EXAMINATION AND COMPARISON OF INFECTIOUS BRONCHITIS VIRUS AND COCCIDIA VACCINES AND VACCINATION METHODS

by

GRACE ASHBY ALBANESE

(Under the Direction of Brian J. Jordan)

ABSTRACT

Of the myriad of diseases affecting commercial chickens, infectious bronchitis and coccidiosis cause the most significant economic losses. Vaccination for both of these pathogens, infectious bronchitis virus (IBV) and *Eimeria* spp., occurs at the hatchery using mass application strategies. IBV vaccines are live attenuated viruses, delivering serotype-specific immunity. The Arkansas IBV serotype is the most frequently detected serotype in the field, so vaccination to protect against disease from this type is commonplace. The current vaccine, ArkDPI, is not efficacious, and an alternative Ark-type vaccine is needed. Ark99, once a commercial IBV vaccine strain, was passaged once in embryonating chicken eggs to produce the ArkGA vaccine candidate. After 60 passages in embryos, the ArkGA vaccine was highly attenuated and provided good protection against Ark-type challenge when vaccinating broiler chickens, and is a suitable alternative to the ArkDPI vaccine.

Like IBV, *Eimeria* spp. vaccines are often applied in the hatchery by mass vaccination methods. Vaccines may be applied using an aqueous spray, or a gel applicator bar may be used, although this technique is newer and not yet validated versus the traditional spray method. Vaccination using both a highly viscous and less viscous gel, applied by bar, was compared with

liquid spray. Oocyst shedding differed slightly between vaccinated groups, although all groups were equally protected from *E. maxima* challenge, indicating that all the vaccine applications tested are effective.

In addition to coccidiosis vaccine application, oocyst species used in vaccines are critical, because coccidia produces only species-specific immunity in chickens. An *Eimeria* type, *E. mivati*, has been contested as a species since its discovery, although it is included in a commercially licensed vaccine. Many claims suggest that *E. mivati* is a variant of *E. mitis*. Next generation Illumina sequencing was used to compare the mitochondrial cytochrome C oxidase subunit I gene sequences of samples containing *E. mivati* with known *Eimeria* sequences. None of the *E. mivati* samples contained sequence that matched with *E. mivati* in GenBank, although all matched with *E. mitis*. This data provides further evidence that *E. mivati* is likely a variant of *E. mitis*, although further genome examination is needed.

INDEX WORDS: Infectious bronchitis virus, Broiler, Vaccination, Arkansas DPI, Arkansas GA, Coccidiosis, *Eimeria mivati*

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DEDICATION

I would like to dedicate this dissertation to my husband, Jack. Thank you for your love, encouragement, and belief in me. I would also like to dedicate this work to my parents, Linden and Susan, and my sister, Savannah. I could not have achieved this work without the love and guidance of my wonderful husband and family. I am incredibly lucky to have you all as my support system and I am so thankful for it.

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CHAPTER 1

INTRODUCTION

Because of large-scale production practices by the United States poultry industry, any prophylactic or vaccine treatments given to commercial chickens must be mass applied. The two most common mass application strategies have traditionally been *in ovo* injection at transfer of eggs from the incubator to the hatcher, and mass spray on day of hatch as chicks are transferred from the hatch baskets into the delivery trucks. Many vaccines cannot be applied *in ovo*, making this technology widely but not ubiquitously used; however, nearly all chickens are spray vaccinated with one or more vaccine types (25).

Infectious bronchitis virus (IBV) is the most common vaccine administered by spray (5, 20). IBV is an enveloped coronavirus with a positive-sense, single stranded, 28 Kb RNA genome. The virus causes infectious bronchitis (IB), a disease characterized by reduced feed conversion, drops in egg production, and ciliostasis that predisposes chickens to secondary infection. Bacterial infection following an IBV outbreak can lead to increased condemnations at the processing plant, which is why the US Animal Health Association Report lists respiratory disease as one of the most significant sources of economic loss to the broiler industry (1, 13). IBV is the main causative agent of respiratory infection in broiler flocks due to its widespread prevalence. The high rate of infection and associated levels of replication provide opportunities for the virus to gain mutations, leading to the emergence of variants and new serotypes of IBV (12, 14, 34). Frequent mutations and emergence of new serotypes makes control of the disease very difficult, creating constant pressure for the development of new vaccines in order to ensure adequate protection of chickens in the field.

Along with spray vaccination for IBV, vaccination against coccidiosis is becoming increasingly more common in the hatchery. Coccidiosis is caused by infection with coccidia, which are single-celled, parasitic protozoa of the phylum Apicomplexa. Coccidia in the genus *Eimeria* infect the lining of the gut of many types of animals, with seven species of *Eimeria* known to only infect chickens: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*. Two other species, *E. hagani* and *E. mivati*, are referenced in the literature, but their existence as separate *Eimeria* species is contested. Ingestion of sporulated *Eimeria* oocysts from the environment leads to the development of the disease coccidiosis. Each *Eimeria* species has a tropism for epithelial cells in a particular region of the intestine. Depending on the species, disease resulting from infection can range from subclinical to intestinal necrosis and mortality (7). Coccidiosis is the most economically significant disease affecting poultry producers worldwide, costing the poultry industry $3 billion annually due to losses in feed conversions and reduced weight gain (2). Additionally, severe infection with *E. maxima* can cause leakage of plasma proteins into the gut lumen and increased intestinal mucus, both of which provide a favorable environment for proliferation of *Clostridium perfringens* (33). Atypical levels of *C. perfringens* replication in the intestine may cause necrotic enteritis, resulting in reduced performance, increased mortality, and additional loss of billions of dollars for producers (36).

Vaccination for coccidiosis at day of hatch is becoming increasingly predominant as the method for disease control. Oocysts are ubiquitous in the environment, so exposure of chickens is inevitable. Traditionally, the disease was managed by administration of anticoccidial drugs. However, with the rising issue of drug-resistant strains of *Eimeria*, ionophores were introduced as anticoccidials that were effective at modulating infection without creating drug-resistant oocysts. Although ionophores are effective, changing consumer preferences for meat from chickens raised

without drugs and governmental regulations imposed on the poultry industry are causing a reduction in the use of chemotherapeutics and ionophores, leaving only vaccine use for coccidiosis control (4, 6, 28). Anticoccidial vaccines contain live, sporulated oocysts of the species that are known to cause the greatest disease challenge in the production environment. Doses vary per the manufacturer, but are typically minimal enough to elicit a protective cell mediated immune response without causing clinical disease (32, 37). The most common application method for coccidia vaccines is to spray day-old chicks using a spray cabinet, akin to IBV. In recent years, the use of a gel diluent for coccidia vaccines has become more widespread. Gel beads containing *Eimeria* oocysts were originally delivered in the feed, and were shown to be protective (17, 18). Now, gel diluent vaccines are administered using a gel applicator bar in the hatchery to allow for more precise control over vaccine coverage (2).

The overall goal of this research is to improve vaccination of day-old chicks in the hatchery and increase the protection level against challenge in the field. Specifically, this research aims to (1) develop a new IBV vaccine of the Arkansas serotype to combat the continual Ark IBV problem in the US poultry industry, and (2) compare coccidia vaccine application methods and contrast the infection, cycling, and protection level from each application type. This research also aims (3) to investigate one of the contested *Eimeria* species, *E. mivati*, and provide further analysis on its status as a distinct species within the *Eimeria* genus.

SPECIFIC AIMS

Specific Aim 1. Recovery of a previously used infectious bronchitis virus Arkansas 99 vaccine.

Of the many IBV serotypes present in the US, Arkansas DPI (ArkDPI) was the most frequently identified over an 11-year period (15), and still is today. This is because ArkDPI shows atypical infection and replication when mass applied by spray, resulting in a "rolling" vaccine reaction post-vaccination (16, 23). The atypical cycling is caused by the presence of multiple populations of ArkDPI in the vaccine bottle, where the major vaccine population does not replicate as well in chickens as does one of the minor subpopulations (21, 26, 27). This results in a very low infection rate, so chickens do not develop adequate immunity and thus are not protected from challenge.

Although ArkDPI is the only Ark strain currently used in the vaccine, it is not the only Arkansas serotype vaccine ever produced. The Ark99 strain was the first Ark-type virus attenuated for vaccine production. It showed good replication and protection characteristics, however, the vaccine caused severe reactions in broilers post-vaccination, making them vulnerable to secondary infection and air sacculitis (10, 19). Because of the severe vaccine reaction, use of Ark99 was discontinued when the milder ArkDPI vaccine was produced. But, the current ArkDPI vaccine does not infect, replicate, and protect adequately. A viable replacement for the ArkDPI vaccine might be a further attenuated Ark99 vaccine, which could potentially eliminate the current issue of rolling vaccine reactions and frequent Ark-type IBV isolation.

Preliminary data indicate that Ark99 is safe when inoculating SPF chicks according to USDA Title 9 Code of Federal Regulations (9-CFR), section 113.327 and section 2.4.1 of the European Pharmacopoeia (EP) guidelines (8, 31). The next step in verifying Ark99 as a vaccine candidate was to determine the infection and replication rate after spray vaccination. In a field trial

by Roh et al. (29), broiler chicks vaccinated with ArkDPI were found to exhibit atypical replication patterns, including multiple peaks of replication, as compared to the desired single peak and then decline in viral load that is typically seen with IBV vaccines. These abnormal replication patterns for ArkDPI are believed to produce a rolling vaccine reaction in the field, and it is necessary to show that Ark99 vaccine will not replicate in these same patterns when spray vaccinating broiler chicks. Additionally, ArkDPI vaccine has a host of genetic subpopulations that can be detected in chickens post-vaccination (21), and these subpopulations influence vaccine efficacy (22, 23). After vaccinating with Ark99, samples should be taken and sequenced to examine the development of subpopulations. After determining that an Ark99 vaccine candidate has a typical IBV replication pattern, a challenge study will need to be conducted to show that Ark99 is protective against homologous Ark-type IBV challenge.

Preliminary vaccine efficacy trials of Ark99 indicated that further embryo attenuation was needed to make Ark99 a suitable vaccine candidate. Ark99 was originally replaced by ArkDPI because of the harsh reaction resulting from Ark99 vaccination. The safety studies that monitor vaccine reaction are carried out in SPF chicks, which are known to be more refractory to the disease than broiler type chickens. After embryo passage to further attenuate Ark99 and sequencing to confirm that no extensive mutations occurred in the spike gene, efficacy and challenge studies were repeated. Vaccine virus nomenclature was changed during passage to Arkansas Georgia (ArkGA), and efficacy and protection studies were performed for ArkGA pass 20 (P20), P40, and P60.

Specific Aim 2. Evaluation of coccidia vaccines using traditional and new vaccine applications.

As stated previously, coccidiosis is the costliest disease to affect poultry producers worldwide. Until very recently, the most common method for vaccination against coccidia in the U.S. was via liquid spray in a hatchery spray cabinet. For chicks to be properly vaccinated, they must ingest a low dose of live oocysts suspended in the spray droplets. When using a liquid spray as a diluent, there is concern that chicks don't ingest the proper dosage of oocysts post-vaccination because the vaccine solution mats into the down, making the oocysts harder to access.

Potential issues with spray application have led to the emergence of gel vaccine suspensions for coccidia. Gel diluents are novel in the US, though they have been used in other countries for some time. There are multiple manufacturers of gel diluents as well as multiple viscosities of individual gel products. When using gel diluents, oocysts remain evenly suspended within the gel, and the application of more stable "beads" of vaccine is thought to increase the available vaccine for ingestion, as well as increase the amount of time the vaccine is available on the chicks. Conversely, there is a question of evenness of application when using a gel application system, where each gel droplet may not contain the same dose of oocysts and each chick may not ingest the same number of droplets. This would lead to uneven dosing of the chicks and chicks that may be missed entirely.

Now that new methods for coccidia vaccine application are in use, research is needed to compare vaccine efficacy between application methods. This research investigated coccidia vaccinations using traditional spray and gel-drop application methods to determine if each method is efficacious and protects against challenge.

Specific Aim 3. Sequence analysis of the proposed coccidian species Eimeria mivati.

There are 7 species of *Eimeria* that are well-known to infect chickens: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*. *Eimeria* species have been defined based on phenotypic characteristics (6, 7) and morphological differences. An additional species, *E. mivati*, has been described in the literature, but its existence as a true separate species is questioned. *E. mivati* was first isolated in 1967 from a litter sample taken from a farm in Zephyr Hills, FL that was experiencing persistent coccidiosis and not responding to treatment. Edgar and Seibold determined the specimens to be a new species based on the region parasitized, the location of parasites in relation to the nuclei of parasitized epithelial cells, oocyst size, sporulation time, prepatent period, and antigenic dissimilarity, all of which were novel compared to the known species of *Eimeria* (9).

Following the discovery of *E. mivati*, many researchers performed studies with conflicting results, either confirming or refuting the existence of the species. Supporters of the new species largely based their claims on studies visualizing infection with electron microscopy (EM). Witlock and Ruff defended *E. mivati* when comparing the intestinal surface damage caused with known species of *Eimeria*, determining that the lesions caused by *E. mivati* were not as severe as those seen in *E. acervulina* infections (38). Fitz-Coy, Edgar, and Mora supported this work with more EM to compare the ultrastructure of schizonts and merozoites of *E. mitis* and *E. mivati*, showing that the differentiation of the two species was supported by the number of merozoites per schizont, the location of schizonts in host cells, and the number of parasites per epithelial cell (11). Although research by Long in 1973 contested the species identification of *E. mivati* and claimed that it should instead be called *E. acervulina var. mivati* as a variant of *E. acervulina*, Ryley and Hardeman later pointed out that the *E. mivati* sample used in that research was likely contaminated with *E.*

acervulina (24, 30). Finally, when studying the phylogenetic relationships among *Eimeria* species that infect chickens using rRNA sequences, Barta et al. recognized the similarity of *E. mitis* and *E. mivati* by placing them in their own clade, but still maintained that they were separate species (3). Conflictingly, when Vrba et al. used the 18S rRNA gene sequence of *E. mivati* identified by Barta et al. and compared it with the 18S rRNA sequence of *E. mitis*, they concluded that there are two types of the 18S rRNA gene in each genome, and both types correspond to known sequences of *E. mitis* and *E. mivati*, suggesting that there is only one species characterized by the presence of these two types of the 18S gene (35). Because of the discrepancies regarding the existence of the species *E. mivati*, further analysis of the *E. mivati* genome is needed to provide insight into its status as a distinct species or a variant strain of *Eimeria*.

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CHAPTER 2. LITERATURE REVIEW

2.1 REVIEW OF THE UNITED STATES POULTRY INDUSTRY

Development of contract farming and vertical integration

Prior to the early 1900's, poultry production as it is known today did not exist. Chickens were raised primarily for family use, with any excess of either eggs or meat being sold at local markets. However, as the century progressed, the sale of chicken and eggs became an increasingly important income source for families. With the discovery of vitamin D in the 1920's and its subsequent inclusion as a feed additive, farmers were able to raise poultry indoors year-round, dramatically increasing their production capabilities (7, 64, 95). By the 1930's, farmers nationwide were routinely selling their poultry products into a rapidly growing commercial market (257).

One of the most noteworthy early innovators of the U.S. poultry industry was Jesse Jewell, a native of Georgia who in the 1930's adapted the concept of vertical integration for use in poultry production. He sold broiler chicks and feed to farmers to grow the chickens to market age, at which time he bought them back and sold them for profit for both himself and the farmers he contracted (260). This practice would eventually become the standard for poultry production in the US (259). After World War II, the U.S. poultry industry's growth only accelerated as integrated production, combined with constantly evolving knowledge of selective breeding, disease prevention, nutrition and new technology, allowed for faster bird growth and lower costs (70, 138).

Today, the modern U.S. poultry industry is entirely vertically integrated, with large companies owning and managing hatcheries, feed mills, transportation, processing, and veterinary care. The grower is contracted to raise the chickens and/or collect the eggs in their facilities, and

receives a profit based on the chicken weight or number of eggs produced for the company (256). In order to ensure that a company's quality practices are maintained by the grower, modern broiler contracts do not guarantee that a grower will receive more chicks after the current flock has been sent to market. Instead, placement of new chicks on the farm is contingent on performance (178, 264). The success of this business model is evidenced by the fact that today, the U.S. poultry industry is a global leader for the production of meat and eggs, and the greatest exporter of poultry meat worldwide (271).

Disease evolution in commercial poultry

As poultry production evolved from small family farms to large, integrated operations, poultry disease prevalence and control shifted along with it. In the late 19th and early 20th centuries, small flocks of chickens were mainly kept as sources of meat and eggs for a single family, and were allowed free range to roam. Maladies such as chronic respiratory disease, white diarrhea, lung fever, pip, and roup were all widely known to cause disease and mortality, although the causative agents and treatment strategies were not yet identified (273). As poultry research became a growing field in the 1900's, pathogens such as *Mycoplasma gallisepticum* and *M. synoviae*, infectious laryngotracheitis virus, *Salmonella pullorum*, Newcastle disease virus, infectious bursal disease virus, and Marek's disease virus, to name a few, were characterized as the agents of common ailments of meat- and egg-type chickens (53, 73, 108, 109, 149, 150, 220, 225). Once these and other pathogens were discovered, methods for diagnosing, treating, and preventing diseases became more widespread and effective, allowing for more expansive growth of the poultry industry.

