-score were not used for gene assembly.

Gene assemblies and phylogenetic trees

For this study, complete length sequences of 18S, ITS1, and CO1 were collected from the non-redundant (nr) NIH sequence database for the confirmed *Eimeria* species known to infect chickens: *Eimeria maxima, Eimeria brunetti, Eimeria necatrix, Eimeria acervulina, Eimeria tenella, Eimeria praecox, and Eimeria mitis*. Sequences labeled *Eimeria mivati* were also included based on the Coccivac B52 vaccine product label. Fastq files containing raw sequence reads generated from nanopore sequencing runs were imported into the *Geneious R9.1.8* bioinformatics program. Sequence reads were then mapped to other known references from the nr database using the Bowtie 2 sequence alignment tool. Parameters were set to high sensitivity with end-to-end alignment. After assemblies were generated, consensus sequences from each assembly were trimmed of primer nucleotides and compared across their respective replicates. Geneious Alignment aligned triplicate consensus sequences, which generated percent identity data. Phylogenetic trees were created with Geneious Tree Builder by global alignment and a cost matrix identity of (1.0/0.0). Jukes-Cantor was the genetic distance model used, and the tree-building method was neighbor-joining. All trees generated use a closely related parasite, *Toxoplasmosis gondii* outgroup, to root the tree and observe evolutionary relationships

RESULTS

Coccidiosis vaccine nanopore NGS reads

Nanopore NGS identified all species on all vaccine labels, whereas species not labeled were not detected. Nanopore NGS, across all runs, produced a total of 10,689,258 sequences. The mean number of sequences assembled across triplicates is shown in Figure 3-2. A total of 2,873,542 reads were assembled across all runs by Bowtie 2. On average, 26.88% of total reads were assembled. Coverage was complete for all gene regions, and the sequence read number was adequate for gene assembly. The mean read number of sequences assembled varied across vaccines and genes, though the proportion of reads attributed to each species across replicates is similar (Figures 3-2;3;4;5). Not all passed sequencing reads were able to be assembled. A large majority of unassembled reads shared homology with prokaryotic genomes.

Repeatability of nanopore NGS for sequencing mixed Eimeria populations

In an attempt to assess the potential of nanopore NGS to accurately and reliably produce high-quality contigs of chicken *Eimeria* species, consensus sequences from our gene assemblies were compared across three runs. Triplicate sequence percent identities and their standard deviations are shown in Table 3-2. Percent sequence identity between nanopore runs is high, with no gene region falling below 97.61% identical across replicate runs. In some cases, basecalls were not specifically made and an ambiguous nucleotide was inserted. A percent identity for an identifying gene region <100% with 0.00 standard deviation indicates shared ambiguous nucleotides between runs, such as S or Y.

Phylogenetic trees of Eimeria

Phylogenetic trees were produced to categorize our vaccine sequences, featuring triplicate consensus vaccine sequences alongside reference sequences for other known *Eimeria* species and *Apicomplexans*. Phylogenetic trees generated using nanopore NGS data are shown in Figures 3-6;7;8. All sequences are complete, and branch labels display the number of substitutions per site to 3 significant digits. Phylogenetic trees assembled with other *Eimeria* sequences collected from the nr database showed that all *Eimeria* sequences branched separately from the *Toxoplasma gondii* outgroup. Additionally, all vaccine sequences are grouped with reference sequences of the same species.

Figure 3-6 shows vaccine 18S gene regions compared with 100 known *Eimeria* 18S reference sequences. Within the 18S *Eimeria* clade are two main branches, 1 with a node containing *E. tenella* and *E. necatrix* sequences and 1 with a node containing all other sequences. Sequences from each species are grouped, including a node containing two branches with sequences labeled *E. mivati* and *E. mitis*. There was a single node that contained *E. tenella* and *E. necatrix*. Although the sequences for each species (except *E. tenella* and *E. necatrix*) formed distinct clades, sequence distances for 18S were low, with less than 0.01 nucleotide substitutions per site between species, with the farthest distance between the *E. maxima* clade and the *E. necatrix*/*E. tenella* clade at 0.002 nucleotide substitutions per site.

Figure 3-7 shows vaccine ITS1 gene regions compared with 45 other *Eimeria* ITS1 reference sequences. Within the ITS1 *Eimeria* clade are two main branches, 1 with a node containing *E. maxima* sequences and 1 with a node containing all other sequences. Sequences from each species are grouped, including a node containing two branches with sequences labeled *E. mivati* and *E. mitis*. Although the sequences for each species formed distinct clades, sequence

distances for ITS1 were higher than 18S and CO1, with 0.661 or fewer nucleotide substitutions per site between species. Additionally, *E. maxima* formed two distinct clades distinguishing between ITS1 and a long-form ITS1 (ITS1 L) gene.

Figure 3-8 shows vaccine CO1 gene regions compared with 46 other *Eimeria* CO1 reference sequences. Sequences from each species were grouped, including a node that contained sequences labeled *E. mivati* and *E. mitis*. Although the sequences for each species formed distinct clades, sequence distances for CO1 were lower than ITS1, with 0.066 or fewer nucleotide substitutions per site between species. Additionally, most CO1 sequences within any given species are identical.

DISCUSSION

In the current study, high-quality sequence data of three identifying gene regions in *Eimeria* were produced using nanopore NGS. Twelve vaccine vials were used (4 vaccines x 3 replicates per vaccine) to isolate gDNA for sequencing runs, and no off-label *Eimeria* species were found. Need more discussion of the main results here. The results demonstrated the repeatability of Nanopore NGS, as the sequences between runs showed high similarity. Moreover, 18S, ITS1, and CO1 genes successfully detected all labeled vaccine species in each run. Consistency in clades was observed across all three genes in the phylogenetic trees. The close clustering of vaccine sequences and the observation of *E. tenella* sharing a node with *E. necatrix* provides insight into their relatedness. McMaster chamber method oocyst counts, done in triplicate, revealed *E. acervulina* as the most numerous species, making up 42-83% of total oocysts depending on the vaccine brand. Additionally, the proportion of *E. tenella* and *E. mivati* populations from counting oocysts were similar to the percentage of sequences identified by nanopore NGS in this study (Figure 3-2). *E. maxima* population counts were comparable for 18S and ITS1 only.

One unexpected finding from the analysis of vaccine sequences was a lack of *E. maxima* CO1 amplicons present in PCR products. Variations in gDNA quality from isolation and flow cell health during runs likely played a general role in mean read number variation. However, we performed CO1 PCR amplification and subsequent nanopore NGS on a laboratory monoculture of *E. maxima* oocysts and confirmed these results (data not shown). In both monoculture and vaccines, nucleotide sequences for *E. maxima* were relatively low. An average of 83 CO1 sequences per vaccine PCR product and a total of 807 CO1 sequences for the monoculture's PCR product were found for *E. maxima*. Additionally, classical oocyst counts using the McMaster chamber method on all vaccines used to generate sequence data showed comparable population

percentages, indicating that it was not an artifact of low *E. maxima* populations in the vaccines. These data are troubling as the CO1 primers have been demonstrated to work for *E. maxima* in the past (15, 16).

One goal of this project was to determine if there was enough sequence variability between the different genes for each species in each vaccine to be able to use sequencing as a way to distinguish between vaccines. The data obtained indicate that vaccine sequences for 18S, ITS1, and CO1 genes are very similar, making it difficult to identify a particular vaccine or strain based on its genetic sequence. However, a shortened version of the ITS gene was identified in Coccivac B52, which is known to contain two strains of *E. maxima*. Additionally, sequences labeled *E. mivati* and *E. mitis* were found to be closely related, but located on different branches of the same node. These results suggest that the *Eimeria* strains present in the coccidiosis vaccines we sequenced are closely related and cannot be distinguished as belonging to a specific vaccine, except for additional species identified in Coccivac B52.

This study demonstrated that the nanopore NGS method has the potential to accurately and reliably distinguish between *Eimeria* species in mixed populations. DNA extraction and PCR amplification steps require less than 12 hours to complete from start to finish, the reagents are relatively inexpensive, and flow cells are reusable. Furthermore, the long-read capability of nanopore NGS gives the potential for *Eimeria* whole genome sequencing and improved assembly of difficult repeat-rich regions. This is all in addition to the most significant advantage of nanopore NGS for species detection: identifying more than one species present by sequencing a sample's entire genetic contents.

Acknowledgments

The authors thank vaccine companies for providing clinical samples. The authors thank Dr. Ramesh Selvaraj, Dr. Dong-Hun Lee, Dr. Eric Shepherd, and Dr. Rami Dalloul for their helpful discussion and direction.

Sources and manufacturers

a. Advent Coccidiosis vaccine, Huvepharma Inc., Lincoln, NE.
b. Coccivac B52 Coccidiosis vaccine, Intervet Inc., Omaha, NE.
c. Immucox 3 Coccidiosis vaccine, Ceva Animal Health Inc., Guelph, Ontario, Canada.
d. Inovocox Coccidiosis vaccine, Zoetis Inc., Kalamazoo, MI.
e. DNAzol, Thermo Fisher Scientific, Warrington, UK.
f. Platinum PCR SuperMix High Fidelity, Thermo Fisher Scientific, Carlsbad, CA.
g. Proteinase K, Roche diagnostics GmbH, Mannheim, Germany.
h. GeneJET PCR Purification Kit, Thermo Scientific Inc., Vilnius, Lithuania.
i. MinIon Mk1B sequencer, Oxford Nanopore Technologies, Oxford, England.
j. Rapid Sequencing Kit, Oxford Nanopore Technologies, Oxford, England.
k. Spot-ON R9 Flow Cell, Oxford Nanopore Technologies, Oxford, England.
l. Flow Cell Wash Kit, Oxford Nanopore Technologies, Oxford, England.
m. Kindly provided by
n. Potassium Dichromate, Janssen Pharmaceutical 3a, Geel, Belgium.

Declaration of conflicting interests

The author(s) have no potential conflicts of interest concerning this article's research, authorship, and publication.

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Table 3-1. A list of pan-species primers used in this study.

Primer	Target	Nucleotide sequence (5'-3')	Approximate amplicon size (bp)	Annealing temperature (°C)	Publication
ER1B1	18S rRNA	ACCTGGTTGATCCTGCCAG	1790	57	*Eimeria* genus (Schwarz et al., 2009)
ER1B10		CCTCCGCAGGTTCACCTACGG			
KM204	CO1	GTTTGGTTCAGGTGGTGGTTG	810	55	*Eimeria* genus (Schwarz et al., 2009)
KM205		ATCCAATAACCGCACCAAGAG			
EF1	ITS1	AAGTTGCGTAAATAGAGCCCTC	500	52	Nested ITS-1 (Lew et al., 2003)
ER1		AGACATCCATTGCTGAAG			

Table 3-2. Percent identity and standard deviation among contigs assembled by Bowtie 2. Sequence percent identity among replicates is high. These data highlight the repeatability of using nanopore NGS for amplicon sequencing of 18S, ITS1, and CO1 DNA regions.