One disease that caused significant production losses in the growing poultry industry was coccidiosis. The agent was identified by Tyzzer in 1929 as a parasite, then named *Eimeria avium*, that was thought to be present in nearly every poultry farm (38). Although this parasite was ubiquitous, moving poultry production into closed houses and increasing the stocking density created further issues with *Eimeria* infection, as the higher doses of oocysts ingested by chickens escalated the pathogenicity. Johnson, another pioneer of early coccidiosis research, showed that *E. avium* was in fact multiple species of *Eimeria* that did not cross-protect, and immunity could be produced by inoculation with low doses of oocysts (39). However, even with new data characterizing the *Eimeria* species infecting chickens and knowledge about development of immunity, coccidiosis continued to be a recurring issue. A major boon for the poultry industry was the discovery that chemotherapeutics could be used as anticoccidials by P. P. Levine in 1939 (40, 209). Although chemotherapeutic treatment was initially effective at curbing coccidiosis-related production losses, their use led to parasite resistance, necessitating an alternative method for control (37). From this, ionophores, which are weak antibiotics that transport ions across cell membranes, were used as an alternative to chemotherapeutics as anticoccidial activity was produced without development of resistance (168, 236). Today, the knowledge that repeated exposure to low levels of oocysts can stimulate immunity is exploited in coccidiosis vaccines, which may be used alone to protect against the disease, or as part of a "bio-shuttle" program that also incorporates ionophores (44, 137, 152). Coccidiosis research has been ongoing over the last century and much has been learned about this disease and its prevention, however, *Eimeria* oocysts are still a constant presence in a chicken production environment, and as such, the costs of prevention, treatment, and control render this disease one of the most significant economic burdens of the U.S. poultry industry (268).

Another costly disease discovered in early research of the U.S. poultry industry was infectious bronchitis virus (IBV). IBV was first identified by Schalk and Hawn in 1931 as a "respiratory disease of baby chicks" (219). As was seen with coccidiosis, the increased stocking densities of commercially integrated poultry production, along with the saturated geographical density of poultry houses in certain regions, created a perfect storm for this highly infectious virus to devastate the industry. Infection caused major production losses in both broiler chickens and laying hens, as both respiratory signs and decreased egg laying and quality were outcomes of this disease (31). After the original characterization of the first serotype of IBV, which was named the Massachusetts (Mass) type, it was found that there were numerous antigenic types of the virus that did not provide cross-immunity (139). Vaccination was developed using live IBV attenuated through serial passage in chicken embryos (74). Although vaccination prevented production losses, constant field surveillance was necessary to detect the IBV types circulating to select the appropriate IBV type-specific vaccines needed. Today, the Arkansas (Ark) type IBV is one of the most commonly isolated serotypes in the field (61, 120). IBV is now known as the most economically important viral respiratory disease of chickens in the US and, like coccidia, this virus is ubiquitous in commercial poultry (52).

2.2 VACCINATION OF COMMERCIAL POULTRY

Introduction

One major contributor to the growth of the modern poultry industry has been the practice of vaccination as a method for disease control. Vaccination can lessen the impact of a pathogenic field challenge by protecting from mortality and reducing clinical disease, thereby preventing economic loss for the production company (1). Vaccination programs are tailored to fit the needs of production complexes in each region, and are designed to account for the climate, geographic density and type of poultry farms, market age, and the disease challenges present in the area (63). The underlying concept behind vaccination is that establishment of herd immunity will reduce the probability of an individual bird or even a flock in a heavily populated region from becoming infected with a field challenge (175). In order to be marketed for commercial use, vaccines must be efficacious, safe, affordable, and suitable for mass application (217).

Types of vaccines

Live vaccines. Mild field strains or pathogens that have been modified by attenuation are commonly used as vaccines to protect against respiratory viruses, such as Newcastle disease virus (NDV) (13), infectious bronchitis virus (IBV) (136), and infectious laryngotracheitis virus (ILTV) (86). Live vaccines stimulate local and systemic immune responses, providing a high level of protection against reinfection and long-lasting immunity (10). Because these vaccines contain live virus, there is potential for clinical disease as an outcome of vaccination if the vaccine dose is too high or if the vaccine is not adequately attenuated (72). Live vaccines are able to replicate in the host as well, creating a risk of reversion to virulence through passage in chickens if the vaccine coverage is not adequate, which is an issue seen frequently with IBV (134). Live vaccines for other

poultry pathogens are used as well, including salmonella, mycoplasmas, and coccidia to name a few (43, 45).

In addition to modified live vaccines, recombinant vectored vaccines are also used for vaccination of commercial poultry. In a recombinant vaccine, a live virus vector expresses an antigenic protein of the pathogen being vaccinated against to safely stimulate immunity against both the vector and the vectored antigen (68, 171, 206). This technique is commonly used with Fowl Pox virus (FPV) and Herpesvirus of turkeys (HVT) vectors expressing antigens for ILTV (86, 250), avian influenza virus (AIV) (194), NDV, and infectious bursal disease virus (IBDV) (12, 22, 104, 247).

Inactivated or killed vaccines. Inactivated or killed vaccines are administered to induce a local and systemic immune response against a pathogen, without the risk for replication that may cause clinical disease and reversion to virulence. However, as these vaccines are killed, they are often not highly immunogenic (102) and require administration in combination with an adjuvant and multiple inoculations to stimulate an immune response (107, 241). These vaccines are commonly used in long lived birds, including layers and breeders (97). In breeders in particular, vaccination with an inactivated vaccine is performed to induce production of antibodies that will be transmitted to the offspring and provide early protection from challenge (63).

Methods for vaccine delivery

Hatchery vaccination. The first opportunity for vaccination in the hatchery is approximately day 18 of incubation, when embryonating eggs are transferred from the incubators to the hatchers. The *in-ovo* vaccination method is used to administer vaccine to embryos as they are transferred from the incubation trays to hatcher baskets (266). This technology was developed

by Embrex in 1995, and allows for automated inoculation of embryos within the egg into the amniotic fluid (265), providing a high degree of control on the vaccination process. This practice has been applied to vaccination for IBDV, Marek's disease virus (MDV), FPV, HVT, and DNA vaccines. Vaccine efficacy is high with this delivery method, and hatchability is not reduced (82, 83, 103, 133, 210, 238).

At day of hatch, broiler chickens may be mass vaccinated using a spray cabinet after they are sorted and counted into baskets. The chick baskets move along a conveyor belt and pass through a spray cabinet, triggering application of vaccine from one or more nozzles or a gel application bar. Vaccine is evenly distributed onto the chicks, and exposure may occur either through oral ingestion, or infection of mucosal membranes via the eyes and nares (136). Spray cabinet vaccination is common for NDV, IBV, and coccidia, with the dose volume, vaccine diluent, and vaccine application method varying per the vaccine type (5, 173, 207, 255).

Field vaccination. In addition to the hatchery, vaccination can take place in the field once chickens have been placed on the farm. Chicks can be sprayed with vaccine once they are placed in the house, ingest vaccine via edible gel, in-feed, or drinking water applications, or be vaccinated via eyedrop (45, 226). Respiratory viruses such as NDV and IBV are commonly boosted in the field two weeks after primary vaccination in the hatchery to provide longer-lived immunity, and can be sprayed or applied via the oculonasal route (77, 121).

2.3 INFECTIOUS BRONCHITIS VIRUS

Infectious bronchitis virus

Avian infectious bronchitis virus (IBV) is a gammacoronavirus that causes infectious bronchitis (IB), an economically significant respiratory disease of commercial chickens (30, 253). In 1931, IB was reported by Schalk and Hawn as the first respiratory disease of chickens in the US. By 1936, the IBV Mass serotype had been identified as the causative agent of this "respiratory disease of baby chicks" (74, 119). Production losses as a consequence of IB include mortality, reduced egg output and quality, reduced weight gain, and condemnations at processing (136).

Disease

IBV is present worldwide (31, 61), and infection often results in upper-respiratory tract disease. Morbidity is nearly 100%, although mortality is usually low in the absence of secondary infections (52). Clinical signs are more severe in young chickens, and often present 5-7 days post-infection with nasal discharge, snicking, tracheal rales, gasping, watery eyes, swollen sinuses, and depression (217). In laying hens, infection of the oviduct can result in reduced egg production and poor egg quality (224).

Initial infection typically occurs in the mucosal membranes of the upper-respiratory tract. Once viremia occurs, the virus will disseminate to other susceptible tissues, including epithelial cells of the reproductive tract, kidneys, and the enteric system (3). Some IBV strains replicate in the proximal and distal convoluted and collecting tubules and collecting ducts of the kidneys, and this causes an increase in mortality (47, 153, 184). IBV has been shown to persist in long-lived chickens, and it has been postulated that the virus persists in the tubular epithelium of the kidneys and in the gut tissues (3, 67, 135).

Molecular and biological characteristics of IBV

IBV is a gammacoronavirus of the family Coronaviridae and order Nidovirales (96). There are four groups of coronaviruses: Alpha (Group I), Beta (Group II), Gamma (Group III), and Delta (Group IV) (76, 147). The 80-100 nm virion is enveloped and pleomorphic, with numerous spike glycoproteins present on the cell surface. IBV is thermo- and ether labile, sensitive to disinfectants, and unstable in warm temperatures (248).

IBV has a positive sense single-stranded RNA genome that is approximately 28 Kb, with 10 open reading frames (162). The genome encodes for a polyprotein 1a and 1ab and four structural proteins: spike, envelope, membrane, and nucleocapsid. Polyprotein 1a and 1ab make up the replication transcription complex, in addition to having a role in viral pathogenesis (23, 98). The polyproteins are post-translationally cleaved by a papain-like protease into 15 non-specific proteins, nsp2-16 (202, 278). The nsps influence virus replication, pathogenicity, attenuation, and code for the RNA-dependent RNA polymerase (8, 54, 75, 112, 117, 187, 205, 251, 277).

The four IBV structural proteins, spike, envelope, membrane, and nucleocapsid, are produced during viral replication from a 5' nested set of subgenomic mRNAs. The spike glycoprotein is responsible for attachment to the host cell and is the major antigen of IBV (141, 190). Because of its role in attachment, spike is one of the major determinants of IBV tissue tropism and therefore plays a role in viral pathogenicity (4, 110, 113, 161). Spike is post-translationally cleaved into two subunits, S1 and S2 (174). S1 contains epitopes that induce neutralizing antibodies, and participates in attachment to receptors on the host cell (33, 143, 204, 222), while S2 is involved in fusion of the virus and host membranes (58, 101, 172, 234, 246). The envelope and membrane proteins interact to facilitate virus budding and assembly (159) and the

nucleocapsid protein is a regulator of virus replication and transcription and viral assembly by regulation of the viral and host cell processes (71, 100, 114, 127, 144, 145).

Virus replication begins with attachment to the host cell receptors via the spike glycoprotein (29). Exposure to acidic pH in endosomes causes rearrangement of the spike protein, initiating membrane fusion by S2 to release the genome into the cytoplasm (262). Transcription of the genomic RNA produces gene 1, the polyproteins 1a and 1ab, which are translated and assemble into the replication transcription complex, including the RdRp responsible for transcription of the nested mRNAs (147). The viral structural proteins S, E, and M are inserted into the endoplasmic reticulum (ER) and are directed by the M protein to accumulate in the Golgi complex. The N protein encapsidates the viral genome, and the E protein is involved in budding (59). Virions are transported to the cell surface in vesicles and released by exocytosis or cell lysis (76).

Immune response

Upon infection with IBV, inflammation triggers the recruitment of heterophils to the site of infection, however, this innate response does not limit virus replication (140) and may contribute to tracheal epithelial cell damage (66, 81). IBV-specific neutralizing antibodies are produced after exposure to the S1 domain of the spike glycoprotein (141, 143), though the S2 and N proteins also have epitopes for inducing cross-reactive antibodies (115, 116). Immediately after infection, IgA production is stimulated in the mucosal membranes (46). IgM is produced up to 8 days post-infection in the serum, and therefore may be used to diagnose acute infection (62). Up to 21 days post infection, IgG may be detected in the serum (186). Although the humoral response to IBV infection is strong and antibody titers may be used to detect exposure and vaccine uptake, it has not been shown to aid in recovery from virus (51). Rather, the humoral response provides

resistance to reinfection via IBV-specific antibodies protecting the tracheal epithelium and preventing viremia (208). Maternal antibodies also provide short-lived protection of chicks after hatch (51, 216).

An important immune response to IBV infection is the cellular immune response (49, 235). Antigen-specific proliferation of T-lymphocytes mediates immunity in the trachea and limit virus replication in the kidney (125). Th1 cytokines TGF, IL-2, and IFN-gamma are detected in the trachea and lung following infection (203). IBV can cause persistent infection, and suppression of cellular immunity will result in re-excretion of the virus (16).

IBV serotypes

When IBV was initially discovered in the 1930's, the Mass type IBV was the only known serotype. Today there are over 20 serotypes and many variants. Serotyping of IBV is based on the spike glycoprotein, which contains epitopes that induce neutralizing antibodies (34). Changes in the spike gene may result in a change in the amino acid sequence of the protein, resulting in a different epitope presentation. Novel epitopes will then induce a different antibody response. There is little cross reaction between serotypes, and new variants appear frequently due to insertions, deletions, and mutations caused by a viral polymerase with poor fidelity and limited proof-reading capabilities, and from recombination that may occur when multiple IBV types infect the same cell (32, 105, 106, 123, 146, 176). In the US, the most common IBV types detected are Ark, Delaware, Conn, and Mass (90, 120). There are also numerous California variants that have been detected since the 1990's, including CAL/CAL99/99, CA/557/03, and CA/1737/04 (84, 123, 188, 189, 221), and many Georgia variants as well, including GA98, which is related to DE072, GA07, GA08, GA/124/11, and GA13 (119, 122, 151, 244). In addition, there are multiple

nephropathogenic IBV strains, including Delmarva strain DMV/1639/11 and some Pennsylvania strains (91, 279).

Vaccines

IBV is controlled through vaccination with a vaccine serotype that is homologous to the field challenge, as there is little to no cross protection between individual serotypes (31). The most commonly used vaccines for IBV are modified live field isolates that have been attenuated through serial passage in embryonating eggs, although there are alternative methods for attenuation that have been studied (89, 122, 217). During repeated rounds of embryo passage, a pathogenic field virus that is highly adapted to infection in chickens will accumulate mutations that result in a higher tropism for embryo infection and replication. Conversely, the outcome of this adaptation is a decreased ability to infect and/or replicate in chicken tissues and therefore a reduced virulence in chickens (17, 24, 74). The first live attenuated IBV vaccine in the USA was produced in the 1950s for the Mass serotype (52). Live attenuated vaccines stimulate both humoral and cellular immunity, resulting in high levels of protection, and can be mass applied by spray (136, 217). Immunity resulting from vaccination with live attenuated IBV vaccines prevents replication of homologous virulent challenge virus within only a short time following vaccination (217). When administered properly, protection may last until 9 weeks post-vaccination, so repeated vaccine boosts are administered in the field in longer lived birds (31). Although live vaccines for IBV confer good protection, there is risk involved, as improper vaccination of chicks can result in back passage of the virus in non-vaccinated birds, creating potential for a reversion to virulence (111, 182). Another concern with the usage of live attenuated vaccines is recombination events between

vaccine serotypes. It is a common practice to give multiple serotypes of IBV in one vaccination (136), providing an opportunity for those viruses to recombine and produce variants (119, 183).

In addition to live attenuated vaccines, there are inactivated oil-emulsion IBV vaccines available that are often used for breeder and layer chickens to maintain circulating IBV antibody titers through the life of the flock. Maintaining antibody titers will help prevent a drop in egg production from IBV (21, 126, 241) as well as promote maternal antibody passage to offspring. These vaccines are inactivated by heat or chemical treatment that renders the virus unable to replicate while leaving the virion and antigen intact to stimulate an immune response. Because there is no replication, the risk for reversion to virulence is not present, however, this lack of replication also reduces the immunogenicity of the vaccine, and therefore an adjuvant must be applied with the vaccine to ensure the proper immune response is generated (10). Often, birds are primed with 2 to 3 inoculations of live vaccine, followed by an inactivated vaccine injected intramuscularly prior to the onset of lay to ensure complete protection (20, 28).

Diagnosis

Diagnosis of IBV can be performed using serological or molecular techniques. The classical method for diagnosis is via virus isolation in 9-11 day-old embryonating chicken eggs. Swabs are taken from the choanal cleft palate, trachea, or cloaca of infected chickens and placed into phosphate buffered saline. Tissue samples from the trachea, conjunctiva, kidney and/or cecal tonsils can also be taken for the same purpose. The samples are then used for inoculation of embryonating eggs via the chorioallantoic sac route. Embryo mortality is monitored, and seven days post-inoculation, IBV-specific embryo lesions are recorded such as stunting, hemorrhage, curled embryos, and urate deposits in the kidneys (217). Serologic methods such as virus

neutralization, hemagglutination inhibition, ELISA, and the agar gel precipitation test are used to monitor for the presence of IBV-specific antibodies and to determine serotype (60). In addition, IBV can be detected molecularly using RT-PCR or qRT-PCR to detect the RNA of the S1 sequence (27, 35, 124, 142).

2.4 INFECTIOUS BRONCHITIS VIRUS: ARKANSAS SEROTYPE

IBV Arkansas Serotype

Although there are numerous IBV serotypes circulating in the field, Arkansas (Ark) type IBV isolates are detected more frequently than any other type in the US, and new Ark variants continue to emerge that can be highly pathogenic (9, 65, 88, 120, 131, 195, 218, 245). Within the Arkansas clade, there are many Ark-like viruses that show high genetic variation, and this may be a reason for the persistence of Ark vaccines in broiler flocks (182).

Arkansas 99 Vaccine

The Arkansas 99 (Ark99) IBV field isolate was detected in 1971 from vaccinated commercial flocks in Arkansas showing respiratory signs. Tissue samples were submitted for serotyping, and cross-neutralization tests against known IBV strains concluded that this serotype was novel. Testing was performed using antisera prepared from field sample 99, resulting in the nomenclature "Arkansas 99" (78). This serotype continued to be detected in the field (132), and commercially available IBV vaccines did not provide protection from the Ark99 IBV isolate (92). A vaccine was developed for commercial use from this Ark99 isolate and it was protective against challenge, however, when used on one day-old broiler chickens, it produced severe clinical signs and exacerbated air sacculitis caused by secondary infection (111).

Arkansas DPI Vaccine

The Arkansas Delmarva Poultry Industry (ArkDPI) IBV strain was isolated and attenuated in the 1980's by serial passage in embryos (4, 25, 89). The ArkDPI vaccine showed reduced pathogenicity compared to Ark99, and was determined to be protective against homologous

challenge (87), leading to its replacement of Ark99 as the predominantly used Ark-type IBV vaccine. However, continued use of the ArkDPI vaccine in the field has highlighted some of its shortcomings. Although the ArkDPI vaccine infects and replicates well in broiler chicks when applied by eyedrop, when applying the vaccine by mass spray, as is required in a commercial setting, vaccine efficacy and protection from challenge are not achieved (212). In fact, it has been shown by Leyson et al. that a 100x dose of sprayed vaccine is required to achieve the same efficacy as an eyedrop dose, which is not consistent with other IBV serotype vaccines (156). Furthermore, because this vaccine does not provide adequate coverage when administered by spray, a rolling vaccine reaction is seen in broilers in the field as the vaccine passes from vaccinated to non-vaccinated birds, resulting in persistence of the vaccine and creating potential for reversion to virulence (121, 193).

This inadequate vaccine efficacy and persistence of ArkDPI in the field is thought to be due to the presence of virus subpopulations in the vaccine. Although subpopulations are not uncommon with IBV (84), the subpopulations within the ArkDPI vaccine impact its ability to infect and replicate in chickens (192, 195, 243). Within the ArkDPI vaccine, there is a subpopulation that is capable of efficient infection in chickens (212). However, it is a minor population in the vaccine and has a spike gene sequence that is different from the consensus spike gene sequence in the vaccine (85, 252), and the dose administered to chicks out of the entire vaccine population is very low (156). A substitution found in vaccine subpopulations in the S1 spike protein at amino acid position 43 from a tyrosine to a histidine, and a deletion amino acid position 344 (182), have been shown to alter the tropism and antigenicity of the ArkDPI virus (154, 155). Research has been performed to improve the ArkDPI vaccine and reduce subpopulations by adapting the virus to growth in chicken embryo kidney cells (93, 94, 276).

However, this new CEK-adapted IBV ArkDPI-derive vaccine is not yet commercially viable, and the poultry industry is in immediate need of an efficacious and protective Arkansas serotype IBV vaccine.

2.5 COCCIDIOSIS

Introduction

Coccidiosis is an economically significant disease of commercial poultry. The disease is caused by infection with *Eimeria*, and the annual worldwide cost of coccidia challenge, treatment, and control is estimated to be at least 3 billion U.S. dollars (19, 268). There are seven species of *Eimeria* known to infect chickens: *E. acervulina, E. maxima, E. tenella, E. brunetti, E. necatrix, E. mitis,* and *E. praecox*. There are two additional proposed species, *E. mivati* and *E. hagani*, that have been described in the literature but are contested as true species. Ingestion of sporulated oocysts from the environment leads to enteric disease in chickens (50), resulting in reduced body weight gain, reduced feed conversion, and in severe cases, mortality. Each of the species of *Eimeria* that infect chickens preferentially infects different regions of the intestinal tract, causing lesions such as thickening of the intestinal wall, bloating of the intestines, petechial hemorrhage, increased intestinal mucous, and necrosis (217). Furthermore, infection with *E. maxima* is thought to be a predisposing factor for necrotic enteritis caused by *Clostridium perfringens* infection (240).

Life Cycle

There are three basic stages of the life cycle of coccidian parasites. The first two, merogony and gamogony, are endogenous, while the final stage, sporogony, is exogenous. When a sporulated oocyst is ingested by a susceptible host, mechanical and enzymatic digestion in the GI tract results in excystation of the sporozoites, which are the infective stage of coccidian parasites. Sporozoites are motile and move by gliding motility. After recognition of target epithelial cells, penetration is achieved by organelles in the apical complex. Once inside of the target cell, a parasitophorous vacuole is formed around the sporozoite, inside of which the sporozoite will begin merogony.

Merogony is the asexual stage of parasite replication, and depending on the parasite, merogony can result in anywhere from 2-100,000 merozoites formed from a single sporozoite. Once mature, merozoites rupture the host cell to penetrate new epithelial cells and begin further generations of merogony. The number of asexual replication cycles that will occur are dependent on the parasitic species. Increasing numbers of merogony generations leads to the development of pathologic lesions (42, 160, 217). After the final round of asexual replication, merozoites enter epithelial cells and transform into gamonts.

During gamogony, either macro- or microgametocytes can be formed. Microgametocytes mature to produce motile microgametes, which fertilize macrogametes producing a zygote that develops into an oocyst. For most coccidian parasites, oocysts rupture the host cell and exit the host through the feces.

The final stage, sporogony, occurs outside of the host. Under appropriate conditions, the immature oocyst undergoes sporulation to form sporocysts containing sporozoites, at which point it is mature and infective to a new susceptible host (15, 217, 263).

Immune response

The chicken immune response to *Eimeria* infection is primarily cell mediated (214, 237). Upon infection, both humoral and cell mediated responses are activated, but the cell mediated response is responsible for development of immunity (6). IgA and IgM are the predominant immunoglobulins secreted during coccidia infection (258), but the ability to limit infection is minimal (275). This was demonstrated when chickens were treated to reduce T cell proliferation and *Eimeria* susceptibility was increased, even with enhanced IgA and IgG coccidia-specific

responses (157). Conversely, bursectomized chickens are resistant to reinfection with *Eimeria* (275).

During primary infection, exposure to newly excysted sporozoites results in inflammation, lymphocyte infiltration, and increased mucous production in the gut. CD4+ T cells recognize APC's in association with MHC class II signaling. The Th1 immune response predominates, and IFN-γ, IL-2, and TNF-β are secreted and activate macrophages, NK cells, and CD8+ cytotoxic T lymphocytes (157). NK cells and macrophages participate in sporozoite destruction (213). Upon reinfection, CD8+ T cells are stimulated and stop parasite proliferation by recognizing infected epithelial cells through MHC class I signaling and destroying them (185). In addition, specific antibodies in the intestinal mucosa act on sporozoites during reinfection by blocking invasion of epithelial cells and enhancing sporozoite destruction in the lumen (158). Protection is species-specific, and immunogenicity differs between the species (79, 177, 214). The primary protective antigen of *Eimeria* is not known, as there are multiple antigens that stimulate humoral and cellular immune responses throughout the stages of infection, including the surface antigen glycoprotein, apical membrane antigen-1, microneme protein, and rhoptry proteins (18, 48, 99, 129, 148, 179, 199, 239, 242, 274).

Control by vaccination

Historically, control of the disease has been managed by chemical anticoccidials and later ionophores as emergence of drug resistant strains of *Eimeria* became prevalent (41). Although ionophore use has shown to be an effective method for management of coccidiosis, FDA restrictions on antibiotic use in agriculture necessitate a different method for disease control (233). Vaccination against coccidiosis has been performed since the mid 1900's, and with decreased

anticoccidial use it has become a primary method for management of this disease (137, 270). Currently, the method for vaccination against coccidia is with the use of live, sporulated oocysts given in a low dose (19). Protection is species-specific (207), and vaccines for broiler chickens contain *E. maxima*, *E. acervulina*, and *E. tenella* in addition to other species that vary based on the pharmaceutical company. Vaccines are given to day-old chickens in a low dose to stimulate immunity, and repeated exposure to oocysts that are shed in the litter and re-ingested will generate a protective cellular immune response (55, 137, 214). To prevent clinical disease following vaccination, highly pathogenic species of *Eimeria* are attenuated for vaccine use, including *E. maxima*. The most widely used method for attenuation is selection of precocious *Eimeria* oocysts that have a shorter prepatent period and a lower fecundity than the wild-type oocysts, producing less cycles of asexual replication in the intestinal epithelium and reducing pathogenicity while still remaining immunogenic (229, 267).

The goal of vaccination with live coccidia vaccines is to mimic a natural infection route, resulting in sporozoite infection of the intestinal epithelial cells. Coccidia vaccines were first administered by adding an oocyst suspension to drinking water (230). It was later found that delivery of oocysts through the eye produced adequate infection as they were transported through the sinuses into the GI tract (57). This led to the use of hatchery spray cabinets for vaccination, as they were already in use for delivery of viral vaccines (43). Spray cabinets are still the most common method for coccidia vaccine administration, allowing for both direct infection through the eye and ingestion of oocysts through preening, which is encouraged by the addition of a dye to the vaccine diluent (26, 269). However, a concern with spray vaccination is a lack of uniform application that may lead to inadequate immunity (43). Gel vaccination techniques were prostrated as a solution to this, initially with slices of gel containing oocysts that were placed in hatch baskets

for chicks to eat as they were waiting for transport to the farm (56). This was followed by gel beads added to the feed on the farm. Both methods of gel vaccination were found to be more efficacious than aqueous spray, resulting in more consistent dosage of oocysts (128). However, use of a spray cabinet has the advantage of rapid, controlled mass vaccination in the hatchery, which both of these gel methods were lacking. Recently, new gel vaccination techniques that administer the vaccine in a gel diluent have become more widely used. Chicks in a basket pass through a cabinet with a gel applicator bar that drops large beads of gel onto the down. This method for vaccination has been shown to be protective (211). However, further research is needed to compare the efficacy and protection of this this new gel vaccination method with the more traditional aqueous spray.

2.6 COCCIDIOSIS: *EIMERIA MIVATI*

Early Isolation and Species Identification

E. mivati was described as a new coccidium of chickens by Edgar and Siebold in 1964. The species, first isolated from a litter sample from a poultry farm in Zephyr Hills, FL in 1959, was described as an unusual and persistent coccidiosis problem. The name *mivati* refers to a Sanskrit word meaning to move or change, and was chosen because changes in location occur during endogenous development in the host and second generation merozoites were noted to move from the duodenal loop, which was the initial site of infection (272), to complete replication in the lower small intestine. Infected regions of the intestine were reddened, swollen, and showed some bloody contents and white plaque-like lesions. Oocysts shed were ellipsoid to broadly ovoid and averaged 15.6 x 13.4 microns. Mild infection did not produce any distinct lesions, although severely affected chickens showed listlessness, anorexia, ruffled feathers, and watery diarrhea, with potential mortality on days 6-7 post-infection (130). The authors addressed the potential confusion with the other small oocyst species, *E. acervulina* and *E. mitis*, by stating that the *E. mivati* oocyst was smaller than both and completed more schizogonous cycles than either species. *E. mivati* also had a different schizont size (164) and location relative to nuclei of parasitized epithelial cells differs, in addition to a distinct developmental pattern of the macrogamete (261). Furthermore, *E. mivati* had a shorter prepatent period, shorter minimum sporulation time, and was more pathogenic than *E. mitis* (249), with *E. acervulina* being more pathogenic than either species and producing distinct intestinal lesions (69, 191, 272). *E. mivati* was also noted to be maintained by passage in chicken embryos (165, 169), which is unique to it and *E. tenella* (166).

Redaction of the Species

In 1973, Long suggested that *E. mivati* should be regarded as a variant of *E. acervulina.* This occurred after a re-examination of the life cycle of both species, as he found that both species had 4 generations of schizogony within the first 4 days of infection. Furthermore, both species were capable of infection of the ceca and in the chorioallantoic membrane of chicken embryos, although only *E. mivati* could carry out a full replication cycle to produce oocysts (167, 232). This claim was contested by Ryley, who showed that the two species could not interbreed, so they must be distinct (215). 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 actually contaminated with *E. acervulina*. The culture studied was then purified of *E. acervulina* via passage in embryonating eggs to produce only *E. mivati* oocysts (198). This monoculture was confirmed through challenge studies that showed a lack of cross protection between the two species and a noted difference in pathogenicity produced from infection with either species (197, 227, 228).

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*, and after examining parameters such as oocyst dimensions, absence of gross lesions, and cross-immunity, they concluded that *E. mivati* was not a species (231). There was still doubt regarding *E. mivati*'s status as a distinct species continuing into the 1990's, when a supposed monoculture strain *E. mivati* was used in drug and vaccine trials (14, 36, 118, 168, 170, 200), but simultaneously other researchers were claiming to be using *E.mivati*/*E. mitis* and did not consider the two species to be distinct (163, 180, 181, 196). One proponent of *E. mivati* as a true 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 (80).

Molecular Analysis

Modern analysis of *Eimeria* species allows for molecular characterization of species in addition to gross and microscopic examination. Initially, species differentiation was based on variations in the 18S ribosomal DNA sequence. In 1997, *E. mitis* and *E. mivati* were found to form a clade based on 18S sequences, but their status as separate species was maintained (11). This was still supported in 2009, when "*E. mivati*-like" and *E. mitis* oocysts were detected on broiler farms in North Carolina based on 18S sequences. However, this distinction was dubious, as the same sequencing picked up both species based on 18S analysis, but only one or the other based on mitochondrial cytochrome C oxidase subunit I (COI) and ribosomal internal transcribed spacer region-1 and -2 sequences (223). In 2011, species differentiation based on COI sequence was found to be more reliable than differentiation using 18S (201). That same year, *E. mitis* was discovered to have two types of 18S RNA, one of which matched with what was previously attributed to *E. mivati* sequence, supporting the theory that *E. mivati* is a variant of *E. mitis* and not a species (254). However, *E. mivati* is still currently identified as a species with its own species-specific lesions internationally (2). This conflicting data about *E. mivati* necessitates further characterization of the genome to provide insight into the relationship with *E. mitis*, and the status of *E. mivati* as either an independent species or variant strain of *Eimeria*.

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CHAPTER 3

ATTENUATION CHARACTERISTICS OF A NOVEL ARKANSAS SEROTYPE INFECTIOUS BRONCHITIS VACCINE THAT IS PROTECTIVE AGAINST CHALLENGE[1]

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ABSTRACT

Almost all commercial poultry are vaccinated against avian coronavirus infectious bronchitis virus (IBV) using live attenuated vaccines mass administered by spray in the hatchery. Although many different types of IBV vaccines are used successfully, the ArkDPI serotype vaccine, when applied by spray, does not infect and replicate predictably like other IBV vaccine serotypes. Because efforts to improve the efficacy of ArkDPI vaccines applied by spray have been unsuccessful, we chose to examine a previous commercial Ark99 vaccine strain for use in a hatchery spray cabinet. Use of the Ark99 vaccine strain was discontinued due to the reactivity, as expressed by mild to moderate disease caused by the vaccine in the host. Further attenuation of the Ark99 vaccine was achieved by passage in embryonated eggs to reduce reactivity. During the attenuation process, egg passages 1, 20, 40, and 60 (designated ArkGA after P1) were sequenced using complete genome Illumina sequencing to identify genetic changes occurring during the attenuation process. Through passaging, the ArkGA vaccine accumulated single nucleotide polymorphisms (SNPs) in regions of the genome associated with viral replication, pathogenicity, and cell tropism, and the genetic population became more stable and homologous. Subsequent egg passages were also examined for infection, replication and attenuation in one-day old broiler chicks. ArkGA P1, P20, and P40 were deemed too reactive and not suitable as a vaccine candidate, but ArkGA P60 appeared to be safe and efficacious when given by spray to broiler chicks, with little or no clinical signs observed. The vaccine also induced good protection from clinical signs and ciliostasis and significantly reduced viral load following homologous challenge compared to non-vaccinated controls as determined by qRT-PCR and virus isolation. These results indicate that the ArkGA P60 vaccine is safe for spray vaccination of broiler chicks and induces suitable protection against challenge with homologous Ark-type virus.

Key words: infectious bronchitis virus, Arkansas DPI serotype vaccine, Arkansas GA serotype vaccine

Abbreviations: Ark99 = Arkansas 99; ArkDPI = Arkansas Delmarva Poultry Industry; ArkGA = Arkansas Georgia; CAS = chorioallantoic sac; C_T = cycle threshold; EID_{50} = 50% embryo infective dose; IBV = infectious bronchitis virus; P = passage; PBS = phosphate-buffered saline; qRT-PCR = quantitative real-time reverse-transcriptase polymerase chain reaction; SPF = specific-pathogen free; U.S. = United States; USDA = United States Department of Agriculture

INTRODUCTION

Avian infectious bronchitis virus (IBV) is a gammacoronavirus that causes an economically significant upper respiratory tract disease in chickens (1). Because of its prevalence and infectivity, nearly all commercial poultry in the U.S. are vaccinated for IBV in a serotype-specific manner (4, 13). Of the vaccines used in the U.S., the Arkansas Delmarva Poultry Industry (ArkDPI) serotype vaccine has been shown to be highly variable in its protective ability, and is frequently isolated from vaccinated chicks (9, 17, 23). It has been shown that the ArkDPI vaccine has an atypical infection and replication pattern when mass applied by spray, and previous data from our laboratory suggests that levels of vaccine virus infection post-vaccination only reach 15-25% (10). Multiple replication cycles also occur in the bird (indicated by viral load in chicks), resulting in "rolling" reactions at different time points post-vaccination. Our work has shown that to achieve an adequate infection rate with ArkDPI post-vaccination and eliminate rolling replication cycles, a 100x dose is required (16, 20).

The atypical infection and cycling observed following ArkDPI vaccination is a result of multiple minor genetic subpopulations in the vaccine bottle. It has been previously shown that several serotypes of IBV vaccines contain genetic subpopulations. The subpopulations are often recovered in chickens following vaccination, even though the vaccines show a typical infection and replication cycle and protect from challenge. With ArkDPI, the major population in the vaccine contains multiple, distinct amino acid changes in the spike protein compared to subpopulations, namely positions 43 and 344, that have been shown to be directly involved in spike protein binding to host tissues as well as being implicated in the development of immunity after vaccination (15, 17). The ArkDPI vaccine major population contains amino acids at these positions in spike that increase binding affinity in the embryonated egg, but decrease binding affinity to mature chicken

cells. This allows the minor populations to infect and replicate in chickens (15, 24), but they are only a fraction of the total genetic population contained in the vaccine bottle. Thus, the infection rate is very low and the time to reach peak infection and replication is delayed (16). For these reasons, chickens do not develop adequate immunity following ArkDPI vaccination. Although using one of the viral subpopulations directly as a vaccine will induce a protective immune response, these populations cannot be maintained through multiple passages in embryonated chicken eggs, which is required to propagate IBV vaccine.