TARGET	ADVENT	COCCIVAC B52	IMMUCOX 3	INOVOCOX
ACERVULINA 18S	99.48 ± 0.05	99.76 ± 0.08	99.78 ± 0.01	99.91 ± 0.03
MAXIMA 18S	98.79 ± 0.04	98.96 ± 0.35	99.15 ± 0.04	99.18 ± 0.01
TENELLA 18S	98.63 ± 0.41	98.96 ± 0.48	97.82 ± 0.08	98.85 ± 0.35
MIVATI 18S	N/A	99.32 ± 0.27	N/A	N/A
ACERVULINA CO1	99.00 ± 0.03	99.96 ± 0.02	100.00 ± 0.00	100.00 ± 0.00
MAXIMA CO1	98.90 ± 0.25	98.14 ± 0.37	99.74 ± 0.02	99.33 ± 0.17
TENELLA CO1	98.20 ± 0.05	99.81 ± 0.07	99.00 ± 0.00	99.93 ± 0.03
MIVATI CO1	N/A	99.32 ± 0.07	N/A	N/A
ACERVULINA ITS1	99.41 ± 0.03	99.43 ± 0.06	99.49 ± 0.00	99.49 ± 0.00
MAXIMA ITS1	98.96 ± 0.00	99.28 ± 0.06	99.00 ± 0.03	97.61 ± 0.00
TENELLA ITS1	99.44 ± 0.03	99.49 ± 0.00	99.44 ± 0.03	99.52 ± 0.03
MIVATI ITS1	N/A	99.06 ± 0.14	N/A	N/A

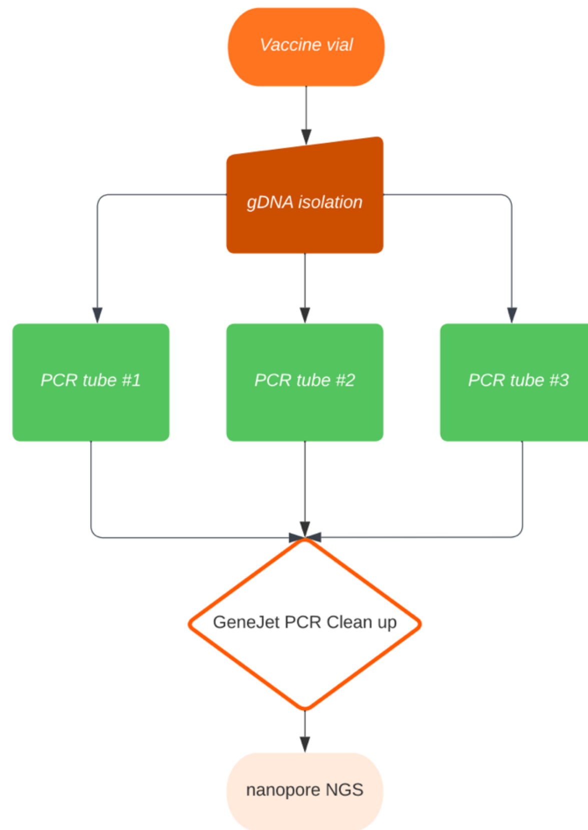
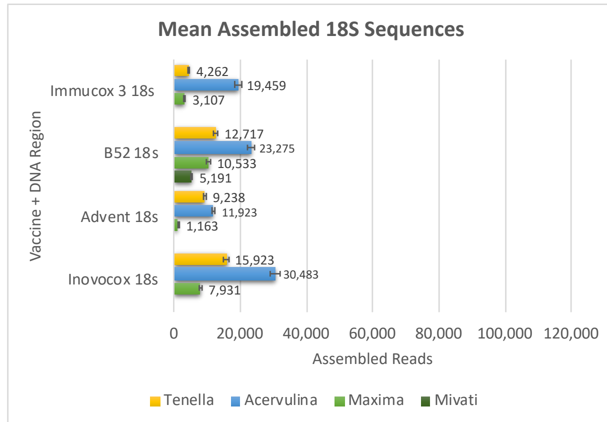
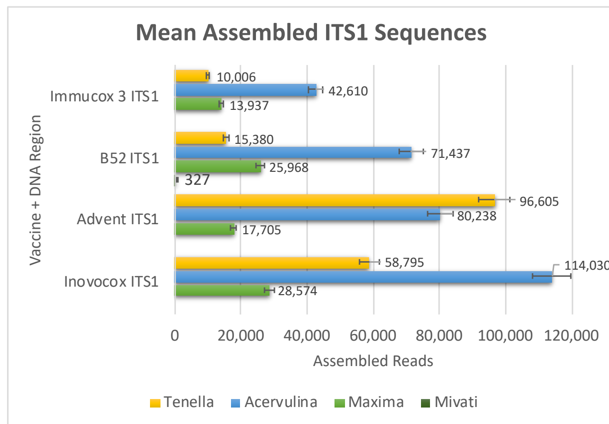


Figure 3-1. A simple workflow for 1 nanopore NGS run. This sequencing workflow was performed in triplicate for 18S, ITS1, and CO1 DNA regions. A new vaccine vial was used for each run.





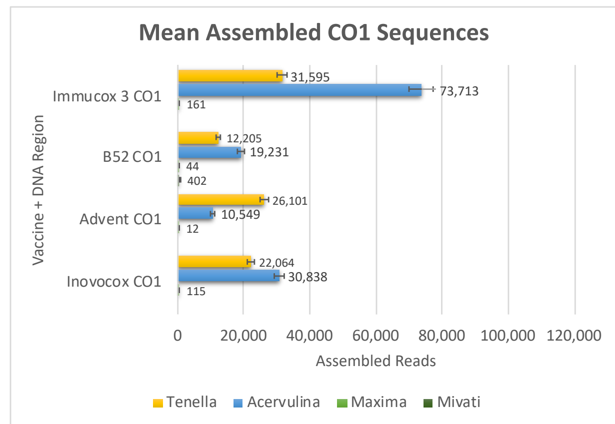


Figure 3-2. The mean number of *Eimeria* sequences assembled by Bowtie 2 from nanopore NGS data. Error bars show standard error across three replicates. Read number varied for each DNA region, and data suggest CO1 in *E. maxima* was not amplified by PCR. Variation in read number may be a result of changes in DNA quality used in the PCR step, number of off-target sequences in the library, and number of nanopores available during a run.

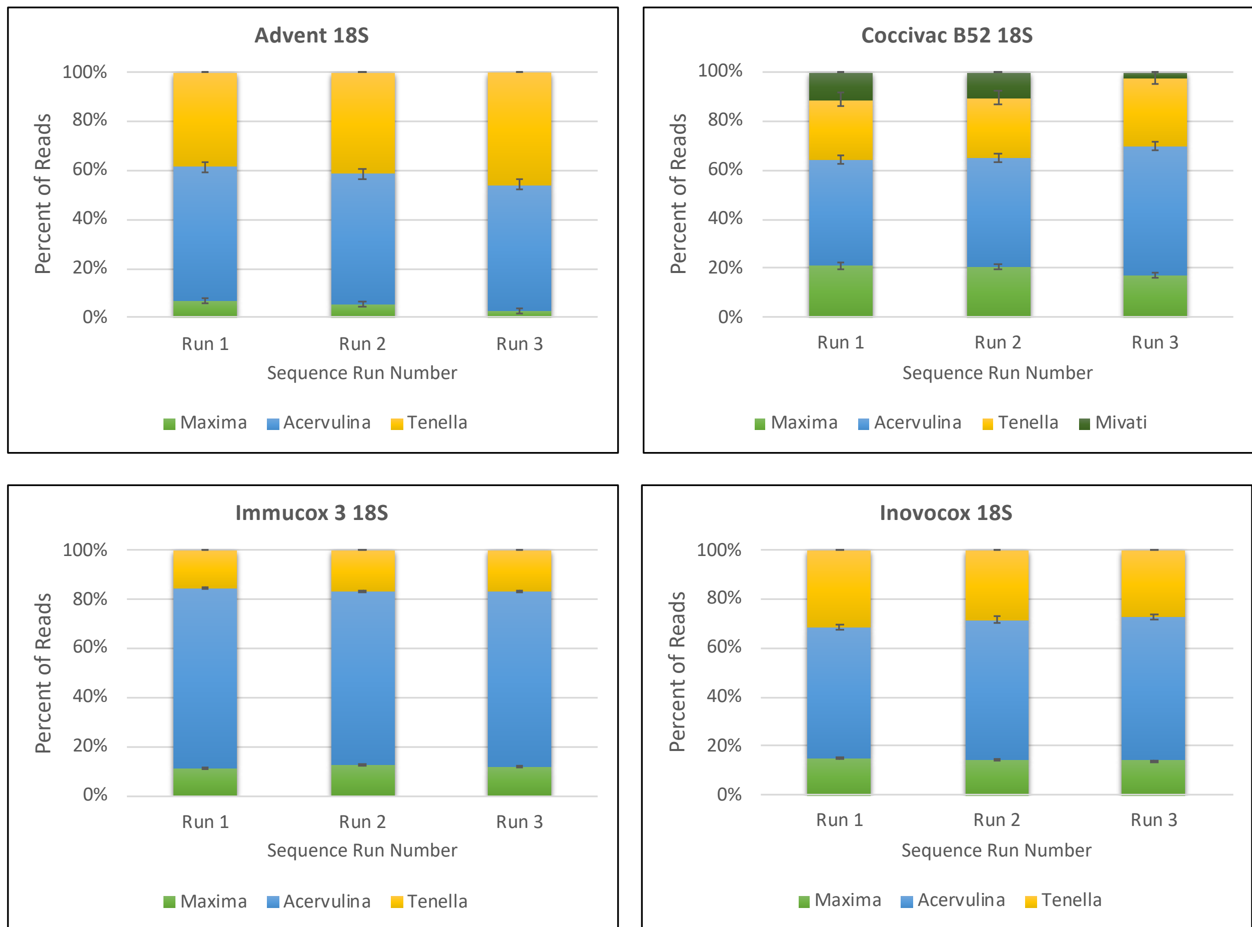


Figure 3-3. The mean number of 18S sequences assembled is shown as a percent out of 100 across replicate runs for the 4 coccidiosis vaccines sequenced by nanopore NGS.

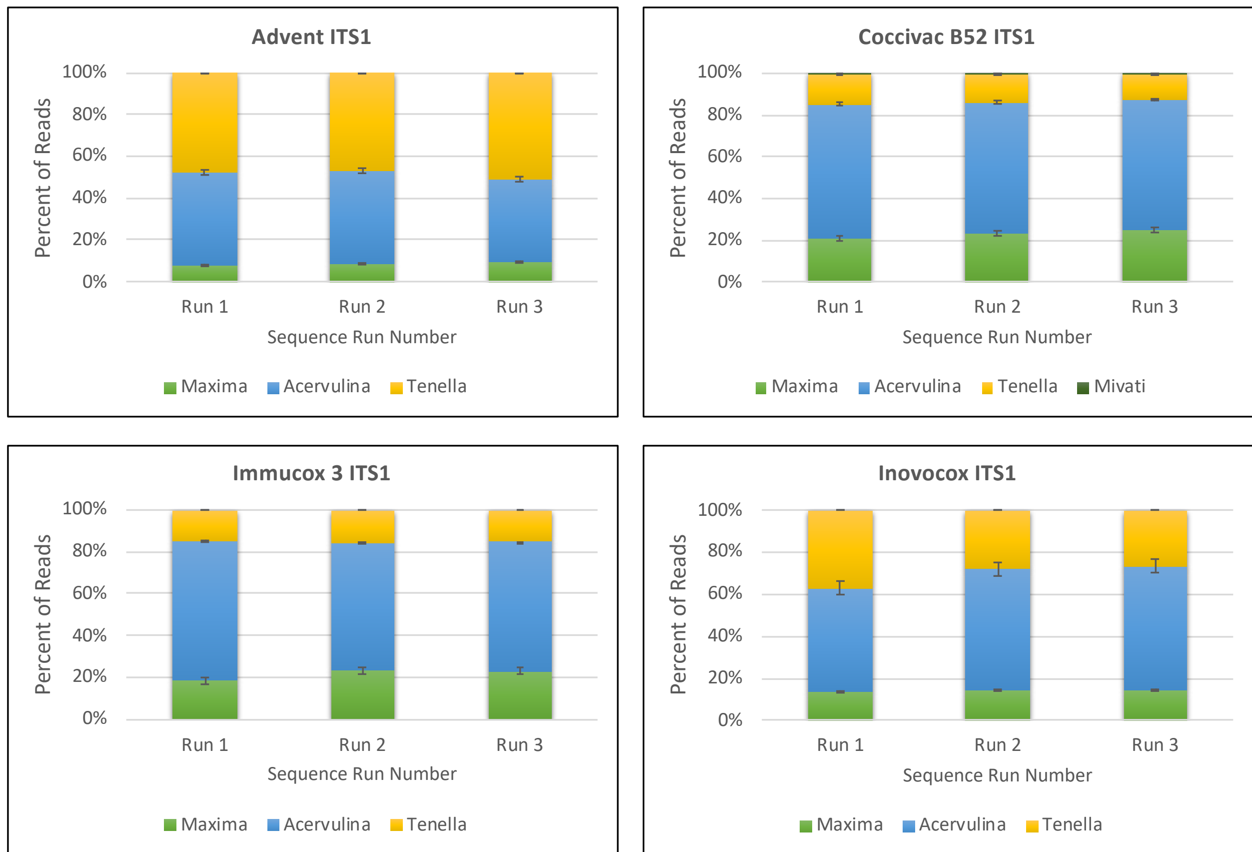


Figure 3-4. The mean number of ITS1 sequences assembled shown as a percent out of 100 across replicate runs for the 4 coccidiosis vaccines sequenced by nanopore NGS.

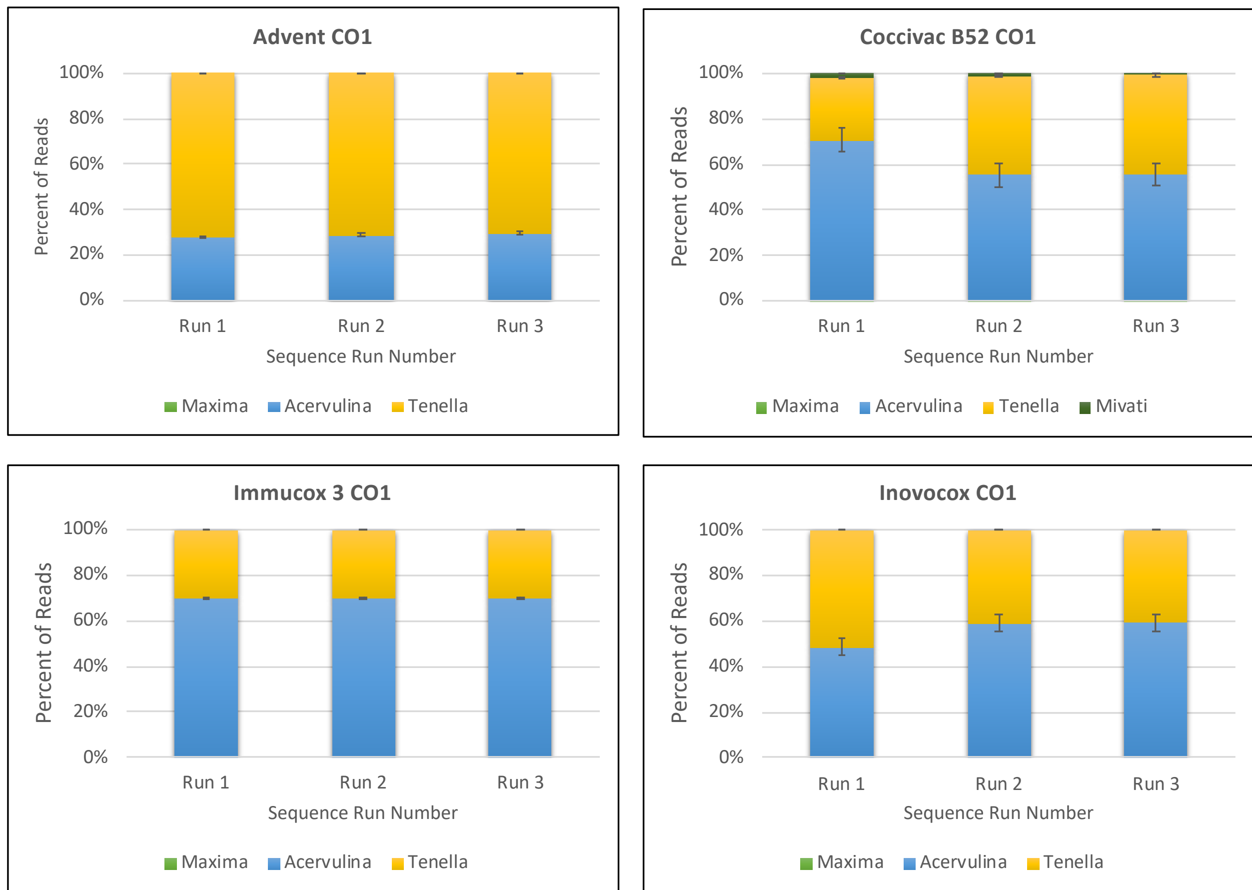


Figure 3-5. The mean number of CO1 sequences assembled shown as a percent out of 100 across replicate runs for the 4 coccidiosis vaccines sequenced by nanopore NGS. Maxima CO1 reads (green) makeup < 1% of total reads assembled across any given sequencing run.

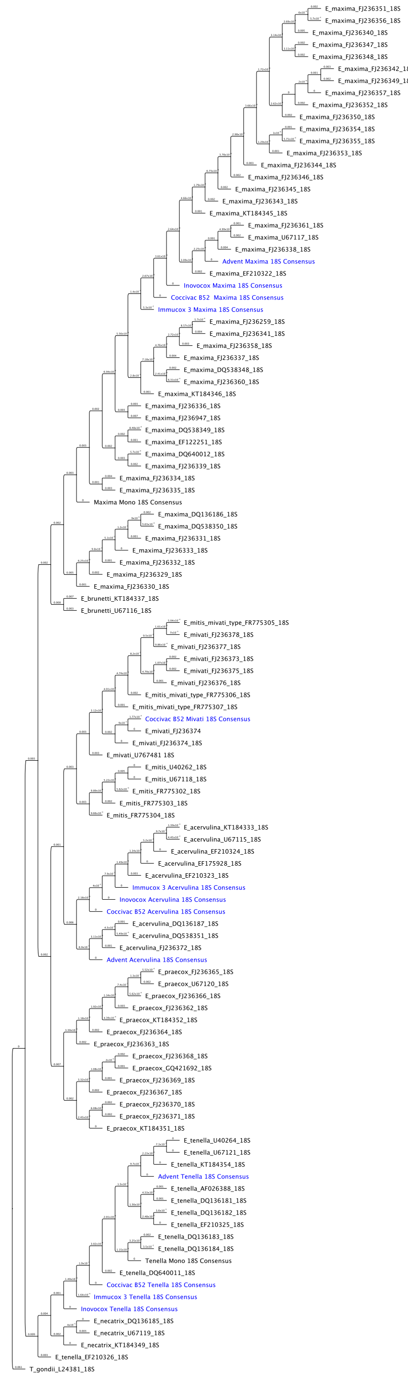


Figure 3-6. Phylogenetic tree of 18S sequences. Included are coccidiosis vaccine sequence contigs (blue) and reference sequences for the 18S gene of *E. maxima, E. acervulina, E. tenella, E. mitis, E. mivati, E. praecox, E. brunetti, E. necatrix, and T. gondii* obtained from GenBank. Branch labels display substitutions per base to 3 significant digits.

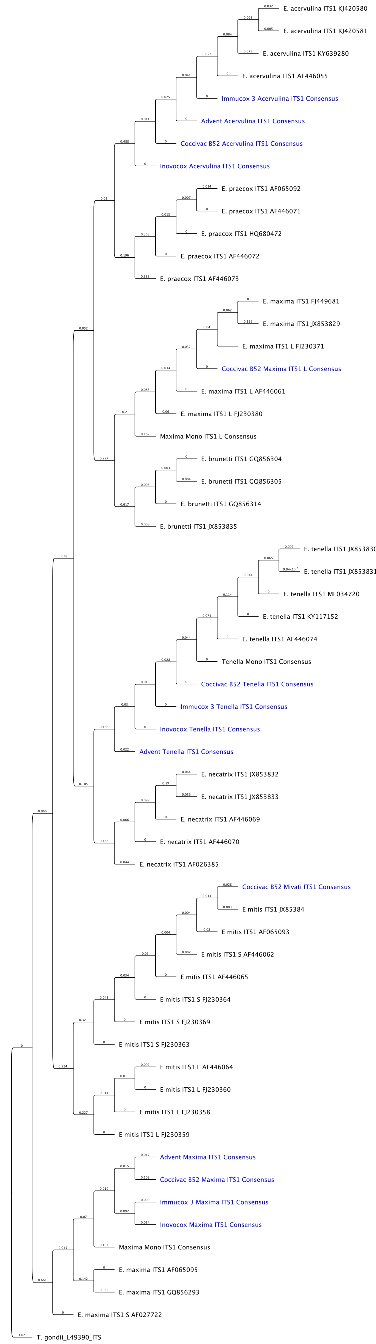


Figure 3-7. Phylogenetic tree of ITS1 sequences. Included are coccidiosis vaccine sequence contigs (blue) and reference sequences for the ITS1 DNA region of *E. maxima, E. acervulina, E. tenella, E. mitis, E. mivati, E. praecox, E. brunetti, E. necatrix, and T. gondii* obtained from GenBank. Branch labels display substitutions per base to 3 significant digits.

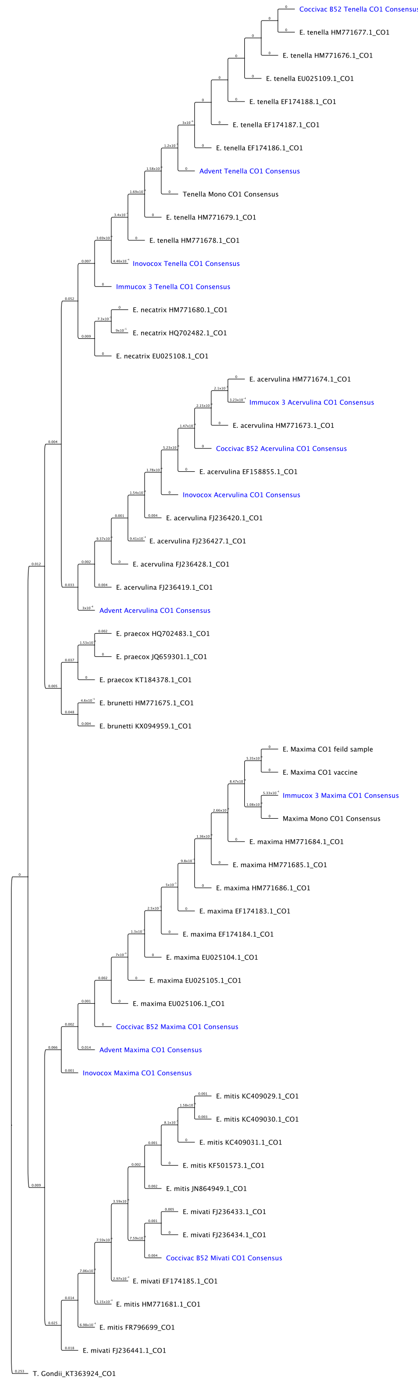


Figure 3-8. Phylogenetic tree of CO1 sequences. Included are coccidiosis vaccine sequence contigs (blue) and reference sequences for the CO1 gene of *E. maxima, E. acervulina, E. tenella, E. mitis, E. mivati, E. praecox, E. brunetti, E. necatrix, and T. gondii* obtained from GenBank. Branch labels display substitutions per base to 3 significant digits.