While ArkDPI is the only commercially available Ark-type IBV vaccine today, it is not the only Ark-type IBV vaccine ever produced. The Arkansas 99 (Ark99) strain was the first Ark-type virus to be attenuated for use as a vaccine. When originally mass applied in the field, it caused a severe vaccine reaction in young broilers, and was therefore discontinued when ArkDPI was developed (8, 12). The purpose of this trial was to confirm the reactivity of the original Ark99 vaccine when administered to 1-day old broilers by spray, followed by evaluation of infection, replication, and protection from challenge of further embryo passages. We also investigated the mechanisms of attenuation of this vaccine during the subsequent embryo passages. This study led to development of a new, more attenuated vaccine designated Arkansas Georgia (ArkGA).

MATERIALS AND METHODS

Vaccine and challenge viruses. Ark99 vaccine is no longer produced nor USDA license maintained by any vaccine manufacturer. An archived reference sample of live Ark99 vaccine was obtained from a commercial source and passaged once in 9-to-11 days of incubation specific-pathogen free (SPF) chicken embryos as described below. The University of Georgia egg-passaged virus, now designated ArkGA, was used for further experimentation. Different egg passages, beginning at egg passage 1 (P1) and going to P60, were used in this study for consecutive experiments. A pathogenic Arkansas serotype challenge virus from our laboratory was also used in this study.

Embryonated chicken eggs and chickens. Specific-pathogen free (SPF) embryonated chicken eggs were purchased from Charles River Laboratories (North Franklin, CT) and incubated to 9-to-11 days of development for use in virus passage, titration, and isolation experiments. Commercial non-vaccinated broiler chickens were used in the vaccination experiments as described below.

Virus attenuation. ArkGA was serially passaged 60 times by inoculating 9-to-11-day-old embryonated chicken eggs via the chorioallantoic sac (CAS) route (7). Inoculated eggs were incubated at 37C for 48 hours, at which point the chorioallantoic fluid was collected for subsequent passage. At every 20^{th} passage the S1 portion of the spike gene was sequenced to detect any mutations.

Virus titration. Viruses were titrated at different egg passage levels using the following protocol: 10-fold serial dilutions of the virus were made in sterile deionized water and each dilution was inoculated into five 10-day-old embryonated SPF chicken eggs (0.1 ml/egg). Inoculated eggs were incubated at 37C for 7-days and embryos were examined for IBV-specific lesions. Embryo mortality within 24-hours post-inoculation was considered nonspecific and not included in virus titer calculations. Virus titers were calculated by the method of Reed and Muench (19) and expressed as the 50% embryo infectious dose (EID_{50}).

Experiment 1: Safety testing of ArkGA. Thirty 1-day-old SPF chicks were vaccinated via the oculonasal route with a dose 10x greater than commercial IBV vaccine manufacturer recommendations of ArkGA P1 vaccine and placed into Horsfal isolation units. Twelve chicks remained non-vaccinated as controls. At 5, 7, and 10 days post-vaccination, all chicks were swabbed in the choanal cleft, and swabs were placed in deionized water and stored at -80C until used for qRT-PCR quantification. In addition, 5 chicks at each swab time point were euthanized and tracheas were collected for ciliostasis scoring via the method described below to evaluate vaccine safety as described by European Pharmacopoeia guidelines (6). Two non-vaccinated chicks were also euthanized at each time point and ciliostasis was evaluated for comparison. At each time point, clinical signs, such as snicks and rales, were recorded.

Experiment 2: Evaluation of infection and replication of ArkGA P1 and protection from challenge. One hundred one-day-old broiler chicks were spray vaccinated with the ArkGA P1 vaccine candidate in a 7ml volume and placed into an isolation house on fresh litter. Ten additional non-vaccinated chicks were placed in Horsfal-Bauer isolation units as controls. At 7, 10, 14, 17,

21, 24, and 28 days post-vaccination, all vaccinated chicks were swabbed in the choanal cleft for qRT-PCR analysis of viral load as previously described. Clinical signs corresponding to vaccine reactions were also recorded on those days. On day 30 post-vaccination, 20 vaccinated and 5 non-vaccinated chickens were challenged with pathogenic Ark-type IBV, while an additional 5 vaccinated and 5 non-vaccinated chickens were held as non-challenged controls. Five days post-challenge, clinical signs were recorded and all chickens were swabbed and euthanized for necropsy. Tracheas were collected at necropsy for ciliostasis scoring.

Experiment 3: Evaluation of infection and replication of ArkGA P20, P40, and P60 vaccine candidates and protection from challenge. Experiment 3 was carried out in the same manner as Experiment 2 for ArkGA P20, P40, and P60 vaccine candidates.

Trial 1. A severe vaccine reaction as evaluated by the presence of clinical signs in broiler chickens caused by the ArkGA P1 vaccine candidate necessitated further virus attenuation by 19 additional embryonated egg passages, yielding the ArkGA P20 vaccine candidate. One hundred one-day-old broiler chicks were spray vaccinated with the ArkGA P1 vaccine candidate in an 18ml volume and placed in a colony type housing unit on fresh litter. Ten additional non-vaccinated chicks were placed in isolators as controls. Swabs were taken at 3, 5, 7, 10, and 14 days post-vaccination to assess viral load and vaccine coverage in chicks, and clinical signs were recorded.

Trial 2. The ArkGA P20 vaccine candidate was passaged an additional 20 times in embryonated eggs to produce the ArkGA P40 vaccine candidate and another vaccination trial was conducted as described in Trial 1.

Trial 3. The ArkGA P40 vaccine candidate was passaged an additional 20 times in embryonated eggs to further attenuate the virus, producing ArkGA P60. One hundred one-day-old

broiler chicks were spray vaccinated with the ArkGA P60 vaccine candidate in an 18ml volume and placed in an isolation house on fresh litter. Ten additional non-vaccinated chicks were placed in isolation units as controls. At 3, 5, 7, 10, 14, 17, 21, 24, and 28 days post-vaccination, all vaccinated chicks were swabbed in the choanal cleft for qRT-PCR analysis of viral load as previously described. Clinical signs corresponding to vaccine reactions were also recorded on those days. On day 30 post-vaccination, 20 vaccinated and 5 non-vaccinated chickens were challenged with pathogenic Ark-type IBV, while an additional 5 vaccinated and 5 non-vaccinated chickens were held non-challenged as controls. Five days post-challenge, clinical signs were recorded and all chickens were swabbed in the choanal cleft palate and euthanized for necropsy. Tracheas were collected at necropsy for ciliostasis scoring.

Virus detection using quantitative real-time RT-PCR. Viral RNA was extracted from 50µl of choanal swab fluid using the MagMAX-96 RNA Isolation Kit (Ambion Inc., Austin TX) on a KingFisher Flex magnetic particle processor (Thermo Scientific, Waltham, MA) per the manufacturer's protocol. Quantitative real-time RT-PCR (qRT-PCR) was conducted using an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA) and the AgPath-ID™ One-Step RT-PCR kit (Ambion Inc.) per the manufacturer's recommendations. Primers and probe for the qRT-PCR were previously published (3) and consist of a forward primer IBV5'GU391 (5'-GCT TTT GAG CCT AGC GTT-3'), a reverse primer IBV5'GL533 (5'-GCC ATG TTG TCA CTG TCT ATT G-3') and a Taqman® dual-labeled probe IBV5'G probe (5' – FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3'). Cycle-threshold (C_T) values above the limit of detection for the assay were considered negative (21). All positive samples were used to determine the total percent positive for each group.

Ciliostasis scoring. The ciliostasis scoring was conducted by examining five rings approximately 1mm thick cut from each chicken trachea representing the proximal, middle and distal portion. Cilia activity was observed with an inverted microscope (Olympus, Center Valley, PA) and scored as: 0, all cilia beating; 1, 75% of cilia beating; 2, 50% of cilia beating; 3, 25% of cilia beating; 4, no cilia beating. Each ring was scored by 3 individuals and the average total score for each trachea was calculated using the formula: 100 - [(total of the individual scores for the group) / (the number of individuals in the group X 20) X 100]. An individual bird is considered protected if the score is greater than 50 (5).

Challenge virus detection in embryonated eggs. Routine virus isolation techniques were used for detection of IBV challenge virus in 9-to-11 days of incubation embryonated SPF chicken eggs. Briefly, 2 ml of ice-cold PBS were added to the choanal swab fluid to match the stipulations of the U.S. Code of Federal Regulations, title IX (9-CFR) (22). PBS from the swabs was filter sterilized and 0.2 ml of each sample were inoculated into the chorioallantoic sac of 6 embryonated chicken eggs. Eggs were candled daily (24-72 hour deaths were discarded) for 7 days and the number of deaths and embryo lesions consistent with IBV infection was recorded.

Sequence analysis of the S1 gene. The S1 portion of the ArkGA spike gene from passages 1, 20, 40, and 60 was amplified and sequenced. Briefly, viral RNA was purified using the Zymo Direct-zol RNA miniprep kit (Zymo Research, Irvine CA). S1 gene sequences were amplified by RT-PCR using the Titan One-Step RT-PCR system (Roche Diagnostics, Indianapolis, IN) and previously published primers: NEWS1OLIGO5’ (11) and Degenerate3’ (14). RT-PCR reactions

were analyzed on a 1% w/v agarose gel and bands of the correct size were excised and DNA was purified from the gel fragment using the GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA). Sanger sequencing was performed by the Georgia Genomics Facility, University of Georgia, Athens, GA. The S1 sequences were assembled and compared using the DNAStar suite of programs (DNAStar, Madison WI).

Post-vaccination choanal cleft palate swab analysis. For ArkGA P1, P20, P40, and P60 vaccination trials, viral RNA from 5 choanal cleft palate swabs was purified from days 7, 10, and 14 post-vaccination for sequencing of the S1 region of the genome as previously described. The S1 amino acid sequences of viral RNA isolated from vaccinated chickens was compared to the S1 sequences of the vaccine virus for P1, P20, P40, and P60 to detect any mutations that occurred after viral replication in chicken tissues.

Genome Sequencing. In addition to sequencing of the S1 region of the viral genome, complete genome sequencing was performed on ArkGA P1, P20, P40, and P60 to detect changes occurring within the viral genome during attenuation and for comparison to pathogenic and vaccine strains of ArkDPI. Virus stock was filtered with a 0.2 μm syringe filter. Viral RNA was extracted from samples using the Direct-Zol RNA MiniPrep Kit (Zymo Research) and treated with DNase I (New England Biolabs). Complementary DNA (cDNA) was synthesized using SuperScript IV (Invitrogen/Thermo Scientific). Double stranded cDNA (dsDNA) was generated from cDNA templates using Second Strand cDNA Synthesis Kit (Applied Biological Materials Inc.). Complete genome sequencing at a 50x depth of coverage was conducted using the Nextera XT DNA Sample Preparation Kit (Illumina) and MiSeq sequencer (Illumina) according to

manufacturer's instructions. De Novo and directed assembly of genome sequences was carried out using the MIRA3 sequence assembler and Geneious r8 program (www.geneious.com). Non-synonymous substitutions in the assembled sequence reads were compared to consensus sequence at 5% of minimum variant frequency using Geneious r8 program.

RESULTS

Experiment 1. Infection and replication characteristics of the ArkGA P1 vaccine candidate were typical of other IBV vaccine types following eyedrop vaccination of SPF chickens (Table 3-1). All vaccinated chickens swabbed were positive for vaccine virus by 5-days post-vaccination, though viral load as indicated by Ct values was not as high as expected. By 10-days post-vaccination, 19/20 vaccinated chickens were positive for ArkGA and the viral load had decreased, indicating that the chickens were beginning to clear the vaccine. No non-vaccinated chickens were positive for ArkGA at any time during this trial. Ciliostasis scores differed between vaccinated and non-vaccinated chickens at all time points (Figure 3-1), though both groups passed the ciliostasis test. On day 10 post-vaccination, when the largest difference in ciliostasis scores between vaccinated and non-vaccinated chickens (53 vs 89) was seen, tracheal rales were also heard in one chicken in the vaccinated group.

Experiment 2. One hundred commercial broiler chickens were vaccinated with the ArkGA P1 vaccine candidate in a 7ml volume by spray using a commercial hatchery vaccine spray cabinet at a dose of $1x10^{3.5}$ EID_{50} per bird. All chickens were swabbed at 7, 10, 14, 17, 21, 24, and 28-days post-vaccination to assess viral load and vaccine infection rate (coverage), and the data are presented in Figure 3-2. Viral load in chickens was high by day 7 post-vaccination, and remained constant until 14-days post-vaccination at which point it began to decrease (Figure 3-2, A). ArkGA P1 vaccine candidate coverage was 100% by day 7 post-vaccination, and remained constant throughout the course of the experiment (Figure 2, B). Clinical signs were also recorded at these time points and tracheal rales were observed in 60% of the chicks vaccinated with ArkGA P1 vaccine candidate at 10 days post-vaccination, which is consistent with previous reports for Ark99.

At 30 days post-vaccination, chickens were challenged with $1x10^{3.4}$ EID_{50} (50% embryo infectious dose) of pathogenic Arkansas serotype virus in a 0.1ml eyedrop application and the data collected at five days post-challenge is shown in Figure 3-3. All groups showed significantly less clinical signs than the non-vaccinated and challenged group (Figure 3-3, A), and all groups passed the ciliostasis test except for the non-vaccinated and challenged group (Figure 3-3, B), as expected. Relative viral load was also significantly reduced in all groups when compared to the non-vaccinated and challenged group. Plotting the Ct values of individual samples taken from each group shows that 4/20 chickens in the ArkGA P1 vaccinated and challenged group were positive by qRT-PCR. (Figure 3-3, C). Virus isolation was not performed for this trial.

Experiment 3. Experiment 3 encompasses three separate trials carried out in the same manner as Experiment 2 for ArkGA P20, P40, and P60 vaccine candidates. However, in this experiment vaccine was applied in an 18ml total volume per 100 chicks. Vaccination experiments for ArkGA P20 and P40 vaccine candidates were ended prior to challenge due to excessive clinical signs post-vaccination.

Trial 1. As seen before, for chicks vaccinated with ArkGA P20, viral load and vaccine coverage were typically high early post-vaccination (Figure 3-4). However, thirty percent of chickens vaccinated with ArkGA P20 showed severe clinical signs (rales) on day 10 post-vaccination, which is reduced from the previous trial but still much higher than what would be accepted by the commercial poultry industry. For this reason, Trial 1 was terminated at this point.

Trial 2. In chicks vaccinated with ArkGA P40, viral load and vaccine coverage were lower at days 3 and 5 post-vaccination than in Trial 1, but reached high levels by day 7 post-vaccination (Figure 3-5). Clinical signs (rales) were reduced to 10% of chicks with tracheal rales at 10 days

post-vaccination, which was less than Trial 1, but was still considered too pathogenic for a commercial poultry vaccine. For this reason, Trial 2 was terminated at this point.

Trial 3. One hundred broiler chickens spray vaccinated with ArkGA P60 with a dose of $1x10^{3.1}$ EID_{50} per bird were swabbed on 3, 5, 7, 10, 14, 17, 21, 24, and 28-days post-vaccination, and viral load and vaccine coverage are shown in Figure 3-6. Viral load in chicks was again high soon after vaccination, though coverage was lower than expected on days 3 and 5 post-vaccination. By 7 days post-vaccination, coverage had reach 93% and peaked at 100% on day 14 post-vaccination. By 21 days post-vaccination, chickens began to clear the vaccine virus, indicated by reduced viral load and coverage (Figure 3-6). Only 3% of chicks vaccinated with ArkGA P60 showed clinical signs (snicks), which was deemed acceptable for an IBV vaccine.

On day 30 post-vaccination, 20 ArkGA P60 vaccinated and 5 non-vaccinated chickens were challenged with $1x10^{3.4}$ EID_{50} of pathogenic Arkansas virus, and 5 vaccinated and 5 non-vaccinated chickens were kept non-challenged as controls. Five-days post-challenge, the birds were euthanized and a necropsy was performed, and the data are presented in Figure 3-7. All groups showed significantly reduced clinical signs (Figure 3-7, A) and viral loads (Figure 3-7, C) compared to the non-vaccinated/challenged group, and all groups except the non-vaccinated/challenged group passed the ciliostasis test (Figure 3-7, B).

When analyzing the individual viral load values, 5/20 of the vaccinated and challenged birds were positive by qRT-PCR. It should also be noted that the ArkGA P60 vaccinated/non-challenged group had 2/5 chickens positive for virus (Figure 3-7, C).

Virus isolation post challenge was consistent with the results found by qRT-PCR (Table 3-2). All of the non-vaccinated and non-challenged group swabs were negative for virus isolation. In the vaccinated/non-challenged group, one of the swabs was found to be positive with an embryo

death at 120-hours post-inoculation. All 5 of the other embryos in this set died by 72-hours post-inoculation however, indicating a possible bacterial contamination in that sample. In the vaccinated/challenged group, 3/19 of the swabs were found to be positive for ArkDPI. All embryos in the 20th swab sample died at 48 hours post-inoculation, so that sample could not be analyzed. All 5 of the non-vaccinated/challenged bird swabs were positive for IBV.

To ensure that virus isolation positives in challenged groups were indeed challenge virus and not residual vaccine, the spike gene of samples from both challenged groups was sequenced. In all instances, sequence matched the Arkansas challenge virus, indicating it was not residual vaccine. In the vaccinated/non-challenged group, no sequence could be obtained from qRT-PCR positive samples.

Sequence analysis of the S1 gene. A comparison of the S1 portion of the spike gene of ArkGA P1 vs ArkDPI reveals at least 20 differences in the amino acid sequence. Of note, ArkGA P1 spike sequence contains a histidine at position 43, which has been previously shown to significantly increase spike protein binding to chicken tracheal tissues, and an asparagine deletion at position 344, which has been shown to influence the ability of antibodies to recognize the protein (15).

The S1 portion of the ArkGA spike gene was sequenced at every twentieth passage during further attenuation to evaluate changes that may have occurred during the embryo adaptation/attenuation process. No amino acid changes were seen in passages 1, 20, and 40, but in passage 60 a serine to asparagine change at position 117 and an arginine to histidine change at position 385 were detected.