CHAPTER 4

USING NANOPORE NEXT GENERATION SEQUENCING TO SEQUENCE AND IDENTIFY AMPLICONS OF CHICKEN BROILER HOUSE *EIMERIA*

Benjamin W. Jackwood[*], Brian Jordan[#]

[*]Department of Poultry Science, University of Georgia, Athens, GA. 30605

[#]Department of Population Health, University of Georgia, Athens, GA. 30605

(E-mail: Benjamin.jackwood@uga.edu)

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ABSTRACT

Eimeria are parasitic protozoans that cause the enteric disease coccidiosis in various animals. Several species of *Eimeria* infecting commercial chickens cause disastrous outbreaks of coccidiosis and result in massive financial loss. Molecular methods exist to detect and differentiate species using polymerase chain reactions (PCR) with or without sequencing. From our previous work, we demonstrated that nanopore NGS deep amplicon reads produce repeatable and accurate sequences of *Eimeria* in live multispecies coccidiosis vaccines, which are pure and relatively clean starting samples. The current study tests the suitability of nanopore NGS to sequence amplicons of identifying gene regions of *Eimeria* in field isolates, which are not pure cultures of species and are relatively dirty samples, and investigates *Eimeria* species present in broiler chicken houses. Field samples were collected from 14 different broiler houses under a variety of coccidiosis control programs and genes of *Eimeria* species were sequenced. Amplicons of Internal Transcribed Spacer-1 (ITS1), Ribosomal 18S DNA (18S), and Cytochrome Oxidase 1 (CO1) genes were generated using pan-species primers and sequenced by nanopore NGS. Bioinformatic tool Bowtie2 was used to align sequences to known references to produce contigs of 8 *Eimeria* species, with 1 being non-chicken-specific. Phylogenetic comparison of contigs shows good applicability of nanopore NGS technology for accurate sequencing and discerning of mixed species of field oocysts. However, no apparent distinction between control program sequences was found using phylogenetic comparison, so further gene examination is needed to determine if such a distinction exists.

Keywords: *Eimeria*; poultry; nanopore NGS; broilers; 18Sr DNA; ITS1; CO1; PCR

INTRODUCTION

Eimeria are intestinal parasites that cause coccidiosis, a disease affecting many vertebrates, including seven known species that parasitize chickens (1-12). These seven species are widespread and can occur in combinations of up to six species on any farm (8, 13, 14). The disease is contracted by ingesting food or water contaminated with sporulated oocysts from the environment and is often characterized by diarrhea, morbidity, weight loss, dehydration, and sometimes mortality (15). Each species infects different regions of the intestinal tract and can cause varying degrees of lesions, including thickening of the intestinal wall, hemorrhages, and necrosis (16). Preventative measures crucial for the control of coccidiosis typically involve the use of vaccines, anti-coccidial drugs, such as sulfonamides or ionophores, and good management practices. Coccidiosis is one of the most economically significant diseases affecting poultry producers worldwide, costing the industry an estimated 14 billion dollars annually due to losses in feed conversion rations and reduced weight gain (17).

The robust nature of *Eimeria* parasites seems to prevent their elimination by practical means. Regular cleaning and disinfection of facilities and equipment can help prevent the spread of coccidiosis, but not eradicate every oocyst. The use of anti-coccidial drugs in the feed or water can help reduce the severity of infection, and vaccines are available for some species of *Eimeria* to provide long-term protection. Nutritional management, along with genetic selection for breeds resistant to coccidiosis, may help maintain the health and immune system of birds to reduce the incidence of disease in the future. However, it is important to implement comprehensive control programs, including a combination of these strategies along with biosecurity, to effectively manage coccidiosis in poultry.

Understanding species and antigenic diversity in field populations is critical for control methods to be successful. Genotyping parasites recovered from field populations will assess the species present in a given location, but may also be able to evaluate their genetic diversity and whether that diversity will impact vaccine efficacy and longevity (18). To date, only one complete genome sequence has been published for each of the seven universally recognized species of *Eimeria* that infect chickens (19), and single nucleotide polymorphism (SNP) haplotyping has been evaluated for *Eimeria tenella* only (20). These two reports represent the only complete genome analysis of *Eimeria* species relevant to commercial poultry and they are from *Eimeria* isolates that have been extensively used in laboratory experiments, not pathogenic field samples.

In our previous study, we used Nanopore NGS to differentiate *Eimeria* species in commercial coccidia vaccines and were able to detect all species present with high sensitivity and specificity. In the current study, we expanded upon this platform and used Nanopore NGA to sequence amplicons of 18S, ITS1, and CO1 genes from oocysts collected from 14 broiler houses subjected to different coccidia control programs. The resulting phylogenetic trees from these sequences identify several *Eimeria* species clustered in predictable groups and provide insight into genetic relatedness. The distinction between two clades of *E. maxima* in 18S and two forms of ITS1, and the identification of two unexpected species, *E. necatrix* and *E. dispersa*, indicate that nanopore NGS shows good potential for use as a diagnostic tool for *Eimeria* differentiation, classification, or surveillance. Although phylogenic analysis of nanopore NGS sequences showed all species grouped with known references of the same species, more investigation needs to be done to probe for reliable genetic markers of oocyst origin.

MATERIALS AND METHODS

Types of field samples

This study used litter/fecal samples obtained from 5 broiler complexes in Georgia, Alabama, and Mississippi as starting material. In total, fourteen broiler houses from these 5 complexes were investigated and were subject to various coccidiosis control programs, including vaccine and chemical. Additionally, we sampled one complex (Mississippi) before and after a chemical-to-vaccine program transition. Houses under chemical programs had no prior history of coccidiosis vaccine use. Each house sample consisted of ten pooled floor samples of fecal material, often mixed with litter. Pooled samples were collected, soaked in deionized water for 24 hours, filtered through a double layer of cheesecloth, and concentrated. Prior to oocyst concentration, parts of the samples were used to calculate OPGs via traditional microscopy with the McMaster chamber method. Oocysts taken from the remaining portion of samples were used for genomic DNA (gDNA) isolation. The laboratory's monocultures of pathogenic field isolates of *E. tenella* and *E. maxima* were also sequenced as controls.

DNA extraction and PCR amplification

gDNA from monocultures and field samples were purified from oocysts utilizing a series of reagents followed by ethanol precipitation. Approximately 20,000 oocysts from each sample were pelleted by centrifuge and resuspended in DNAzol. Oocysts were homogenized in 1.4 mm ceramic bead tubes at 6 m/s for 40 seconds. The resulting cell lysate was incubated in 50 µl Proteinase K with a 160 µg/ml concentration at 65°C for 15 minutes. The mixture was cooled on ice for 1 minute, followed by additional centrifugation. Finally, gDNA was isolated from the

supernatant by ethanol precipitation. Isolated gDNA was used alongside pan-species primers to amplify 18S, ITS1, and CO1 genes for all chicken-specific *Eimeria*.

A list of previously published pan-species primers was used (previous research; Chapter 3 above). PCR reactions for each gene region were done in triplicate using Platinum PCR SuperMix High Fidelity and then pooled together in one library. Each 40 µl reaction included ~100 ng of gDNA, 25 pmol forward and reverse pan-specific primers, and an adequate amount of Platinum PCR SuperMix High Fidelity, according to the manufacturer. Thermal cycling was done with an initial denaturing step at 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, an annealing temperature specified previously for 30 seconds, and 72°C for 90 seconds. A final extension step at 72°C for 10 minutes was performed. The amplification of the PCR product was checked by gel electrophoresis in 2% agarose gels stained with 0.5 g/mL ethidium bromide. The 5 µl PCR product used for gel electrophoresis was not used for nanopore NGS sequencing. The remaining triplicate PCR products for each identifying gene region were pooled if successful bands were produced. The amplicons were cleaned and prepared for sequencing using the GeneJET PCR Purification Kit according to the manufacturer's instructions and eluted into 20 µl of a buffer.

Nanopore NGS sequencing

In the study, 14 separate house samples plus two monocultures with three identifying gene regions resulted in 48 sequencing runs (16 samples x 3 genes = 48). All samples were individually sequenced using the ONT MinIon Mk1B sequencer, prepared with a Rapid Sequencing Kit following the manufacturer's instructions, and loaded onto an R9 version, Spot-ON Flow Cell. R9 Flow cells were washed following protocols associated with the ONT Wash Kit after each use. The MinKnow software program operated the MinIon Mk1B sequencer. Output fast5 and fastq

files containing fasta sequences of passed reads were generated with a minimum Q-score of 7 (~80% base call accuracy) and fast base calling. The raw sequence data were imported into *Geneious* to create gene assemblies and phylogenetic trees. Sequence reads failing to meet the minimum Q-score were not used for gene assembly.

Gene assemblies and phylogenetic trees

Complete length sequences of 18S, ITS1, and CO1 were collected from the nr sequence database for confirmed *Eimeria* species that infect chickens: *Eimeria maxima, Eimeria brunetti, Eimeria necatrix, Eimeria acervulina, Eimeria tenella, Eimeria praecox, and Eimeria mitis*. Additionally, sequences for 18S, ITS1, and CO1 genes obtained in a previous study by nanopore NGS of oocysts in various US-brand coccidiosis vaccines were used. Sequences labeled *Eimeria mivati* were included based on the Coccivac B52 vaccine product label. fastq files containing raw sequence reads generated from nanopore sequencing runs were imported into the *Geneious R9.1.8* bioinformatics program. Sequence reads were then mapped to other known references from the nr database using the Bowtie 2 sequence alignment tool. References used to map sequence reads included both chicken and non-chicken specific *Eimeria* species. Assembly parameters were set to high sensitivity with end-to-end alignment. After assemblies were generated, consensus sequences from each assembly were trimmed of primer nucleotides, and phylogenetic tree models were created with Geneious Tree Builder by global alignment and a cost matrix identity of 93% similarity (5.0/-9.026168). Tamura-Nei was the genetic distance model used, and the tree-building method was UPGMA. All trees generated use a closely related *Toxoplasmosis gondii* outgroup to root the tree and observe evolutionary relationships.

RESULTS

Average OPG counts from all field samples tested are shown in Figure 4-1. Nanopore NGS resulted in a total of 1,606,557 passed sequence reads being assembled from field samples. On average, 32.53% of total reads were assembled. Coverage is complete for all gene regions, and the sequence read number is adequate for gene assembly (Figures 4-2;3;4). Sequencing amplicons from the *E. tenella* monoculture resulted in 2,022,756 passed sequence reads, of which 381,876 were assembled, or 18.87%. Sequencing amplicons from the *E. maxima* monoculture resulted in 1,111,447 passed sequence reads, of which 237,995 were assembled or 21.41%.

Phylogenetic trees from all sequences show that the field *Eimeria* were identified as known and grouped with their respective species (Figures 4-6;7;8). All sequences branch separately from the *Toxoplasma gondii* outlier sequence. In broiler house samples where NGS identified a species by one gene, it was also identified by the other two genes. All chicken *Eimeria* species were identified except *E. brunetti*. *E. acervulina* was present in all houses, while E. *necatrix* was identified in only two houses, and *E. dispera,* a non-chicken-specific species, was identified in 3 houses. Sequences labeled *E. mivati* or *E. mitis* shared a node on all trees. Likewise, while forming distinct clades, sequences of *E. tenella* and *E. necatrix* shared a node on all trees. Monoculture sequences for *E. tenella* and *E. maxima* group with their respective species for all genes. Field sequences, regardless of the control program, were highly similar.

There were two main branches within the 18S *Eimeria* clade (Figure 4-6). One branch contained sequences labeled *E. tenella* and *E. necatrix; the* second contained all other sequences. *E. maxima* forms two separate clades which share a node. Interestingly, US brand vaccine sequences and our US-sourced field samples both group to only one of the 18S clades. All field *E. acervulina* 18S sequences were identical to the vaccine sequences, except for one sample taken

from a farm on a chemical control program before the vaccine program transition, which had a distance of 0.003 substitutions/bp.

Within the ITS1 *Eimeria* clade (Figure 4-7), there are two main branches, 1 with a node containing *E. maxima* sequences and 1 with a node containing all other sequences. *E. maxima* formed two distinct clades distinguishing between ITS1 and a long-form ITS1 (ITS1 L) gene/isoform. ITS1 sequence distances of ≤ 0.158 substitutions/bp separated *Eimeria* species clades, with only *E. maxima* from Coccivac B52 identified by the shorter ITS1. All other vaccine and field sample sequences were assembled under ITS1 L only.

Within the CO1 *Eimeria* clade (Figure 4-8), sequence distances for CO1 were lower than ITS1, with ≤ 0.034 nucleotide substitutions/bp between species. Additionally, many CO1 sequences within any given species are identical. CO1 sequences from *E. tenella* formed two distinct clades, with vaccine sequences grouping on one clade and field samples grouping on the other.

DISCUSSION

Using Nanopore NGS we were able to identify many different species of *Eimeria* in field litter samples from multiple poultry farms. The high-throughput, parallel sequencing capabilities of nanopore NGS make it ideal for sequencing all DNA present. It is unknown if the pan-species primers used in this study are able to amplify other strains of *Eimeria*, but several sequences from our field sample libraries share the most homology with unexpected strains of *Eimeria*.

One unexpected finding from the field sample sequence analysis was the presence of *E. dispersa* oocysts. *E. dispersa* was found in 3 houses in a complex using a vaccine program. This species is not in any commercial vaccine nor considered a chicken species of *Eimeria*. *E. dispersa* is traditionally a turkey stain, and was first isolated in Britain (21). Our lab group was able to detect multiple full gene-length sequences of *E. dispersa* by subjecting contigs from non-chicken-specific gene assemblies to the Basic Local Alignment Search Tool (BLAST). These data suggests that nanopore NGS is sensitive enough to detect small *Eimeria* sub-populations that may be missed by conventional microscopy or sequencing. It also may indicate that oocysts can be transiently present in commercial poultry houses, where mechanical vectors, such as local wildlife, likely introduced *E. dispersa* into the houses. This is supported by the fact that the houses where *E. dispersa* was detected were all on the same farm, demonstrating a farm-wide contamination.

Likewise, oocysts identified by nanopore NGS as *E. necatrix* were also present in two houses, one from a complex using a vaccine program and one from a complex using a chemical program pior to transitioning to a vaccine. On the one hand, finding *E. necatrix* in a broiler complex is surprising as it is an *Eimeria* species typically associated with long-lived birds like layers, not broilers that live for only 5-7 weeks. On the other hand, *E. necatrix* is included in broiler-breeder and layer vaccine programs, and with the geographic overlay of breeder and broiler complexes it

may indicate a level of cross-contamination. Another surprising finding was identifying *E. praecox* in 8 of the 14 houses samples. *E. praecox* is not included in coccidiosis vaccine formulations, and it is unknown what effect, if any, *E. praecox* had on the performance at these farms. However, body weight loss and market increases in food conversion ratio (FCR) by *E. praecox* have been observed before (22), and surveys in North and South America have also revealed *E. praecox* to be present in a non-trivial percentage of farms (9).

Phylogenetic comparison of field samples taken from broiler farms under different coccidiosis control programs revealed no apparent distinction in vaccine or reference sequences. Since no statistical analysis for sequence homology exists, it is difficult to know if the coccidiosis control method influenced sequence identity. *E. acervulina*, which had the highest sequencing depth and was present in all samples, showed no distinct grouping between control programs or vaccines. Many contigs of *E. acervulina* are identical or near identical. Still, the potential for distinguishing between vaccine and field origin oocysts may be more subtle than phylogenetic comparison and require more work to identify genetic markers such as Single Nucleotide Polymorphisms (SNPs). Additionally, CO1 sequences for field samples of *E. tenella* were grouped on a separate clade from vaccine sequences of CO1. While this observation did not hold true for ITS1 or 18S, it is noteworthy that the reference sequence used for aligning both the vaccine and field sequences (*E. tenella* CO1 EF174186.1) did not group together with the vaccine sequences in the same clade. Based on this observation, it can be inferred that the formation of our contigs was more influenced by the sequencing depth than the reference sequence used for alignment.

In conclusion, nanopore NGS was able to identify multiple *Eimeria* species present in litter samples collected from farms using numerous coccidia control programs. Most *Eimeria* identified are common in commercial broiler production, but other species of *Eimeria* were also identified

in these samples. These data indicate that Nanopore NGS is comparably rapid and specific, adding to conventional identification methods to solidify correct species identification and minor species that may be missed using traditional methods.

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Figure 4-1. A simple workflow for 1 nanopore NGS run. This sequencing workflow was performed in triplicate for 18S, ITS1, and CO1 DNA regions.

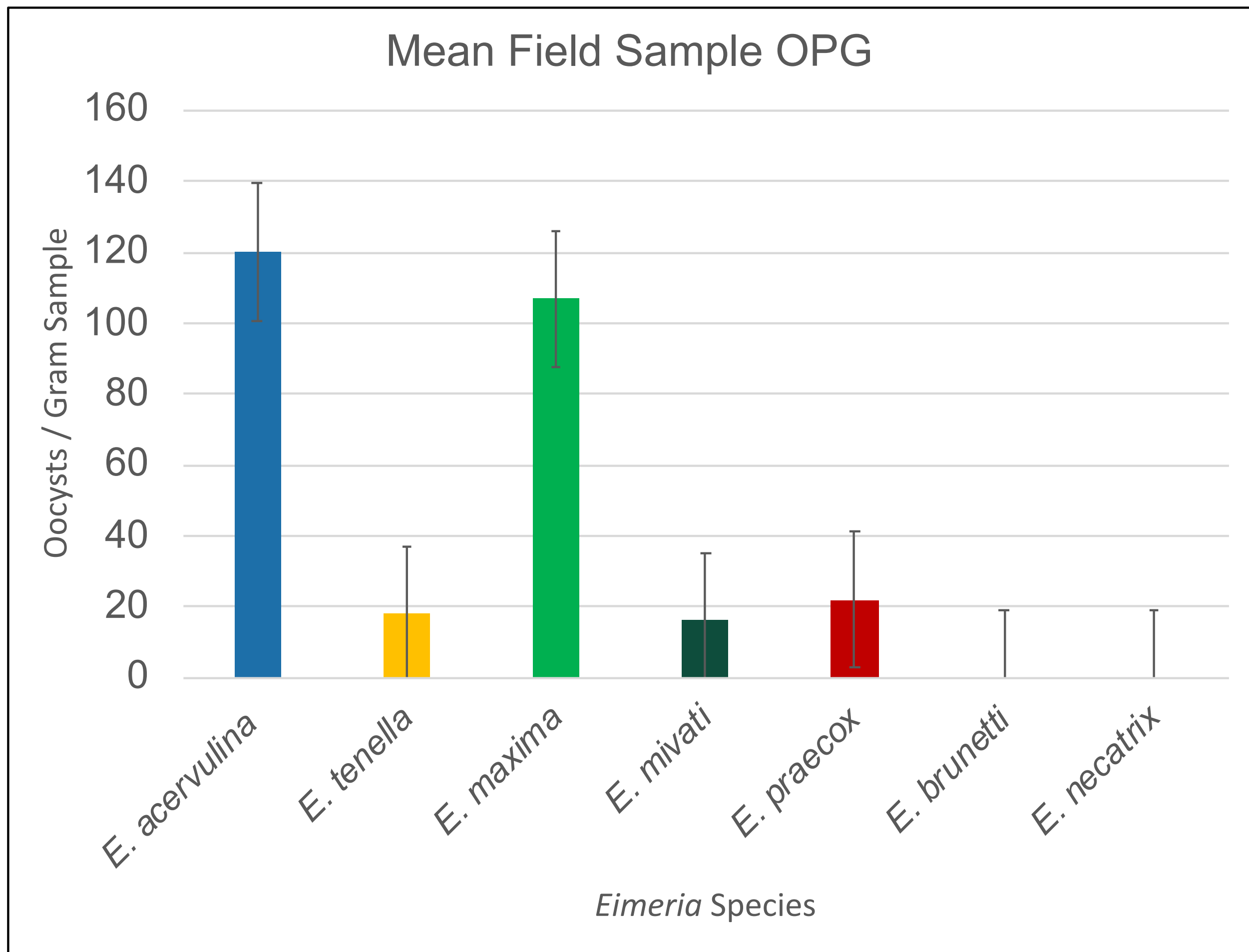

Figure 4-2. The mean number of oocysts per gram (OPG) of field samples for all sample types. Error bars show standard error. *E. brunetti* and *E.necatrix* were not identified in any sample. OPG counts were performed by traditional microscopy using the McMaster chamber method.

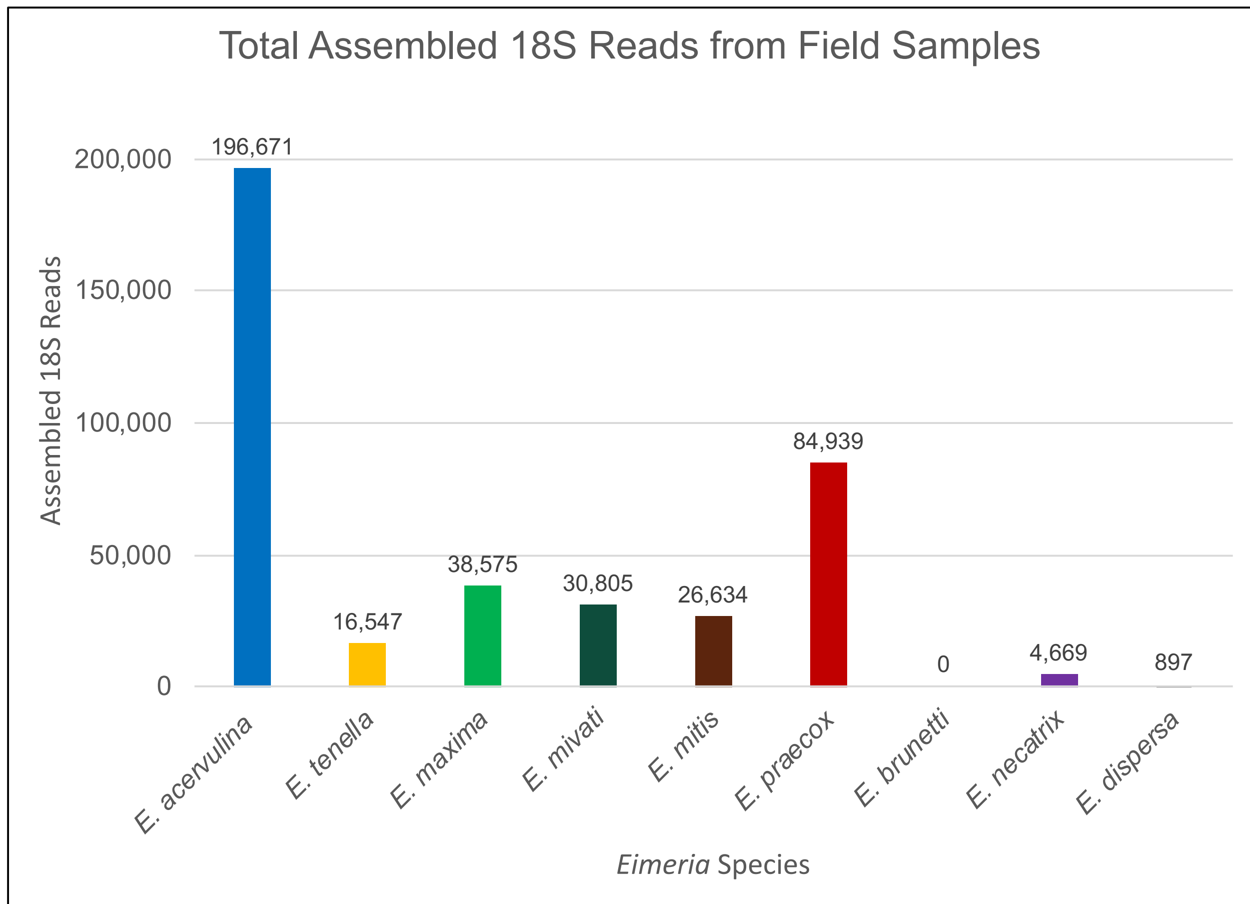

Figure 4-3. The total number of 18S sequences assembled by Bowtie 2 for *Eimeria* species from all field samples in this research.