Analysis of the ArkGA P1, P20, P40, and P60 S1 sequence isolated from vaccinated birds. During the ArkGA P1, P20, P40, and P60 vaccination trials, swabs collected from 5 chickens on days 7, 10, and 14 post-vaccination were analyzed for S1 sequence comparison with the vaccine, and results are shown in Table 3-3. No difference was observed between the ArkGA P1 vaccine virus and the virus re-isolated from vaccinated chickens. When analyzing P20 and P40, at position 198 a substitution from lysine to lysine/threonine was observed in the virus isolated from the swab, as well as a glycine to glycine/aspartic acid substitution at position 200. An additional mutation occurred in P40 at position 130, where a serine was present in the vaccine and a serine or asparagine could be seen in the swab sequence. These mutations were not maintained in P60, however in this passage new mutations occurred in the vaccine sequence compared to previous passages. As noted previously, in the ArkGA P60 vaccine, an asparagine was present at position 117 compared to all other passages containing a serine. The same occurrence was seen at position 385, where a histidine was present in P60 vs an arginine in this location for all earlier passages. Although both of these amino acid changes were seen in the P60 vaccine sequence, the virus re-isolated from swab material showed a reversion to the sequence shown in the previous vaccine passages, with a serine at position 117 and an arginine at position 385.

Whole genome sequencing of ArkGA P1, P20, P40, and P60. Figure 8 shows the consensus sequences of ArkGA P1, P20, P40, and P60 as compared to the original pathogenic Ark99 virus and the ArkDPI vaccine and pathogenic viruses. In all passages, the ArkGA viral genome was highly different from the ArkDPI virus genome, though serotypically they are the same virus type. Over the 60 additional passages performed in our laboratory, the ArkGA vaccine accumulated 14 single nucleotide polymorphisms (SNPs) compared to the Ark99 pathogenic virus. When

examining these SNPs, a SNP emerged in the leader sequence of the genome in ArkGA P40 and was maintained in P60. In addition, two SNPs emerged in the nsp3 protein gene region in ArkGA P60, and a large number of SNPs occurred in the spike protein gene.

In addition to comparing the consensus sequences of the ArkGA passages, whole genome sequencing was also used to evaluate the frequency of variant SNPs found in each passage (supplemental data). The number of variant SNPs in the Ark99 pathogenic virus was low and infrequent (Supplemental Table 3-1). As the virus was passaged in embryonated eggs, the number of variant SNPs increased to P60. Initially during passaging, the frequency of the variants increased (Supplemental Tables 3-2 - 3-4), however a decrease in variant frequency was seen in ArkGA P60 (Supplemental Table 3-5) as the population became more adapted to embryos.

DISCUSSION

When comparing the ArkGA vaccine candidate to the current ArkDPI vaccine, many differences are observed. Comparison of the S1 region of the spike protein gene between ArkGA and ArkDPI shows that the two viruses, while serotypically related, are genetically distinct and distinguishable. Comparing the pathogenic version of each virus to the attenuated viruses shows that the ArkGA spike gene appears to be more stable following embryonated egg passage (2 vs 24 amino acid changes). All of this data indicates that the ArkGA vaccine candidate is a better genetic match to pathogenic Ark viruses than the ArkDPI vaccine virus.

When evaluating the whole genomes of the ArkGA passages and comparing them to the Ark99 and ArkDPI pathogenic field strains and the ArkDPI vaccine, the same trend is seen as with comparison of S1. Throughout passaging, the ArkGA vaccine candidates remained highly similar to the Ark99 pathogenic field virus, whereas they are highly different from both the ArkDPI vaccine and field virus. Over the further attenuation process of the ArkGA vaccine candidate, SNPs accumulated over time that may have reduced the pathogenicity of the virus. In particular, the changes seen in the leader sequence and within the spike protein may have contributed to attenuation of the virus, as these regions are associated with viral replication, pathogenicity, and tropism (2, 18, 25). Furthermore, during passaging the frequency of variation was reduced, indicating that the major population within the vaccine virus became more dominant over time.

Different passages of the ArkGA vaccine were evaluated for infection, replication, vaccine reaction, and efficacy in broiler chicks. Experimental vaccine and challenge trials showed that the ArkGA P1 vaccine had suitable infection, replication, and induced adequate protection from challenge but was too pathogenic, causing a severe vaccine reaction in the majority of chicks. Further passages in embryonated eggs reduced the severity of the vaccine reaction to 30% for P20,

10% for P40, and 3% for P60. This further attenuation did not adversely affect infection or replication characteristics of the vaccine, as the relative viral load in chicks did not change throughout the trials. ArkGA P60 vaccine coverage was slightly less than expected early post-vaccination, but reached 100% by day 14. This may be attributed to the S1 amino acid changes seen between the vaccine and swab sequences in P60, as the S1 sequences re-isolated from chickens had reverted to the more pathogenic P1 sequence. In the ArkGA P1 vaccine trial a higher vaccine coverage was seen, indicating that these amino acid positions may have an impact on rate of infection. This could be improved by giving a higher dose of the vaccine. High infection and replication patterns in all trials stand in contrast to the infection and replication cycles of the ArkDPI vaccine, which shows very low infection rates and multiple replication cycles during the life of the bird following spray vaccination (20). The ArkGA vaccine at P1 and P60 was effective at protecting chickens from a pathogenic Ark IBV challenge. Clinical signs and viral loads post-challenge were significantly lower than non-vaccinated and challenged groups, and all vaccinated birds passed the ciliostasis test. Again, this stands in contrast to previous ArkDPI vaccine and challenge experiments that showed that chickens were clearly not protected from challenge after ArkDPI vaccination by spray (20).

The attenuated ArkGA vaccine described herein is a significant improvement over the current commercially available ArkDPI vaccine when comparing infection and replication following spray application and induction of protective immunity following homologous challenge. The ArkGA (P60) is also genetically distinct, making it possible to distinguish the ArkGA vaccine from the ArkDPI vaccine or pathogenic viruses. Further molecular investigation is needed to fully evaluate the 2 amino acid changes seen in the S1 gene in passage 40 and 60, but these changes do not seem to impact the effectiveness of the vaccine. In conclusion, the ArkGA

vaccine developed herein is safe when given to 1-day old broilers by spray, and it induces an efficacious immune response against homologous challenge.

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TABLES

Table 3-1. Experiment 1. ArkGA P1 vaccine candidate detection in SPF chickens by real-time PCR during safety testing.

Group	Day 5	Day 7	Day 10
Vaccinated	30/30 (31.48±.39)[A]	25/25 (32.41±.49)	19/20 (34.90±.46)
Non-Vaccinated	0/12	0/10	0/8

[A] Number in parentheses represents average Ct value.

Table 3-2. Experiment 3. Trial 3. Pathogenic Ark-type challenge virus detection in embryonated eggs. Data are represented as the number embryos positive per total for classic IBV signs 7 days post inoculation.

Group	Chicken	ArkDPI
Non-Vaccinated/Non-Challenged	1	0/6[A]
	2	0/6
	3	0/6
	4	0/6
	5	0/6
Vaccinated/Non-Challenged	6	0/5
	7	0/6
	8	0/6
	9	0/6
	10	1/1
Vaccinated/Challenged	11	0/6
	12	0/4
	13	0/5
	14	0/6
	15	0/6
	16	0/6
	17	0/4
	18	0/6
	19	0/4

	20	0/5
	21	0/4
	22	1/6
	23	0/6
	24	0/3
	25	3/6
	26	0/5
	27	1/6
	28	0/3
	29	0/6
	30	0/6*
Non-Vaccinated/Challenged	31	3/6
	32	1/5
	33	1/6
	34	3/6
	35	5/5

[A] Number of embryos positive per total for classic IBV signs 7 days after inoculation. *All 6 embryos died 48 hours post-inoculation, presumably from bacterial contamination. Embryos did not show lesions of IBV when examined post-death.

Table 3-3. S1 amino acid sequence comparison of ArkGA vaccine virus passages and viral RNA isolated from vaccinated chickens. Included are amino acid positions in the S1 sequence that exhibited variation among the different ArkGA passages. All other amino acid positions remained consistent throughout passaging.

ArkGA Passage	S1 Amino Acid Position	Vaccine Amino Acid	Swab Amino Acid
P1	117	S	S
	130	S	S
	198	K	K
	200	G	G
	385	R	R
P20	117	S	S
	130	S	S
	198	K	K/T
	200	G	G/D
	385	R	R
P40	117	S	S
	130	S	S/N
	198	K	K/T
	200	G	G/D
	385	R	R
P60	117	N	S
	130	S	S

198	K	T
200	G	D
385	H	R

FIGURES

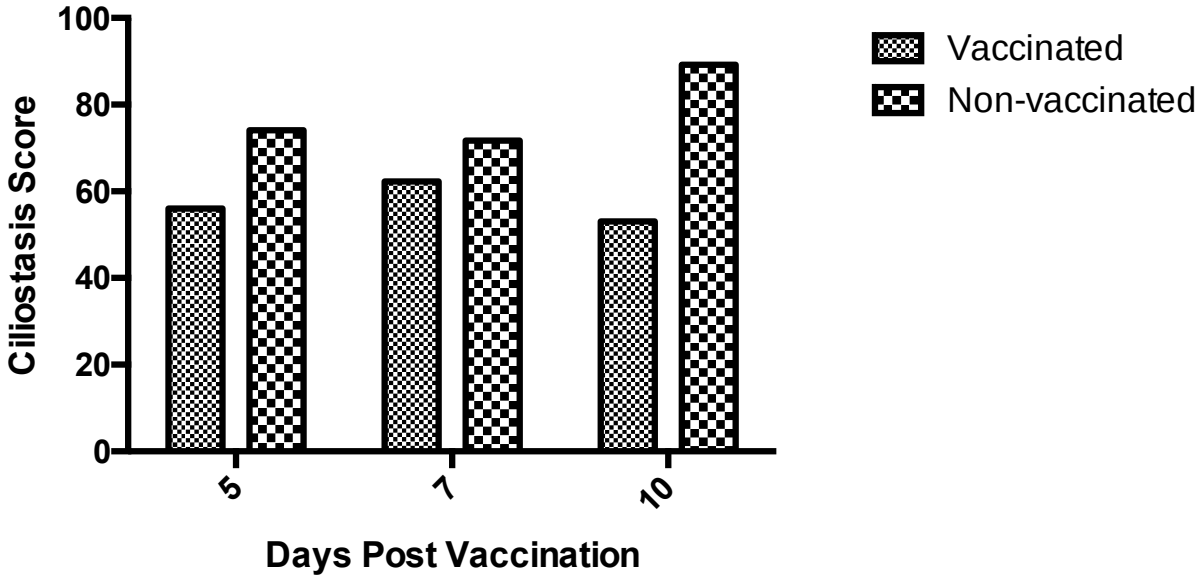

Figure 3-1. Experiment 1. Comparison of ArkGA P1 vaccinated and non-vaccinated ciliostasis scores in SPF chickens during safety testing. Ciliostasis scores were calculated as previously described where a score above 50 is considered passing.

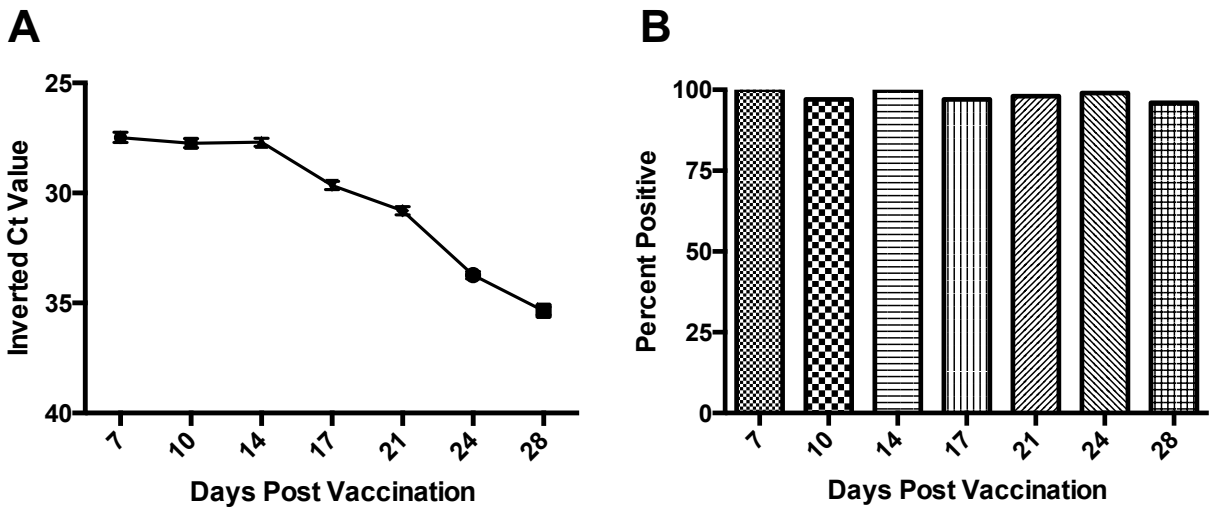

Figure 3-2. Experiment 2. Viral load in chickens (A) and vaccine coverage (B) after spray vaccination with ArkGA P1 vaccine candidate. Ct = cycle threshold.

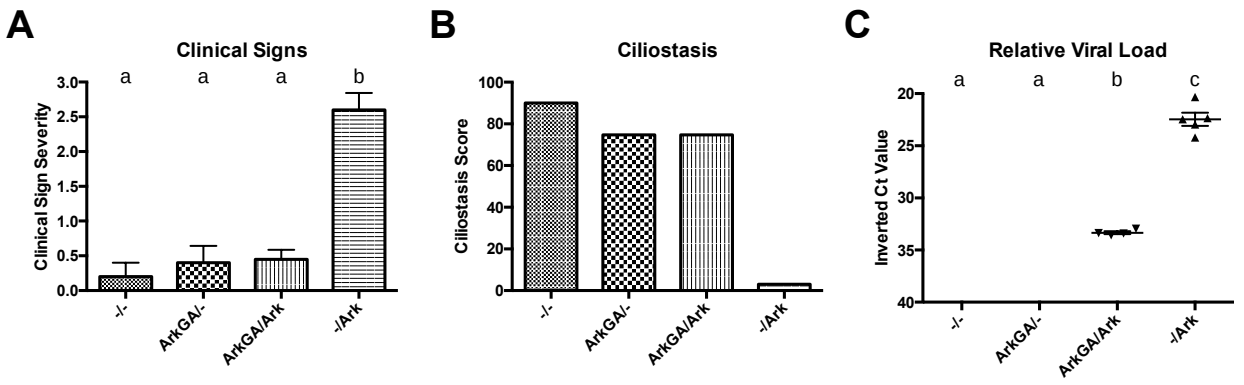

Figure 3-3. Experiment 2. Clinical signs, ciliostasis scores, and viral loads in chickens post-challenge. Clinical sign scores were calculated based on severity where 0 = negative, 1 = mild signs, 2 = watery eyes and some mucus in the nares, and 3 = watery eyes, mucus in the nares and trachea (tracheal rales). Ciliostasis scores were calculated as previously described where a score above 50 is considered passing. Ct = cycle threshold.

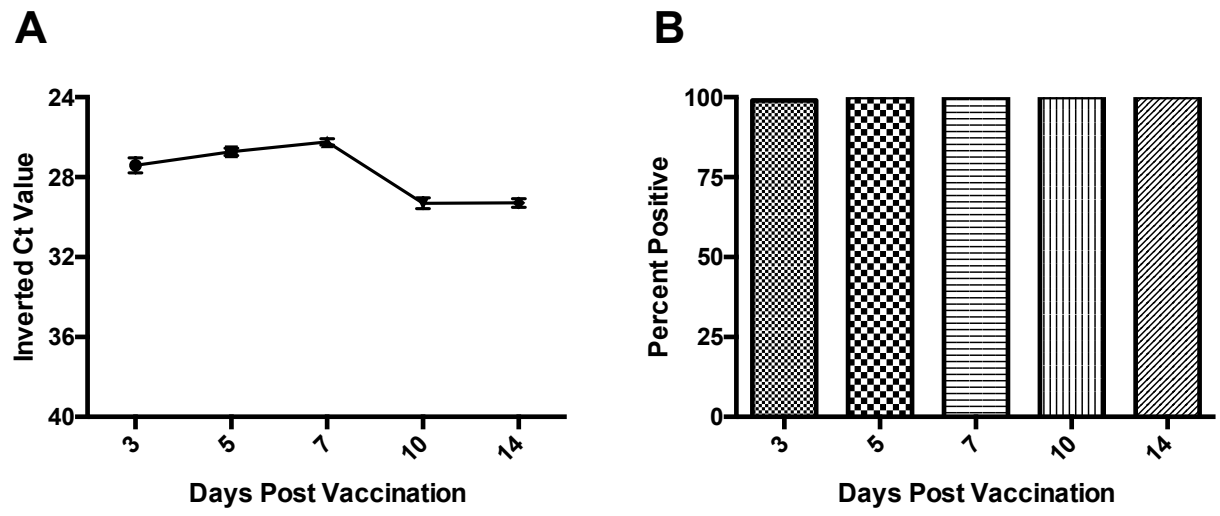

Figure 3-4. Experiment 3. Trial 1. Viral loads in chickens (A) and vaccine coverage (B) post-vaccination with ArkGA P20. Ct = cycle threshold.

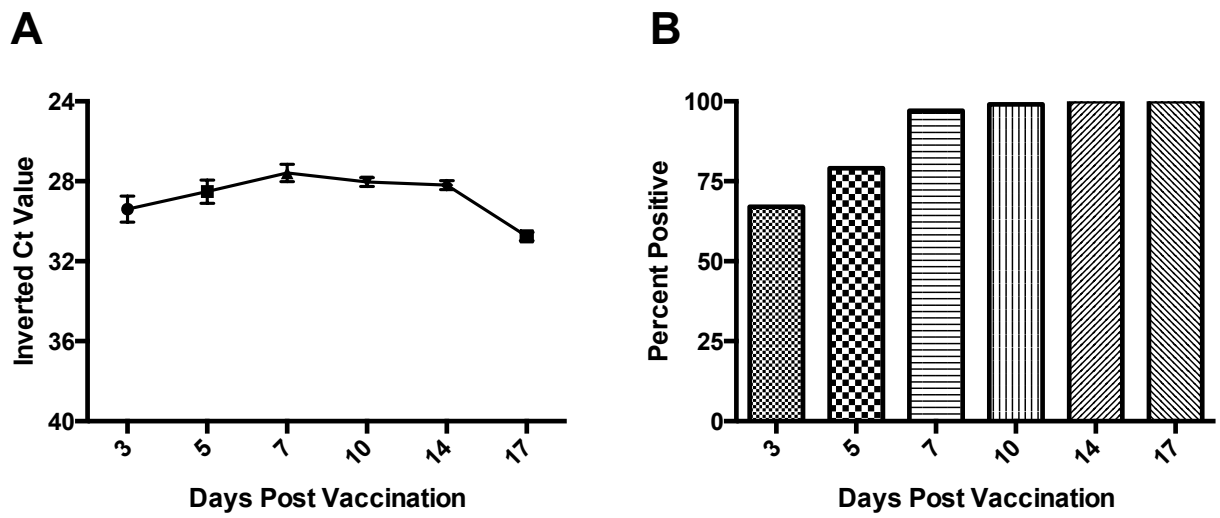

Figure 3-5. Experiment 3. Trial 2. Viral loads in chickens (A) and vaccine coverage (B) post-vaccination with ArkGA P40. Ct = cycle threshold.

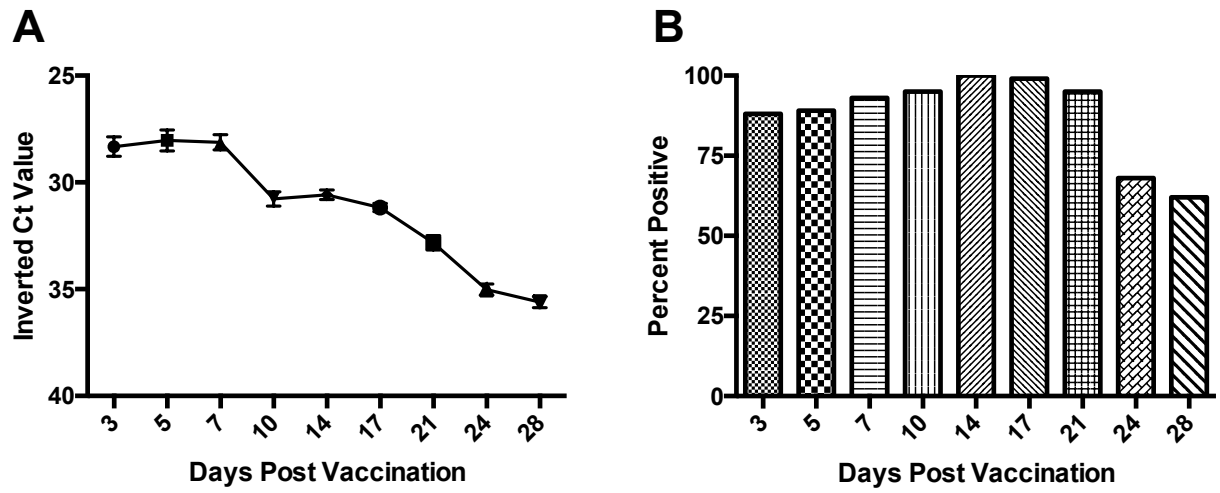

Figure 3-6. Experiment 3. Trial 3. Viral loads in chickens (A) and vaccine coverage (B) post-vaccination with ArkGA P60. Ct = cycle threshold.

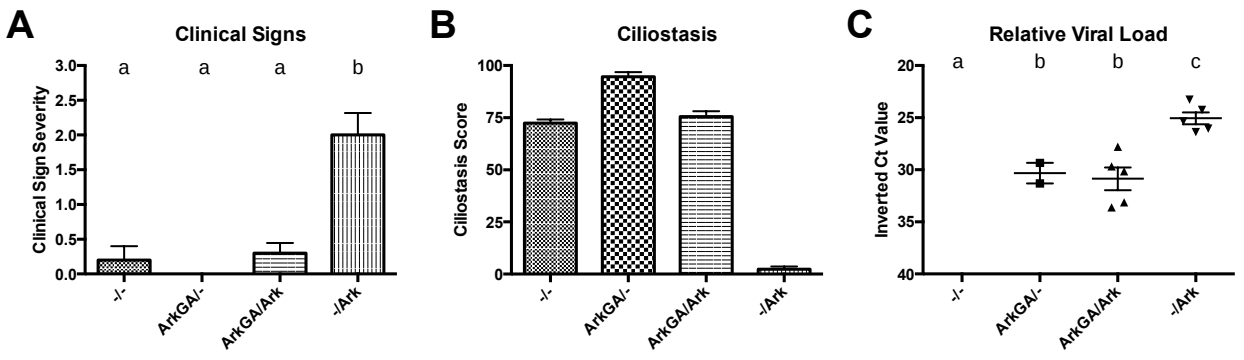

Figure 3-7. Experiment 3. Trial 3. ArkGA P60 vaccinated and non-vaccinated clinical signs, ciliostasis scores, and viral loads in chickens post-challenge. Clinical sign scores were calculated based on severity where 0 = negative, 1 = mild signs, 2 = watery eyes and some mucus in the nares, and 3 = watery eyes, mucus in the nares and trachea (tracheal rales). Ciliostasis scores were calculated as previously described where a score above 50 is considered passing. Ct = cycle threshold.

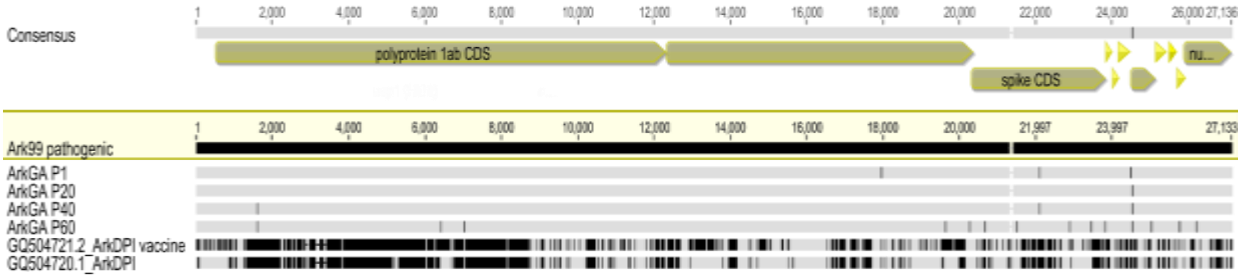

Figure 3-8. Consensus sequences during attenuation of the ArkGA IBV vaccine candidate in comparison to Ark99 pathogenic field strain, ArkDPI pathogenic field strain, and ArkDPI vaccine genomes.

SUPPLEMENTAL MATERIALS

Supplemental Table 3-1. Ark99 pathogenic virus strain SNPs.

Protein	CDS Position	Change	Codon Change	Consensus Sequence Frequency	Variant Frequency
Polyprotein 1a	2,216	C → T	CCT → CTT	94.9%	5.10%
Polyprotein 1a	7,964	A → T	TAC → TTC	66.10%	33.90%
Polyprotein 1a	9,875	T → C	ATG → ACG	93.50%	6.50%
Polyprotein 1a	11,141	A → G	AAT → AGT	90.70%	9.30%
Polyprotein 1ab	12,542	T → C	TTA → TCA	94.90%	5.10%
Polyprotein 1ab	12,968	C → T	ACG → ATG	94.90%	5.10%
Polyprotein 1ab	15,152	C → A	ACT → AAT	88.50%	11.50%
Polyprotein 1ab	16,573	A → T	AAT → TAT	92.20%	7.80%
Polyprotein 1ab	17,517	G → A	ATG → ATA	94.00%	6.00%
Spike	116	G → A	GGT → GAT	91.40%	8.60%
Spike	409	G → C	GCT → CCT	94.20%	5.80%
Spike	815	C → T	ACT → ATT	89.20%	10.80%
Spike	965	A → T	TAT → TTT	94.60%	5.40%
Spike	2,296	T → C	TTT → CTT	90.10%	9.90%
Membrane	26	C → T	TCG → TTG	93.40%	6.60%
Membrane	28	G → A	GAG → AAG	91.60%	8.40%

Supplemental Table 3-2. ArkGA P1 (Ark99 vaccine) SNPs.

Protein	CDS Position	Change	Codon Change	Consensus Sequence Frequency	Variant Frequency
Polyprotein 1a	52	C → T	CTC → TTC	62.60%	37.40%
Polyprotein 1a	277	G → T	GGT → TGT	60.40%	39.60%
Polyprotein 1a	286	C → T	CCT → TCT	92.50%	7.50%
Polyprotein 1a	1,207	C → T	CAC → TAC	93.40%	6.60%
Polyprotein 1a	2,807	G → T	TGC → TTC	88.50%	11.40%
Polyprotein 1a	8,422	A → G	ATC → GTC	70.20%	29.80%
Polyprotein 1a	8,849	C → T	GCT → GTT	62.60%	37.40%
Polyprotein 1a	10,866	C → T	ACT → ATT	85.80%	14.20%
Polyprotein 1ab	14,444	C → T	TCA → TTA	89.10%	10.90%
Polyprotein 1ab	17,822	A → G	AAA → AGA	67.20%	32.80%
Polyprotein 1ab	19,644	C → A	CAC → CAA	88.90%	11.10%
Polyprotein 1ab	19,757	A → C	AAA → ACA	91.50%	8.50%
Spike	982	A → G	ATG → GTG	92.90%	7.10%
Spike	1,038	T → G	TTT → TTG	47.80%	52.20%
Spike	1,153	C → T	CGT → TGT	87.70%	12.30%
Spike	1,763	G → A	GGA → GAA	90.90%	9.10%
Spike	1,886	A → T	AAG → ATG	80.20%	15.40%
Spike	1,885	A → C	AAG → CAG	91.70%	8.30%
Spike	2,285	T → C	GTT → GCT	53.30%	46.70%
Envelope	308	A → G	GRA → GGA	51.20%	48.80%
Membrane	7	A → G	RAT → GAT	51.20%	48.80%
Nucleocapsid	617	A → G	GAT → GGT	59.70%	40.30%

Supplemental Table 3-3. ArkGA P20 SNPs.

Protein	CDS Position	Change	Codon Change	Consensus Sequence Frequency	Variant Frequency
Polyprotein 1a	1,106	A → C	GAG → GCG	52.90%	47.10%
Polyprotein 1a	2.202	G → T	TTG → TTT	94.90%	5.10%
Polyprotein 1a	5,300	G → T	CGT → CTT	92.30%	7.70%
Polyprotein 1a	7,701	G → T	TTG → TTT	53.30%	46.70%
Polyprotein 1a	10,342	C → T	CTT → TTT	91.90%	8.10%
Polyprotein 1a	10,816	G → A	GTT → ATT	94.90%	5.10%
Polyprotein 1a	11,686	G → A	GTC → ATC	88.00%	12.00%
Polyprotein 1ab	15,308	C → T	CCA → CTA	94.90%	5.10%
Polyprotein 1ab	17,035	C → T	CAT → TAT	91.90%	8.10%
Polyprotein 1ab	19,542	G → A	ATG → ATA	93.70%	6.30%
Spike	1,709	C → T	CCT → CTT	72.10%	27.90%
Spike	2,563	G → A	GTG → ATG	71.50%	28.50%
Nucleocapsid	289	G → C	GGA → CGA	83.10%	16.90%
Nucleocapsid	806	C → T	ACA → ATA	77.60%	22.40%
Nucleocapsid	916	G → A	GTC → ATC	77.20%	22.80%
Nucleocapsid	922	C → T	CGT → TGT	76.70%	23.30%
Nucleocapsid	1,218	G → C	GAG → GAC	94.80%	5.20%

Supplemental Table 3-4. ArkGA P40 SNPs.

Protein	CDS Position	Change	Codon Change	Consensus Sequence Frequency	Variant Frequency
Polyprotein 1a	1,106	C → A	GCG → GAG	94.80%	5.20%
Polyprotein 1a	6,527	A → C	GAT → GCT	71.40%	27.70%
Polyprotein 1a	10,393	C → T	CCC → TCC	56.00%	44.00%
Polyprotein 1ab	19,114	C → T	CCT → TTT	62.90%	37.10%
Polyprotein 1ab	19,757	A → C	AAA → ACA	67.80%	32.20%
Spike	350	G → A	AGC → AAC	79.70%	20.30%
Spike	389	G → A	AGC → AAC	55.00%	45.00%
Spike	593	A → C	AAA → ACA	72.80%	27.20%
Spike	599	G → A	GGT → GAT	72.00%	28.00%
Spike	1,154	G → A	CGT → CAT	67.40%	32.60%
Spike	1,709	C → T	CCT → CTT	52.90%	47.10%
Spike	1,750	T → C	TTT → CTT	61.00%	39.00%
Spike	2,563	G → A	GTG → ATG	56.50%	43.30%
Spike	3,032	C → T	TCT → TTT	68.40%	31.60%
Spike	3,107	A → G	GAT → GGT	88.50%	11.50%
Spike	3,112	G → A	GAG → AAG	83.60%	16.40%
Nucleocapsid	289	G → C	GGA → CGA	59.00%	41.00%
Nucleocapsid	787	A → G	AAT → GTT	91.50%	8.50%
Nucleocapsid	806	C → T	ACA → ATA	83.20%	16.80%
Nucleocapsid	916	G → A	GTC → ATC	84.90%	15.10%
Nucleocapsid	922	C → T	CGT → TGT	84.80%	15.20%
Nucleocapsid	1,120	G → T	GAT → TAT	92.50%	7.50%
Nucleocapsid	1,202	A → G	GAT → GGT	86.80%	13.20%
Nucleocapsid	1,218	G → C	GAG → GAC	74.10%	25.70%
Membrane	517	C → A	CCG → ACG	81.40%	18.60%
Membrane	539	G → A	CGT → CAT	80.40%	19.60%

Supplemental Table 3-5. ArkGA P60 SNPs.

Protein	CDS Position	Change	Codon Change	Ref. Frequency	Var. Frequency
Polyprotein 1a	293	C → T	GCA → GTA	92.00%	7.60%
Polyprotein 1a	2,807	G → T	TGC → TTC	88.00%	12.00%
Polyprotein 1a	3,029	A → G	AAA → AGA	93.90%	6.00%
Polyprotein 1a	4,204	T → G	TTT → GTT	79.70%	20.30%
Polyprotein 1a	4,712	A → C	GAA → GCA	92.80%	7.20%
Polyprotein 1a	6,527	C → A	GCT → GAT	62.80%	36.80%
Polyprotein 1a	7,701	G → T	TTG → TTT	94.30%	5.70%
Polyprotein 1a	8,141	A → C	GAC → GCC	90.80%	8.70%
Polyprotein 1a	10,393	C → T	CCC → TCC	94.00%	6.00%
Polyprotein 1ab	12,131	G → T	CGG → CTG	93.60%	6.40%
Polyprotein 1ab	13,471	A → G	ATC → GTC	93.10%	6.60%
Polyprotein 1ab	14,014	G → A	GAA → AAA	91.50%	7.90%
Polyprotein 1ab	15,856	A → G	ACA → GCA	89.40%	8.70%
Polyprotein 1ab	18,704	A → G	AAT → AGT	94.60%	5.20%
Polyprotein 1ab	19,114	T → C	TTT → CTT	81.40%	18.60%
Polyprotein 1ab	19,118	C → T	GCG → GTG	92.70%	7.10%
Polyprotein 1ab	19,757	C → A	ACA → AAA	76.00%	24.00%
Spike	158	C → T	TCT → TTT	91.30%	8.30%
Spike	350	A → G	AAC → AGC	81.00%	18.70%
Spike	510	T → G	AAT → AAG	93.40%	6.50%
Spike	593	A → C	AAA → ACA	90.40%	9.60%
Spike	599	G → A	GGT → GAT	89.50%	10.20%
Spike	1,010	G → A	AGT → AAT	93.10%	6.70%
Spike	1,154	A → G	CAT → CGT	74.60%	25.30%
Spike	1,757	T → A	GTT → GAT	92.80%	7.10%
Spike	2,563	A → G	ATG → GTG	87.80%	12.00%
Spike	3,032	C → T	TCT → TTT	92.60%	7.20%
Spike	3,107	A → G	GAT → GGT	90.00%	9.60%
Spike	3,112	A → G	AAG → GAG	81.00%	19.00%
Spike	2,166	T → C	TTT → CTT	94.60%	5.20%
Membrane	539	A→ G	CAT → CGT	79.70%	20.00%
5a	178	C → A	CCC → ACC	94.00%	5.30%
5b	55	G → A	GTT → ATT	78.40%	21.60%
Nucleocapsid	289	C → G	CGA → GGA	81.20%	18.30%
Nucleocapsid	1,218	G → C	GAG → GAC	81.10%	18.10%

CHAPTER 4

EVALUATION OF A COCCIDIA VACCINE USING SPRAY AND GEL APPLICATIONS[1]

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ABSTRACT

Coccidiosis is an economically significant disease of poultry caused by species of *Eimeria*, a parasitic protozoan. Disease can result in poor feed conversion, reduced weight gain, and can lead to the development of necrotic enteritis. For prevention of coccidiosis, poultry are commonly vaccinated with live, sporulated oocysts mass applied with a vaccination cabinet in the hatchery. Traditionally, coccidia vaccines have been applied by coarse spray in a water based diluent, however, new technology using gel diluents has entered the United States market. Gel diluents can have variable viscosities and are "dropped" onto chicks with an applicator bar. It is thought that gel droplets remain intact on the birds for longer than water based droplets, allowing more time for preening and ingestion of oocysts. In this experiment, the efficacy of a commercial coccidia vaccine applied with a water based diluent, a more viscous gel diluent, and a less viscous gel diluent was compared. Fecal samples were collected at multiple time points post-vaccination to quantify vaccine oocyst shedding. Shedding in the first cycle (days 5-8 post-vaccination) was correlated to the number of oocysts received from each application method. However, a decrease in shedding was seen for the more viscous gel group in the second cycle (days 12-15 post-vaccination). Chickens were challenged with *Eimeria maxima* oocysts and 7 days post-challenge body weight gains and gross and microscopic lesions were recorded to evaluate protection levels for the different vaccine applications. All vaccinated groups appeared to be protected based on body weight gain and lesion scoring. The results of this project indicate that all vaccine applications are effective at protecting against *Eimeria maxima* challenge when using a proper dose of vaccine that allows for repeated oocyst cycling in the litter post-vaccination.