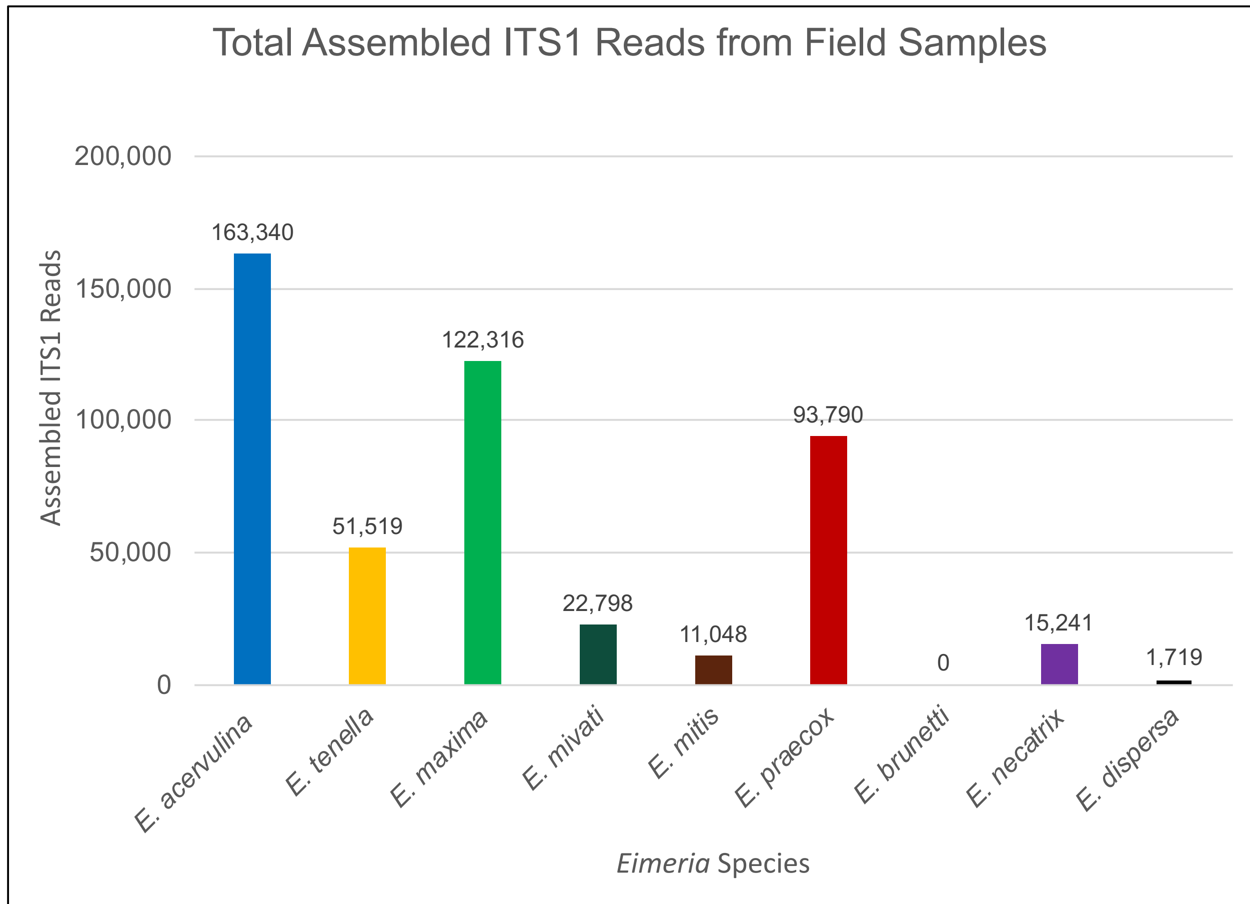

Figure 4-4. The total number of ITS1 sequences assembled by Bowtie 2 for *Eimeria* species from all field samples in this research.

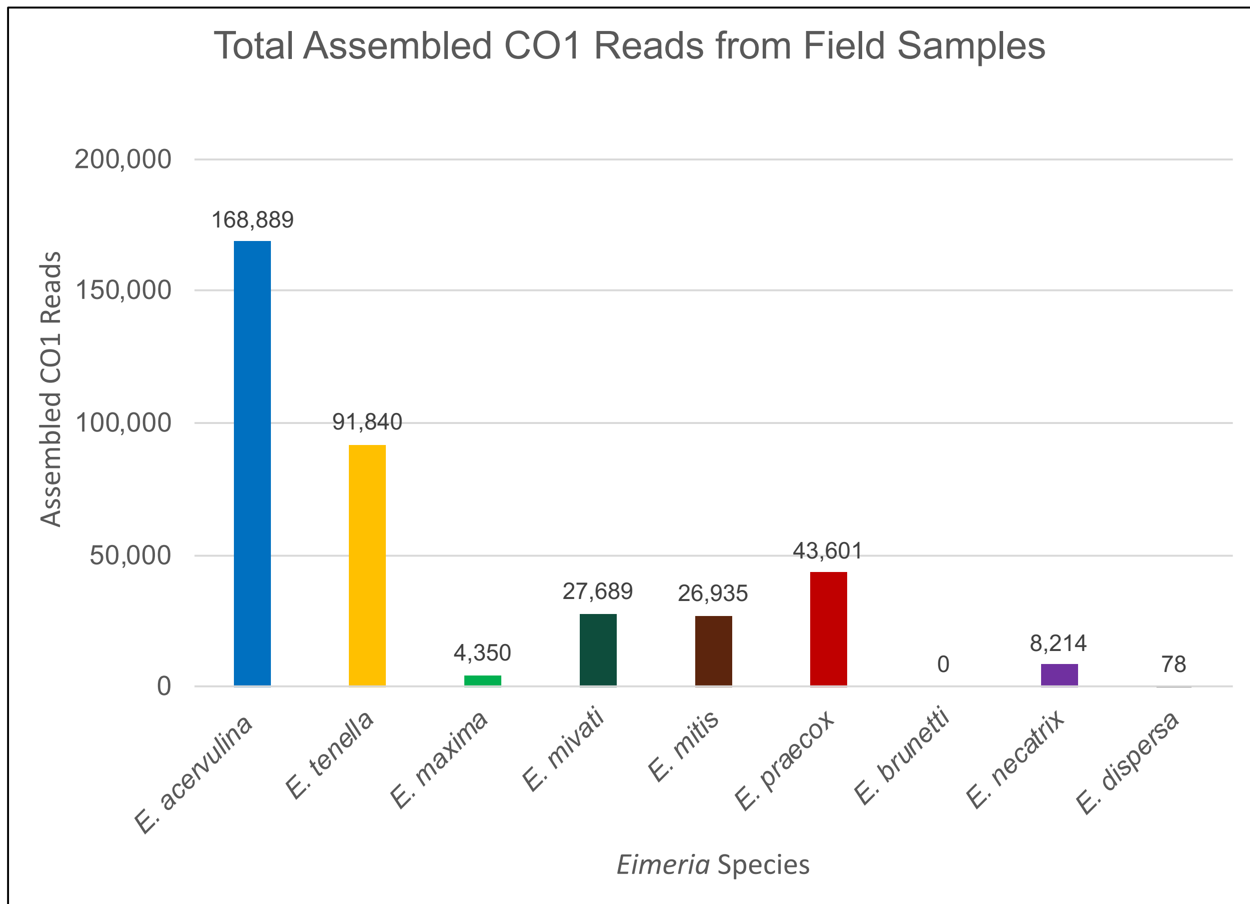

Figure 4-5. The total number of CO1 sequences assembled by Bowtie 2 for *Eimeria* species from all field samples in this research.

- US Coccidiosis vaccine
- A - Chemical program
- B - Vaccine program
- C - Chemical transition
- D - Vaccine transition

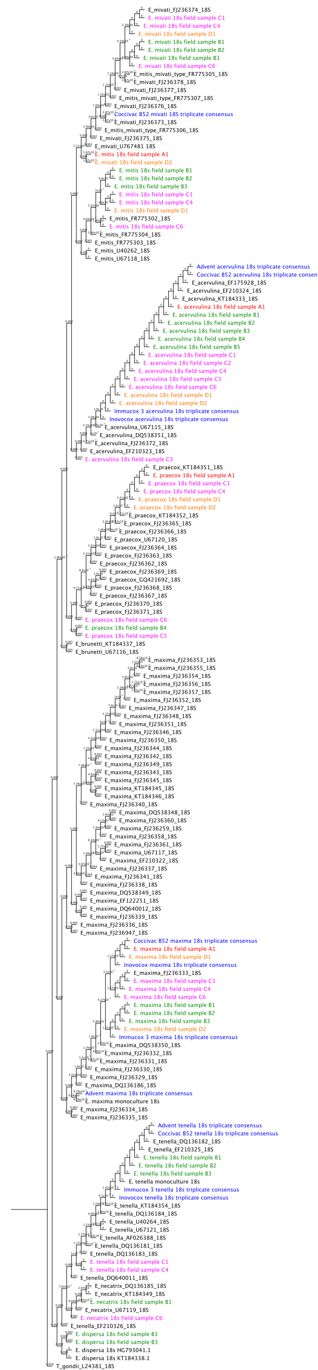

Figure 4-6. Phylogenetic tree of 18S sequences. Included are *Eimeria* sequences detected in the field, monoculture, and vaccine samples along with reference sequences for the 18S gene of *E. maxima, E. acervulina, E. tenella, E. mitis, E. mivati, E. praecox, E. brunetti, E. necatrix, and T. gondii* obtained from GenBank. Branch labels display substitutions per base to 3 significant digits.

- US Coccidiosis vaccine
- A - Chemical program
- B - Vaccine program
- C - Chemical transition
- D - Vaccine transition

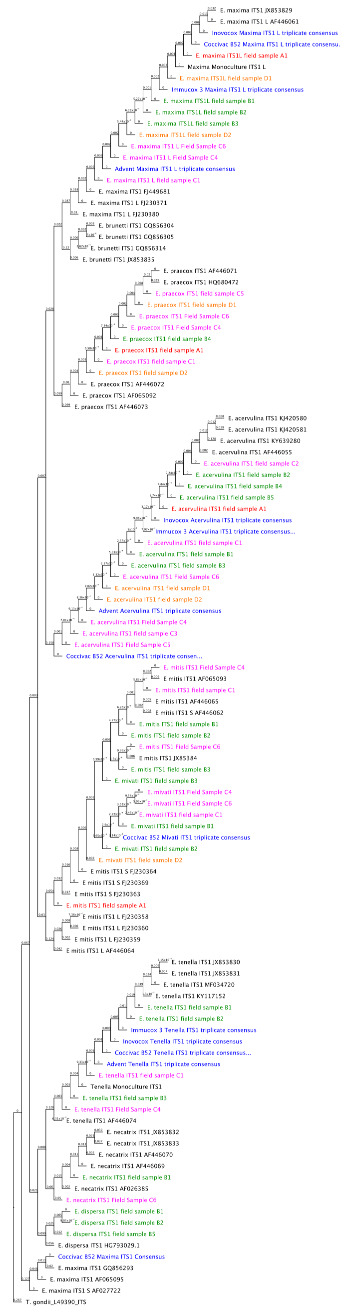

Figure 4-7. Phylogenetic tree of ITS1 sequences. Included are *Eimeria* sequences detected in the field, monoculture, and vaccine samples along with reference sequences for the ITS1 DNA region of *E. maxima, E. acervulina, E. tenella, E. mitis, E. mivati, E. praecox, E. brunetti, E. necatrix, and T. gondii* obtained from GenBank. Branch labels display substitutions per base to 3 significant digits.