Key words: coccidiosis, *Eimeria*, broiler, vaccination

Abbreviations: % CV = percent coefficient of variation; *E.* = *Eimeria*; LV = less viscous; MV = more viscous; NE = necrotic enteritis

INTRODUCTION

Coccidiosis is an enteric disease of commercial poultry caused by *Eimeria*, which are coccidian species belonging to the apicomplexan phylum. Ingestion of sporulated *Eimeria* oocysts from the environment leads to the development of enteric disease. Each of the seven species of *Eimeria* that cause disease in chickens preferentially infects different regions of the intestinal tract and can result in lesions of varying severity, including thickening of the intestinal wall, petechial hemorrhages, and necrosis, to name a few (7). Worldwide, the costs arising from coccidia challenge, treatment, and control are estimated to total at least 3 billion US dollars annually (1, 2, 29). Not only is coccidia infection alone an expensive burden for the poultry industry, but infection with *Eimeria maxima* is known to be a predisposing factor for necrotic enteritis (NE) caused by *Clostridium perfringens* infection (26). Lesions resulting from the subclinical form of NE cause reduced nutrient absorption and feed conversion, and cost producers $6 billion in 2015 (27).

Eimeria infection occurs when a susceptible chicken ingests a sporulated oocyst from the environment (2, 3, 5-7, 24). For every oocyst ingested, it is estimated that several hundred thousand may be produced, which are then available for ingestion and infection of other chickens in the poultry house (22). Historically, coccidiosis has been treated via the use of anticoccidials, including ionophores and chemicals. Although anticoccidial treatments are effective in protecting against disease outbreaks, development of drug resistance, current external pressures on the industry, and regulatory changes have producers turning towards vaccination (2, 4, 18, 23). Coccidia vaccines contain live, sporulated oocysts of varying mixtures and concentrations of *Eimeria* species, and they are given in a low dose to initiate an immunologic response in the bird (10, 25, 30). Infection with *Eimeria* is self-limiting, as oocysts produced in the intestine are not capable of auto-infection of the host chicken and must be excreted and re-ingested for further

infection (9, 28). The immune response to *Eimeria* infection is species-specific and requires multiple exposures to oocysts of each *Eimeria* sp. to achieve sufficient protective immunity (21). Thus, vaccine companies rely on the concept of "vaccine oocyst cycling" in the litter to gain protective immunity in a poultry flock (17).

The traditional method of coccidia vaccine application is in a water-based spray using a hatchery spray cabinet. Vaccine coverage is essential, as chicks that do not ingest oocysts at day of hatch will later be exposed to oocysts in the litter, and this higher dose of oocysts can result in clinical infection and gut lesions. Gel vaccination technology for coccidia has been posited as an alternative to spray vaccination. Gel beads containing *Eimeria* oocysts have been shown to be protective when delivered in the feed (11, 14, 15). Now, coccidia vaccines in gel diluents are being applied to day-old chicks at the hatchery with a gel applicator bar (19). There are multiple manufacturers of gel diluents as well as multiple viscosities of individual gel products. Some gels are only slightly more viscous than water, but create more stable droplets on the chicks when applied. Other gels are extremely viscous and create very defined and gelatinous drops on chicks. The higher viscosity of gel diluents compared to water spray may increase the available vaccine for ingestion.

Now that alternative methods for coccidia vaccine application are in use, research is needed to confirm that vaccines are still as efficacious with these new application methods as with traditional application. This study aimed to compare the same commercial vaccine applied by the traditional spray method and using a gel application method with both high and low viscosity gels. During the experiment, post-vaccination oocyst shedding was recorded for two cycles along with evaluation of protection from challenge when vaccinating with both methods.

MATERIALS AND METHODS

Coccidia Vaccine. The commercially available coccidia vaccine Coccivac®-B52 (Merck Animal Health) used in these experiments contains live, sporulated oocysts that were administered on day of hatch at a dose specified by the manufacturer.

Coccidia Challenge. Eimeria (*E.*) *maxima* oocysts of the APU1 strain were generously donated by Dr. Mark Jenkins (13). Oocysts were stored at 4C in 2.5% potassium dichromate. Pathogenic dose was determined by administration of varying doses of sporulated oocysts to 16-day old broiler chickens prior to the start of this experiment. For experimental challenge, oocysts were enumerated to obtain a dose of 5×10^4 oocysts per bird and diluted in deionized water. Challenge was administered 16 days post-vaccination via the oral gavage route.

Experimental Animals. Non-vaccinated broiler chickens were used to provide a relevant model to commercial poultry operations. Day 19 broiler chicken embryos were purchased from a commercial source and hatched at the Poultry Diagnostic and Research Center (Athens, GA). Chicks were randomly assigned to one of the experimental groups. Animal care and use protocols have been approved by the University of Georgia Institutional Animal Care and Use Committee.

Experimental Design. This experiment compared coccidia vaccine infection and oocyst cycling following multiple application methods and protection from *Eimeria maxima* challenge. All experimental groups consisted of 100 one-day-old broiler chicks that were vaccinated with the same commercially available coccidia vaccine at the same dosage. Chicks in Group 1 were vaccinated using a traditional spray application with a water based diluent. In Group 2, chicks were

vaccinated using the commercially available more viscous (MV) gel vaccine diluent Hydrodrop gel (ClearH_2O®, Westbrook, ME) applied by a gel bar applicator. In Group 3, chicks were vaccinated using the commercially available less viscous (LV) gel diluent CEVAGEL® (Ceva Animal Health) Dry Gel Powder with the same gel applicator bar as was used for the MV gel. In Group 4, chicks were vaccinated by oral gavage to serve as a positive vaccination control. One hundred chicks remained unvaccinated to serve as positive and negative challenge controls in Groups 5 and 6.

To evaluate vaccine oocyst cycling in the litter post-vaccination, each group of chicks was placed on fresh litter in separate colony-type houses. Four days post-vaccination, 20 chicks from each vaccinated group were removed for individual chick placement in Horsfall isolators. Feces from each chick in each group were collected on days 5-8 to evaluate the first cycle of vaccine oocyst shedding. After fecal collection on day 8 post vaccination, these chicks were removed and euthanized. This was repeated 11 days post-vaccination for fecal collection on days 12-15 to evaluate the second cycle of vaccine oocyst shedding. Oocyst counts were recorded as both *E. maxima*-specific and total (*E. maxima*, *E. acervulina*, *E. tenella*, and *E. mivati*) using a McMaster counting chamber.

Sixteen days post-vaccination, each group was reduced to 20 birds per group. All birds in all groups excluding the negative challenge control, Group 6, were challenged with 5×10^4 pathogenic *E. maxima* oocysts via oral gavage. In addition, a pre-challenge body weight was obtained for each bird. Seven days post-challenge, birds were weighed, humanely euthanized, and evaluated for gross lesions. In addition, slide smears were taken from the mid-intestines for oocyst count scoring and segments of the mid-intestine were collected and placed in formalin for histological microscopic lesion scoring.

Vaccination Procedure. Individual vials from the same lot of Coccivac®-B52 vaccine were prepared for use in different application methods. For the oral gavage method, the vaccine was diluted in sterile deionized water to reach a concentration of 1 dose per 0.5 ml. The spray, MV gel, and LV gel were all prepared to apply 100 doses of vaccine per chick basket. The spray method required dilution of the vaccine in sterile deionized water. The MV gel diluent was mixed with vaccine using a paddle mixer. The LV gel diluent Dry Gel Powder was added to 2.5 L sterile deionized water and mixed with a blender until combined, at which time the manufacturer's dye and the vaccine were added. For direct comparison of the two gel diluents, both the MV and LV gels were applied using the same gel applicator bar (Merck Animal Health). The spray application dispensed 24 ml of vaccine suspension per 100 chicks. The MV gel was dropped from the gel applicator bar to apply 25 ml of gel diluent onto a basket of 100 chicks. The LV gel also dispensed 25 ml per chick basket.

Coccidia Vaccine Dose Determination for each Application Method. To confirm that each vaccination method was applying the same dose of oocysts, a sample was taken from the gavage solution, the spray nozzle, and the gel applicator bar for both gels. Sporulated oocysts in each solution were counted using a McMaster chamber (8), and the *E. maxima* and total oocysts/ml were enumerated using the formula: (# oocysts)*(dilution factor)*(6.67). The oocysts/ml and the volumes of the gavage, spray, and gel solutions that were administered during application were used to obtain the oocysts/dose for each method.

Oocyst Enumeration from Fecal Samples using the McMaster Counting Method. Oocysts were enumerated using a McMaster counting chamber in a method based on one described by Conway and McKenzie (8). After collection, feces of each bird were weighed and resuspended in deionized water at a volume of 10x the fecal weight and allowed to soak overnight at 4C in 500 ml bottles. The next day, the bottles were shaken vigorously, and the fecal suspensions were filtered through a double layer of cheesecloth. For each sample, filtrate was collected into a 15 ml centrifuge tube and centrifuged for 5 minutes at 1500 rpm to pellet the solids. The supernatant was discarded and the pellet was resuspended in a saturated salt solution to a volume of 15 ml. After inversion of the tube, a sample was removed with a transfer pipet and a McMaster counting chamber was filled. Oocysts within the chamber were counted as *Eimeria maxima* and total. The oocysts/g of fecal material was calculated as (# oocysts/0.15)*10, where each oocyst counted is equivalent to 67 oocysts per gram of sample. In cases where the oocysts were too numerous to count, ten-fold dilutions of the oocyst suspension were made in saturated salt water until the oocysts reached a countable concentration in the McMaster chamber. When dilutions were made, the dilution factor was applied to the # oocysts before calculating the oocysts/g of fecal material.

Gross Lesion Scoring. Eimeria maxima gross lesions in the midgut were scored for all experimental groups 7 days post-challenge according to a method first described by Johnson and Reid (16). The midgut was identified by the presence of the Meckel's diverticulum, and scores were assigned on a scale of 0-4, with 0 being no lesions present, 1 showing small numbers of petechiae on the serosal surface of the intestine, 2 showing more numerous petechiae and orange intestinal contents, 3 showing thickening of the intestinal wall and ballooning with or without

pinpoint blood clots and mucus, and 4 showing bloody intestinal contents, ballooning, and a greatly thickened wall.

Oocyst Count Scoring. Eimeria maxima oocyst counts were scored for all experimental groups 7 days post-challenge. Following gross lesion scoring, a smear of the midgut of each bird was applied to a microscope slide and viewed under a 10X objective lens. The score system of Goodwin et al was used, in which 0 = no oocysts seen, 1 = 1-20 oocysts per field, 2 = 21-50 oocysts per field, 3 = 51-100 oocysts per field, and 4 = TNTC (12).

Microscopic Lesion Scoring. Microscopic lesion scoring followed the method described by Goodwin et al (12). A 2.5 cm portion of the jejunum proximal to the Meckel's diverticulum was collected from 5 birds in each experimental group and immersed in 10% buffered formalin. Portions of each intestinal segment were cut parallel to the longitudinal axis and placed into coded cassettes for processing through graded ethanols and xylene and embedding in paraffin. Three μm sections of deparaffinized formalin-fixed mid-intestine were placed onto glass slides and stained with hematoxylin and eosin for scoring by a pathologist. *Eimeria maxima* was scored based on the presence of developmental stages in the intestinal material. The microscopic lesion score is the sum of A+B. "A" represents the distribution of developmental stages of *E. maxima* along the intestinal segment. Four fields were viewed at a 10X objective, and the scoring system for distribution is as follows: 0 = no parasites, 1 = parasites in one field, 2 = parasites in two fields, 3 = parasites in three fields, and 4 = parasites in all four fields. "B" represents the severity of *Eimeria maxima* infection within the four examined fields, where 0 = parasites in 0% of the villi, 1 = parasites in <25% of the villi, 2 = parasites in 25-50% of the villi, 3 = parasites in 51-75% of the

villi, and 4 = parasites in >75% of the villi. The initial microscopic lesion scores could range from 0 to 8, but to compare to gross lesion scores and oocyst count scores the microscopic lesion score was divided by 2 to give a final score range of 0-4.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism software (La Jolla, CA) using an alpha of 0.05. Oocyst per gram shedding statistical comparisons were analyzed by two-way ANOVA with Holm-Sidak multiple comparisons testing. Prism software was also used to calculate % coefficient of variation for total and *E. maxima* shedding of each group and each time point. Pre-challenge mean body weight, post-challenge body weight gain, and post-challenge lesion scores were all analyzed by comparison of the means with SEM.

RESULTS

Vaccine Doses. Vaccine doses of sporulated oocysts for each vaccine application method are shown in Table 4-1 from samples taken directly from the applicator mechanism. The dose was highest in the MV gel group with 1751 oocysts/dose, followed by the gavage, LV gel, and finally the spray with 1089 oocysts/dose. When calculating only sporulated *E. maxima* oocysts per dose, the order was the same as that of the total with MV gel providing the highest dose of 567 oocysts/dose, then gavage and LV gel with 501 and 500 oocysts/dose, and lastly spray with 449 oocysts/dose.

Cycle 1 Oocyst Shedding. During the first cycle, the birds vaccinated by gavage were shedding higher numbers of total oocysts at all time points (Figure 4-1, A). Excluding the gavage group, there was no significant difference in total oocyst shedding between vaccinated groups until day 8, when the MV gel group was shedding significantly higher total oocysts per gram than the spray group, while the LV gel group was not significantly different from either group (Figure 4-1, B). The percentage of chickens vaccinated by gavage that were shedding oocysts in cycle 1 was higher than that of all other groups, peaking at 100% on days 6-8. Of the other vaccine application methods, only the less viscous gel group reached 100% of chickens shedding oocysts at day 6. Ninety-five percent of the chickens vaccinated by the MV gel were shedding oocysts on days 6 and 7. The spray vaccinated chickens peaked at day 6 with 95% of chickens shedding oocysts (Figure 4-1, C). The percent coefficient of variation (% CV) for total oocyst shedding in the first cycle shows that the variation of oocyst numbers shed by the gavage group was lowest, followed by the LV gel, MV gel, and finally the spray group showing the highest variation (Table 4-2).

Eimeria maxima oocyst shedding data for the first cycle of fecal collection followed a similar trend to the total oocyst shedding, with the chickens vaccinated by gavage shedding higher *E. maxima* oocysts per gram than any of the other experimental groups (Figure 4-1, D). Excluding the gavage group, the chickens vaccinated with the MV gel method were shedding significantly higher *E. maxima* oocysts per gram than both other experimental groups at day 7 (Figure 4-1, E). The percentages of chickens shedding *E. maxima* in each group were lower than those for the total oocyst shedding, peaking at 7 days post-vaccination with 85% of the chickens vaccinated by gavage shedding *E. maxima*. In the other groups, the MV gel group had the highest percentage of chickens shedding *E. maxima* oocysts with 65% shedding on days 7 and 8 (Figure 4-1, F). The variation in numbers of *E. maxima* oocysts shed by the MV gel group was the lowest, and %CV increased with the gavage, followed by the LV gel and spray groups (Table 4-3).

Cycle 2 Oocyst Shedding. In the second cycle, the gavage group total oocyst shedding became more consistent with that of the other groups (Figure 4-2, A). When excluding the gavage data, the spray group was shedding significantly higher total oocysts per gram than the MV gel group on day 12, and significantly higher oocysts than both MV and LV groups on day 13. By day 14, shedding from all groups had begun to decline (Figure 4-2, B). Although the numbers of oocysts per gram differed between the groups, there was 100% shedding of total oocysts for the birds in each group on all days in cycle 2 except for day 12 (Figure 4-2, C). The %CV for total oocyst shedding in cycle 2 was decreased in all groups compared to the first cycle, and variation was lowest in the spray vaccinated group and highest in the MV gel group (Table 4-2).

Eimeria maxima shedding during the second cycle was quite low and did not show the same increase in shedding from cycle 1 that was seen with the total oocyst shedding (Figure 4-2,

D). Other than day 15, where the chickens vaccinated by spray were shedding significantly higher *Eimeria maxima* oocysts per gram than the other experimental groups, there was very little *E. maxima* oocyst shedding (Figure 4-2, E). In addition to the low *E. maxima* oocyst numbers being shed during the second cycle, the percentage of chickens positive for shedding *E. maxima* in each group was low during days 12-14, with an increase in the percent positive at day 15 (Figure 4-2, F). The %CV for the second cycle of *E. maxima* oocyst shedding was increased in all groups compared to the first cycle. Variation was highest in the gavage vaccinated group and lowest in the spray group (Table 4-3).

Pre- and Post-Challenge Body Weight. There was no significant difference in the mean body weight recorded 16 days post-vaccination between any of the vaccinated groups or the non-vaccinated control group (Figure 4-3, A). The body weight gain recorded 7 days post-challenge did not show a significant difference between any of the vaccinated groups and the non-vaccinated/non-challenged group. However, the MV gel vaccinated group body weight gain was also not significantly higher than the non-vaccinated/challenged group (Figure 4-3, B).

Gross Lesions. None of the vaccinated groups differed significantly when evaluating gross lesion scores, with all vaccinated groups having gross lesion scores below 1. The gavage, more viscous gel, and less viscous gel groups also all did not differ significantly from the non-vaccinated/non-challenged group. The non-vaccinated/challenged group showed significantly higher gross lesion scores than all other groups, with a mean score greater than 2 (Figure 4-4, A).