- US Coccidiosis vaccine
- A - Chemical program
- B - Vaccine program
- C - Chemical transition
- D - Vaccine transition

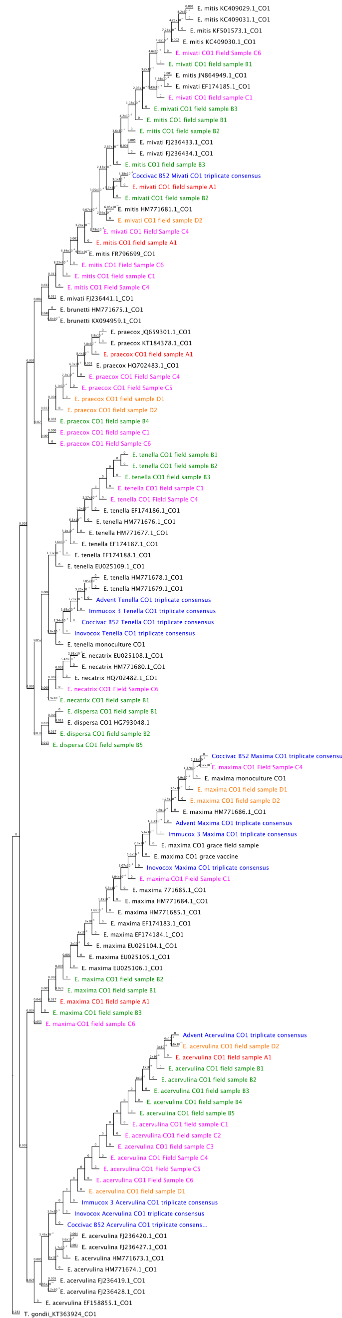

Figure 4-8. Phylogenetic tree of CO1 sequences. Included are *Eimeria* sequences detected in the field, monoculture, and vaccine samples along with reference sequences for the CO1 gene of *E. maxima, E. acervulina, E. tenella, E. mitis, E. mivati, E. praecox, E. brunetti, E. necatrix, and T. gondii* obtained from GenBank. Branch labels display substitutions per base to 3 significant digits.

CHAPTER 5

SUPPLEMENTATION OF *EIMERIA* GENE ASSEMBLIES THROUGH A BIOINFORMATIC BLASTn PIPELINE

Ben Jackwood[1] Benjamin Lorentz[2] Brian Jordan[2]

[1]Department of Population Health, [2]Department of Poultry Science, University of Georgia, Athens, Ga.

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ABSTRACT

Although sequencing and other high-throughput data production technologies are increasingly affordable, data analysis and interpretation remain significant factors in the cost of bioinformatics research. An explosion of genomic sequences generated by high-throughput Next Generation Sequencing (NGS) technologies has made computational tools necessary to accurately describe data contents and empower linkage to existing resources. Here we present a complete workflow that can be executed in a Linux environment for a bioinformatic pipeline consisting of two Shell scripts and one Python code, created to be used with the Georgia Advanced Computing Resource Center (GACRC) computing cluster. In this study, 18,726,323 sequences (Figure 5-3) generated by nanopore NGS from *Eimeria* gene amplicons were analyzed by the nucleotide Basic Local Alignment Search Tool (BLASTn) to find regions of local similarity between sequences. The pipeline outputs a database of all taxa returned in any BLASTn queries and counts the number of times a species was the subject of a successful BLASTn query. Pipeline outputs validate our gene assemblies of *Eimeria* sequences from previous studies and provide potential to be used in future omics studies.

INTRODUCTION

Over the past decade, nucleotide sequencing technologies have made tremendous progress in throughput, speed, and reduction of sequencing cost (1-4). However, the volume of data generated by NGS technologies becomes challenging, requiring high computational power and execution time. This leads to a significant bottleneck in computational biology and bioinformatics (5, 6). Bioinformatics is the intersection of biology and computer science, using software programs on biological data for various applications. A bioinformatics pipeline is a series of software algorithms that processes large biological data sets and generates interpretations from such data. Bioinformatic pipelines enable life scientists to analyze biological data through automated multi-step processes constructed by individual programs or databases. One example program is BLASTn, an algorithm and tool for comparing primary biological sequence information, such as nucleotides of DNA or RNA sequences.

To supplement *Eimeria* gene assemblies from our previous work and create a novel molecular analysis tool for the poultry industry, we constructed a bioinformatic pipeline using the BLASTn tool (NCBI) to identify all taxa in a given sequencing run and quantify their numbers into a simple list. From our previous work, one unexpected finding from the analysis of vaccine *Eimeria* sequences was a lack of *E. maxima* CO1 in our PCR products. We performed CO1 PCR amplification and subsequent nanopore NGS on a laboratory monoculture of *E. maxima* oocysts and replicated these low results. Still, further confirmation that *E. maxima* CO1 sequences were not present was needed to rule out the possibility that Bowtie 2 could simply not assemble the sequences present.

The pipeline consists of two parts. In the first step, a simple script performs a nucleotide BLASTn search for all sequences it is given against the NCBI nucleotide sequence database.

Parameters for runtime, memory, file names and email notifications are set within the script. Modules for the BLAST+ software and the current NCBI database are loaded and directed to our sequence files. The resulting output text file contains BLASTn results of all successful sequence queries. The second part of the pipeline parses through the text file of BLASTn results to develop and quantify a taxonomy database of successful hits. The data provided by this pipeline are an example of a real-life use case that will have practical utilities for researchers involved in sequencing and studying new genomes.

MATERIALS AND METHODS

Raw nanopore NGS data in the form of FASTQ files from 64 runs using an Mk1B nanopore NGS device (see previous Chapters) were used as the starting input for the workflow. FASTQ files were uploaded to the Sapelo2 computer nodes at the Georgia Advanced Computing Resource Center (GACRC) along with a shell script we created (Figure 5-1) capable of running BLASTn searches on the NCBI nucleotide database. Nanopore NGS reads, consisting of both passed and failed reads based on a Phred score of 7, were separated based on the sequence run. Each gene sequence was then individually queried against the local database via the blastn command of NCBI-blast +, and the significant alignments were directed into a separate text file. The completion of running these reads with the script shown in Figure 5-1 resulted in a text file containing BLASTn results from our nanopore NGS data. An example of one entry from a resulting text file is shown in Figure 5-2.

In the second step of the pipeline, our custom python 3 script parsed out the BLASTn results in text files and created a database of all species with successful queries. Upon running the script, the accession number with the lowest e-value was chosen for each BLASTn entry and recorded into a database that stores the scientific name and tax-id from NCBI. After each batch job, the output database was used as an input for the following batch job to limit the time and resources required to parse results. Data were then produced in an organized and visually accessible layout, that could be used for sequence library interpretation.

RESULTS AND DISCUSSION

Eimeria species accounted for most taxonomic counts from all nanopore NGS runs in this trial, and taxonomic counts for each identifying gene region exceeded the number of assembled reads for that gene region. The mean percent identity and query coverage for each species was low, often below 80% compared to the subject query. Low percent query coverage and higher taxa count than assembled reads suggest that our sequence libraries contain large amounts of partial reads. For the CO1 gene, taxa counts for coccidiosis vaccines were higher than the number of reads assembled by Bowtie 2 (Figure 5-4). The BLASTn workflow identified more sequences of CO1 as *E. maxima* than were assembled by Bowtie 2. However, the largest difference was 4,275 sequences of *E. maxima* CO1 compared to 483 assembled reads of *E. maxima* in Immucox 3. In addition to chicken *Eimeria* species, the created databases listed several microorganisms and genomic chicken DNA present in field samples. The final database consisted of 1,310 unique NCBI accession numbers.

The bioinformatics pipeline used to identify and quantify taxa in our nanopore NGS sequence libraries concisely extracts practical data from millions of sequences. Results suggest our sequence libraries contain large amounts of partial reads indicated by low query coverage and taxa count to assembled read ratio. This is likely a consequence of using both assembled and non-assembled reads as input for the pipeline since partial reads are not likely to be assembled by Bowtie 2 using our parameters. In fact, many of the taxa from the final database could not be assembled by Bowtie 2 under a reference sequence. In hindsight, the percent identity and query coverage could be improved using only assembled reads with a Phred quality score greater than 7. Relatively few taxa count for CO1 sequences of *E. maxima,* which validates our previous low assembly coverage for that gene.

The bioinformatics pipeline outlined in this paper offers an automated workflow tool for identifying and quantifying nanopore NGS sequence libraries. It provides a potential for future use in widening the bottleneck of NGS data processing. Since no complex environment set-up is expected of the user, anyone without strong knowledge or background in computer science could easily use the scripts to analyze their data by simply changing the input file name. Results can be extracted from Sapelo2 using one short Linux command, and raw nanopore NGS reads require no data pre-processing.

Usage information has been documented in this paper, and additional documentation, as well as all test cases and datasets used in this paper, can be found in the GitHub repository, at https://github.com/lorentzben/jackwood_blast_parser.

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```
#!/bin/bash

#SBATCH --partition=batch
#SBATCH --job-name=Jackwood_test_blast
#SBATCH --nodes=2
#SBATCH --ntasks=8
#SBATCH --time=96:00:00
#SBATCH --mem=64gb

#SBATCH --mail-user=""
#SBATCH --mail-type=BEGIN,END,FAIL


ml BLAST+/2.12.0-gompi-2020b
ml ncbiblastdb/20220404
#blastn -num_threads 16 -query FA008363_pass_d1f96d95_24.fasta -out results.out -db nt
blastn -db nt -query FA008363_pass_d1f96d95_24.fasta -out result.out -outfmt "7 delim=,
qacc sacc sblastnames evalue bitscore qcovus pident" -max_target_seqs 10 -num_threads 8
```

Figure 5-1. Example code in the shell script required to BLASTn sequences.

```
# BLASTN 2.12.0+
# Query: ad44cd20-b5ba-43a1-b247-553d119b3ea9
runid=d1f96d9539c9def8d4d9af38d7e732d9b4be12e2 read=84157 ch=68
start_time=2022-02-22T19:24:14Z flow_cell_id=FAO08363
protocol_group_id=Jemco_H1_CO1_0222022 sample_id=Jemco_H1_CO1
# Database: nt
# Fields: query acc., subject acc., evalue, bit score, % query coverage
per uniq subject, % identity
# 10 hits found
ad44cd20-b5ba-43a1-b247-553d119b3ea9,FR796699,7.92e-66,263,63,96.835
ad44cd20-b5ba-43a1-b247-553d119b3ea9,FJ236441,7.92e-66,263,63,96.835
ad44cd20-b5ba-43a1-b247-553d119b3ea9,FJ236434,7.92e-66,263,63,96.835
ad44cd20-b5ba-43a1-b247-553d119b3ea9,EF174185,1.02e-64,259,63,96.203
ad44cd20-b5ba-43a1-b247-553d119b3ea9,KX094963,3.68e-64,257,63,96.203
ad44cd20-b5ba-43a1-b247-553d119b3ea9,KX094962,3.68e-64,257,63,96.203
ad44cd20-b5ba-43a1-b247-553d119b3ea9,KX094961,3.68e-64,257,63,96.203
ad44cd20-b5ba-43a1-b247-553d119b3ea9,JN864949,3.68e-64,257,63,96.203
ad44cd20-b5ba-43a1-b247-553d119b3ea9,HM771681,3.68e-64,257,63,96.203
ad44cd20-b5ba-43a1-b247-553d119b3ea9,FJ236433,3.68e-64,257,63,96.203
```

Figure 5-2. Example of a BLASTn result entry found in the text file output. The entry includes nanopore sequence read ID, BLASTn query subject accession number, e-value, bit score, percent coverage, and identity for 10 hits.

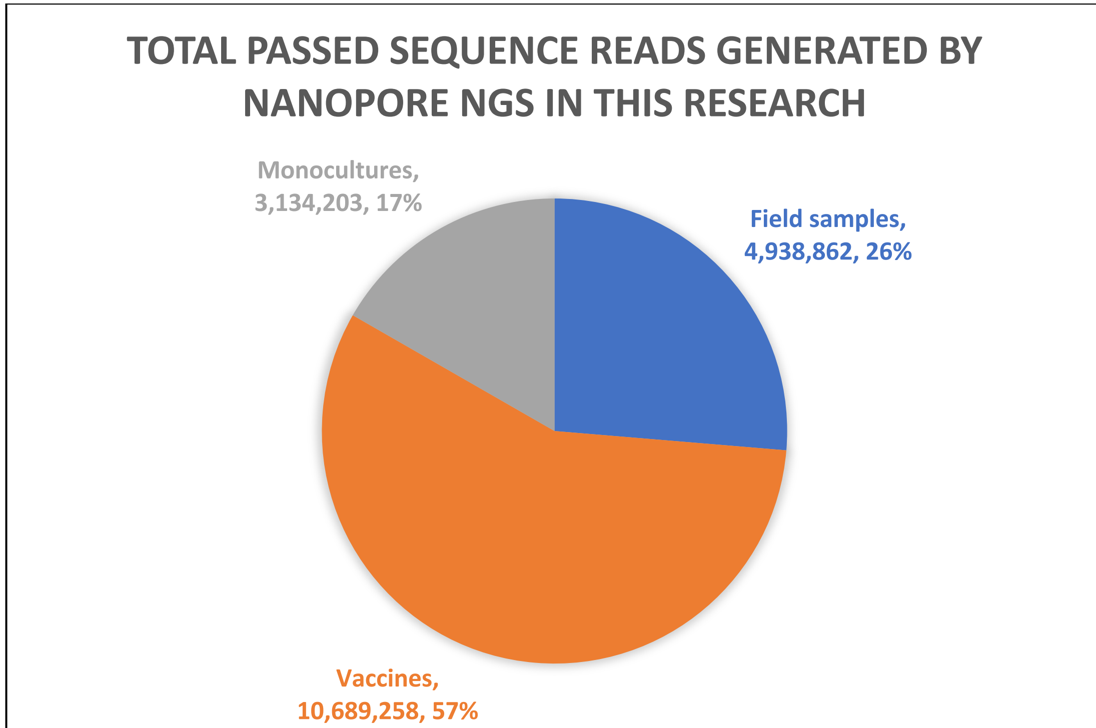

Figure 5-3. The total number of passed sequence reads generated by nanopore NGS from research projects 1 and 2, shown as a pie chart. The sum of passed sequenced reads from all nanopore NGS runs equals 18,726,323 sequences.

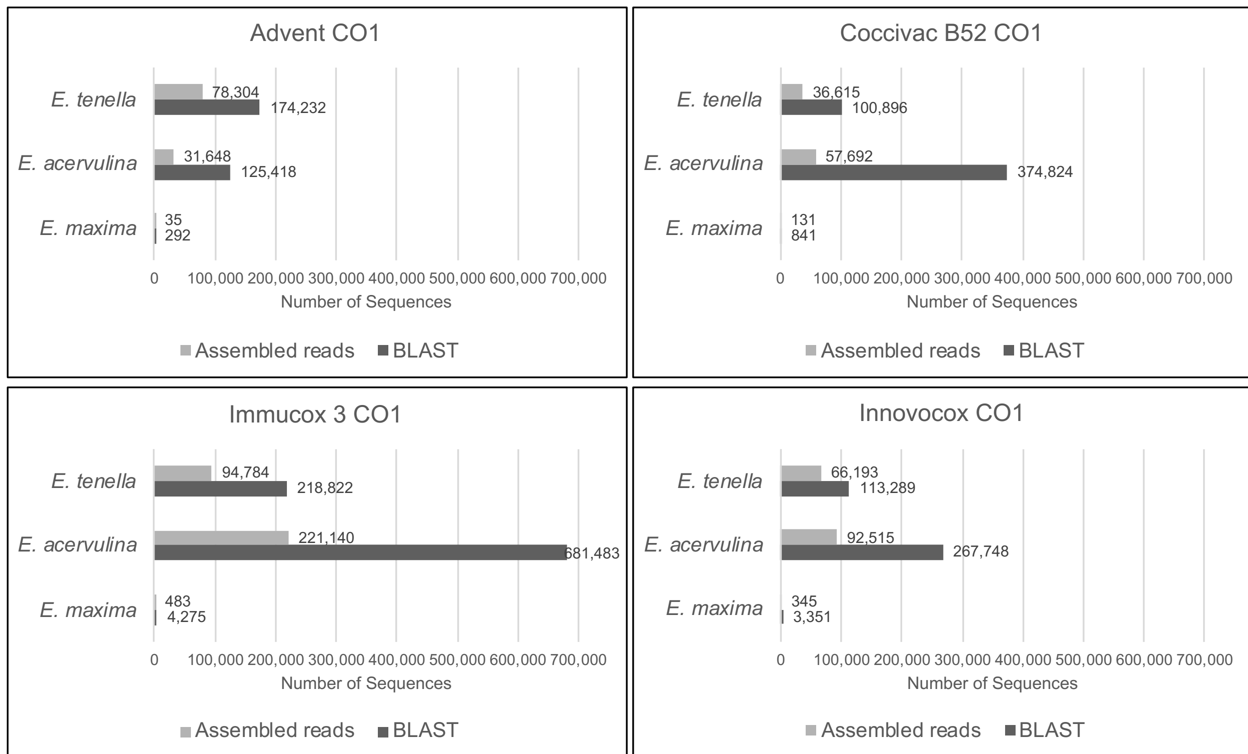

Figure 5-4. The total number of CO1 reads assembled by Bowtie 2 compared to the total number of taxonomic counts from the BLASTn workflow for coccidiosis vaccines. Assembled reads consist of only passed sequence reads with a Phred score >7, while BLASTn counts incorporate all reads sequenced by nanopore NGS.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Eimeria cause one of the most economically significant diseases of commercial chickens, labeled coccidiosis. Control of these parasites and the resulting disease is essential for the continued success of the poultry industry. Coccidia are commonly vaccinated against on day of hatch in the hatchery with live vaccines. Coccidia vaccines are often applied using a spray cabinet, although other application methods exist. Coccidia species do not cross-protect, so vaccines contain live, sporulated oocysts of the species most likely to cause a challenge in the environment. Coccidia vaccines contain live, sporulated oocysts ingested by the chickens and stimulate a protective immune response in the intestines. It is speculated that cycling coccidiosis vaccine strains replace challenge oocysts, which may be highly pathogenic, with strains known to be susceptible to anticoccidials or precocious. Vaccine-origin oocysts are shed by vaccinated birds and may persist in the environment. The goal of this research was to develop a potential diagnostic assay using molecular identification to distinguish and differentiate *Eimeria* species in mixed populations with the potential to distinguish between field and vaccine isolates. To help manage this highly impactful disease, this research aimed to (1) assess the ability to use Nanopore NGS to produce high-quality sequences of ITS1, CO1, and 18S coccidia genes, (2) produce high-quality sequences of these genes in field samples, and (3) generate phylogenic trees of all consensus sequences and supplement gene assemblies through novel bioinformatic tools.

The first aim of this dissertation research was to assess the ability of Nanopore NGS

to produce high-quality sequences of three identifying gene regions. One drawback of nanopore NGS when it was first introduced was its high error rate. Additionally, only one complete genome of each species, obtained by Sanger sequencing, is published. This scarcity of genetic information leads to a reliance on species identification by traditional microscopy or necropsy through morphological or biological traits. Species traits helpful for traditional identification often overlap, and molecular identification methods would further improve confidence in making correct distinctions. Several PCR assays targeting identifying genes have been published, but no robust diagnostic assays have been put forth to evaluate species in mixed populations.

Coccidiosis vaccines offer clean samples for DNA isolation and PCR with the added benefit of knowing the species present based on the manufacturer's label. For this project, multiple coccidiosis vaccines were sequenced by nanopore NGS to determine if this technology is accurate and repeatable enough to develop a diagnostic assay for *Eimeria* species differentiation. High throughput of nanopore NGS allowed for high amplicon sequencing depth. Assembling reads with Bowtie 2 under known references gave contigs for species listed on the vaccine labels. The Nanopore NGS technology provided sequences for all of the species present in each sample, and it was found that two forms of the ITS1 gene could be assembled for *E. maxima* in Coccivac B52. Together, genomic data and the similarity of replicate sequencing runs indicate that nanopore NGS is an accurate, repeatable, and inexpensive method to supplement non-molecular coccidia identification methods.

The second aim of this dissertation research was to produce high-quality sequences of these genes in field samples. This experiment aimed to assess the ability of nanopore NGS to

identify Eimeria in field samples with unknown species and oocyst number, as well as investigate the potential to differentiate vaccine and field strain origins. Various field samples from broiler farms utilizing separate coccidiosis control programs were used. Nanopore NGS was able to sequence and identify several field *Eimeria* in addition to unexpected non-chicken specific species. This experiment demonstrated that nanopore NGS could be used on samples of fecal droppings containing oocysts, and sequence coverage and depth are high enough to differentiate species accurately.

The goal of creating phylogenetic trees with contigs of coccidiosis vaccines and field samples from this work was their comparison to database sequences of other *Eimeria* species. The data provided valuable insights into the genetic relatedness among the samples. The vaccine sequences were found to be closely grouped together, while the 18S region showed two clades of *E. maxima*, and two forms of ITS1 distinguished them. Surprisingly, two unexpected species, *E. necatrix* and *E. dispersa*, were identified, indicating the potential of nanopore NGS as a diagnostic tool for *Eimeria* differentiation, classification, or surveillance. Although the phylogenetic analysis of nanopore NGS sequences showed all species grouped with known references of the same species, further investigation is required to identify reliable genetic markers of oocyst origin.

As stated previously, DNA sequencing costs have dramatically decreased, allowing for an explosion of new sequencing data for many organisms. One consequence of generating large sequencing data sets is the bottleneck surrounding gene assembly, analysis, and data interpretation. The creation of novel bioinformatic tools is needed to remove this bottleneck. This final dissertation project aimed to create a bioinformatic BLASTn pipeline to

supplement existing gene assemblies and improve data interpretation of future projects. A fully automated 2 step bioinformatic pipeline was designed to use the GACRC Sapelo2 computing cluster at UGA. The pipeline consists of shell scripts containing Linux-based commands and python code, which subject raw nanopore sequences to the BLAST+ software and parse through successful queries. Output data list taxa counts, strain information, percent identity, and query coverage. Although these data provide further confidence that Bowtie2 assemblies of CO1, ITS1, and 18S coccidia genes are accurate, further testing of more datasets is needed to understand how this tool may be implemented for diagnostic purposes.

A molecular assay capable of distinguishing between various chicken *Eimeria* species would be highly advantageous for the poultry industry. Our work demonstrated the suitability of nanopore NGS to sequence amplicons of identifying gene regions in *Eimeria* and investigate species present in broiler chicken houses under various coccidiosis control programs. The resulting data showed good applicability of nanopore NGS technology for sequencing and discerning mixed species populations from vaccine and field samples. Although these data seem to provide insight to molecular *Eimeria* relatedness, further analysis is needed to characterize these parasites.

Future directions for this project will likely include changes to materials and methods, DNA target sequences, project scope, and experimental approach. The quality of nanopore NGS data relies heavily on the quality of DNA or RNA sequenced. The DNA isolation method from this work is adequate for deep sequencing of PCR amplicons. However, future objectives requiring long reads, such as whole genome sequencing or single oocyst DNA isolation, will benefit from an improved method that ensures high-quality starting material of all the nucleic

acids present. In addition, the entire genome sequencing of all Eimeria species important to chickens will provide the maximum sequence data for correct species classification. Increasing the scope of this project will likely provide insight into novel gene targets, improving the ability to identify chicken Eimeria beyond those used in this study. Finally, using a bioinformatic BLAST pipeline for diagnostic purposes will require a precise protocol for implementation. It is still being determined what the best practices for this tool are. However, it will likely be helpful for future sensitivity assays where the exact number of off-target sequences are identified from gene assemblies and providing BLAST information from unassembled reads. By subjecting reads that fail to assemble under a known reference to a BLAST pipeline, researchers can pull information from large sequence data sets that would otherwise not be investigated with the possibility of identifying novel variants.