Oocyst Count Scores. *E. maxima* oocyst enumeration from mid-intestine scrapings showed that the vaccinated groups did not differ significantly in oocyst count scores from each other or from the non-vaccinated/challenged group, and all scores were below 2. The non-vaccinated/non-challenged group had a score of 0, which was significantly lower than all other groups (Figure 4-4, B).

Microscopic Lesions. Microscopic lesion scores ranged from 2.5-3 for all challenged groups, with none of the *E. maxima* challenged groups differing significantly. The non-vaccinated/non-challenged group had a score of 0 which was significantly lower than all other groups (Figure 4-4, C).

DISCUSSION

The influence of each application method is shown in the cycle 1 total oocyst shedding. The birds vaccinated by gavage were shedding higher numbers of total and *E. maxima* oocysts than any of the experimentally vaccinated groups in the first cycle. When vaccinating by oral gavage, the oocyst suspension is deposited directly into the crop, resulting in more efficient delivery of the oocysts to the intestinal tract. The spray, MV gel, and LV gel methods require chicks to actively ingest oocysts both from preening and from pecking in the hatchery basket, resulting in a potential loss of vaccine. The MV gel vaccinated chickens shed higher total and *E. maxima* oocysts in the feces than either of the experimental groups in the first cycle, which can be correlated to the higher doses of oocysts for that application method.

During the second cycle of fecal oocyst shedding, the total oocysts shed increased tenfold for the gavage, spray, and less viscous gel groups. This was expected as the birds ingested the higher doses of oocysts present in the litter following the first cycle of shedding. However, the birds vaccinated by the more viscous gel method were shedding significantly lower numbers of total oocysts than the other vaccinated groups, although 100% of the birds in that group were shedding oocysts at all time points except for day 12. Although temperature in the colony room of each group was constant, it is possible that there was reduced humidity in the more viscous gel room, leading to lower sporulation rates of the oocysts shed in the first cycle and therefore lower doses of oocysts ingested by those birds.

Like the data for the first cycle of *Eimeria maxima* oocyst shedding, the numbers of oocysts shed during the second cycle was quite low in all groups except for day 15, when the spray group was shedding significantly higher oocysts per gram than the other groups. In addition, the percentage of birds in each group positive for *E. maxima* shedding was low until day 15. It is

possible that the start of shedding for the second cycle was delayed, and higher numbers would have been seen had fecal collection continued beyond 15 days. This trend can be seen in Figure 4-2, F, where the percentage of chicks shedding *E. maxima* is increasing every day.

E. maxima was deemed to be the most appropriate species of *Eimeria* to use as a challenge, as it is a component in the development of necrotic enteritis, and is therefore of extreme relevance to the poultry industry (31). Pre-challenge body weights showed no significant difference between any of the groups, including the non-vaccinated birds. This is interesting considering that a common industry concern regarding the use of live coccidia vaccines is reduced body weight gain, but the effect of vaccination on body weight was not seen in this experiment. Following challenge, the mean body weight gains of the vaccinated groups were not significantly different from the non-challenge controls, indicating protection. The mean body weight of the negative control group was significantly increased compared to the non-vaccinated challenged control group, showing that the challenge had an influence on body weight for non-vaccinated birds. Interestingly, the MV gel group, which showed significantly reduced oocyst shedding in the second cycle compared to the other groups, did not significantly differ in average body weight gain from the non-vaccinated, challenged control group. This illustrates the importance of re-exposure to oocysts in the litter in order to achieve complete protection.

When evaluating gross lesions, scores for the vaccinated groups were low, and the gavage, more viscous gel, and less viscous gel group scores all were statistically the same as the non-vaccinated/non-challenged group, indicating protection from challenge. The spray group did not have significantly different scores from the other vaccinated groups, but did have a significantly higher mean gross lesion score than the negative control group. However, since it was not different from the other vaccinated groups it is reasonable to claim that protection was achieved. The oocyst

count scores and the microscopic lesion scores of the vaccinated groups were all statistically the same as the mean score of the positive challenge control group. However, interpretation of these scores is difficult, considering that the birds were all kept on litter during the 7 days post-challenge, and could have continued to cycle vaccine oocysts during this time, rendering it nearly impossible to distinguish between vaccine and challenge oocysts present in the mid-intestine. This makes the body weight and the gross lesion scores the most reliable method for evaluating protection.

This experiment demonstrates that vaccine application method can influence the dosage of oocysts per chicken. Even though all vaccines were mixed so that each chick basket would receive the same dosage of oocysts, there was a difference in the number of oocysts collected from each application method. The gel application methods had oocyst numbers in each dose consistent with the gavage preparation (where vaccine is not mass applied). Contrastingly, there was a loss of oocysts during vaccination for the spray method, which is consistent with other reports showing that infectious bronchitis virus vaccine is lost when applied by spray (20). While these differences in total oocysts delivered to chicks varied between application methods, there was no difference in body weight gain or protection from challenge between birds vaccinated using water spray, more viscous gel bar, less viscous gel bar, or gavage. This demonstrates that when these methods are used properly and chickens are exposed to an appropriate dosage of coccidia vaccine, protection will be achieved, regardless of the vaccine application.

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TABLES

Table 4-1. Oocysts per dose for each vaccinated group

Experimental Group	**Oocysts/dose**	
	Total	*Eimeria maxima*
Gavage	1634	501
Spray	1089	449
More Viscous Gel	1751	567
Less Viscous Gel	1418	500

Table 4-2. Percent coefficient of variation (%CV) for total oocyst shedding of each group at each time point

Experimental Group	%CV							
	Cycle 1				Cycle 2			
	D5	D6	D7	D8	D12	D13	D14	D15
Gavage	194.0	131.7	115.2	152.2	113.5	116.7	67.6	302.8
Spray	334.5	156.2	314.1	172.7	98.5	129.1	79.7	53.6
More Viscous Gel	272.1	140.3	100.5	315.0	231.0	166.4	184.8	120.3
Less Viscous Gel	238.6	101.5	116.7	167.4	75.9	152.4	92.8	143.3

Table 4-3. Percent coefficient of variation for *E. maxima* oocyst shedding of each group at each time point.

Experimental Group	%CV							
	Cycle 1				**Cycle 2**			
	D5	D6	D7	D8	D12	D13	D14	D15
Gavage	0.0	244.2	131.3	161.7	447.2	347.3	181.9	171.2
Spray	0.0	368.6	280.5	308.1	0.0	0.0	196.0	119.9
More Viscous Gel	0.0	0.0	123.4	246.6	431.9	447.2	0.0	282.4
Less Viscous Gel	0.0	411.3	269.3	251.3	447.2	447.2	0.0	122.5

FIGURES

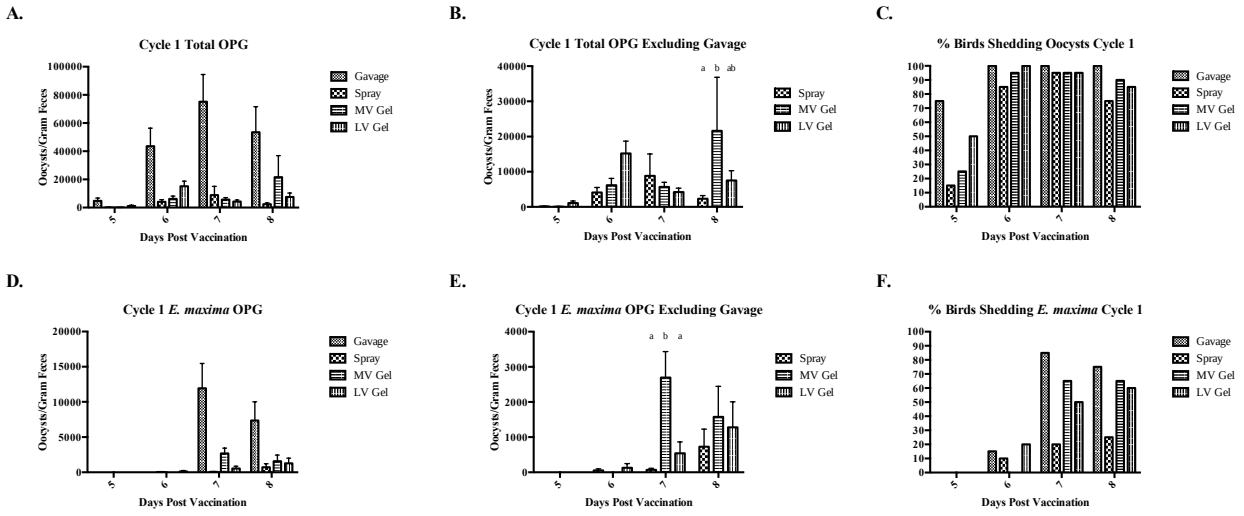

Figure 4-1. Total and *Eimeria maxima* oocyst shedding data for the first cycle post-vaccination. The data shown are oocysts per gram of feces shed by the chickens in each group on each day of the cycle. Each bar represents a group that was vaccinated by a different method. A – total oocysts per gram shed. B – total oocysts per gram shed without the gavage vaccinated group. C – % of birds in each group positive for shedding total oocysts. D – *Eimeria maxima* oocysts per gram shed. E – *Eimeria maxima* oocysts per gram shed without the gavage vaccinated group. F – % of birds in each group positive for shedding *Eimeria maxima* oocysts.

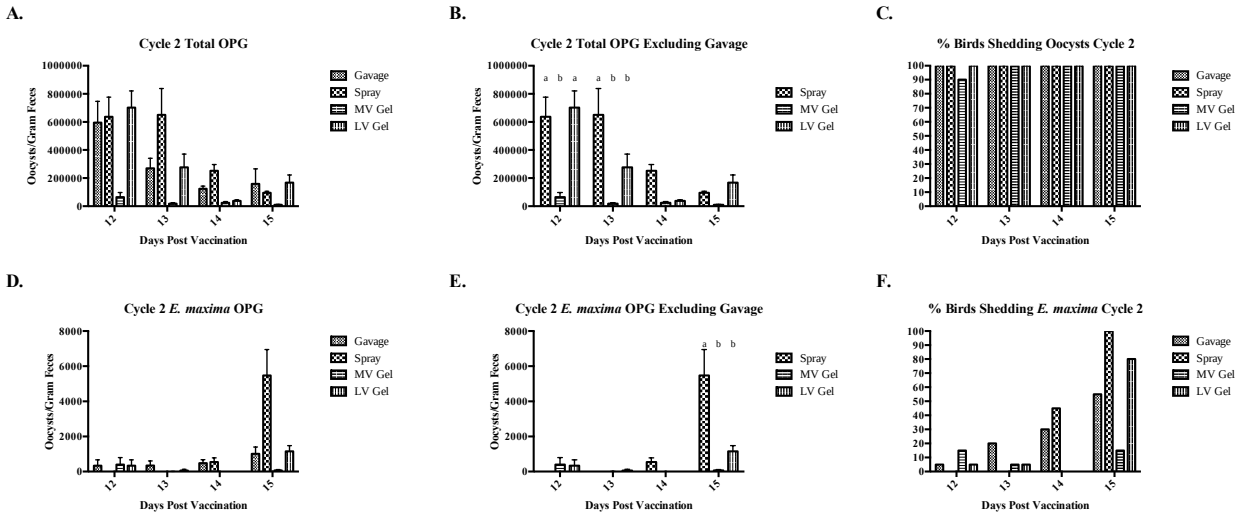

Figure 4-2. Total and *Eimeria maxima* oocyst shedding data for the second cycle post-vaccination. The data shown are oocysts per gram of feces shed by the chickens in each group on each day of the cycle. Each bar represents a group that was vaccinated by a different method. A – total oocysts per gram shed. B – total oocysts per gram shed without the gavage vaccinated group. C – % of birds in each group positive for shedding total oocysts. D – *Eimeria maxima* oocysts per gram shed. E – *Eimeria maxima* oocysts per gram shed without the gavage vaccinated group. F – % of birds in each group positive for shedding *Eimeria maxima* oocysts.

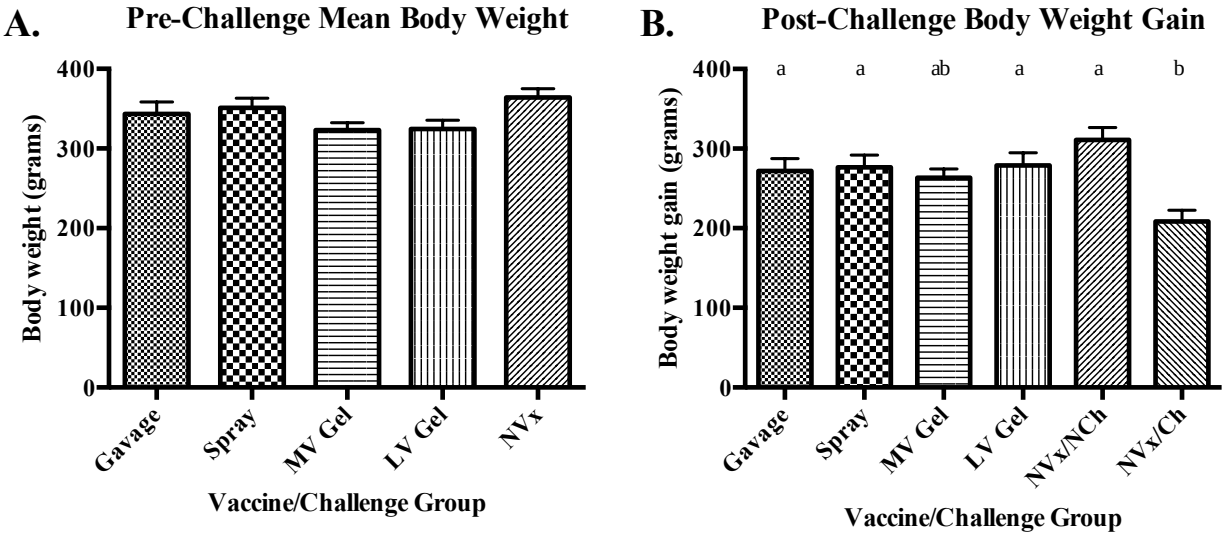

Figure 4-3. Mean body weight 16 days post-vaccination (A) and mean body weight gain 7 days post-challenge with *E. maxima* (B).

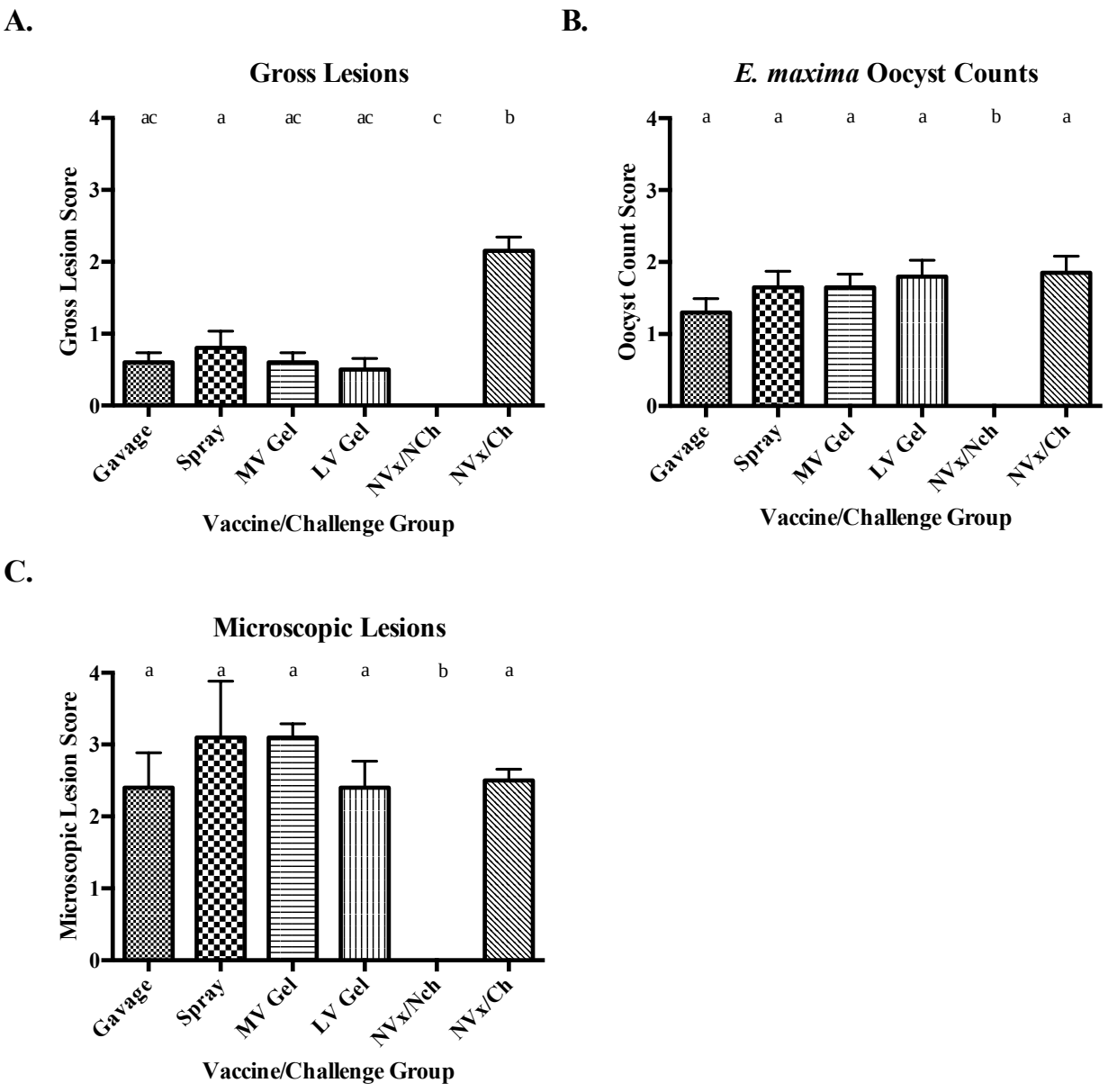

Figure 4-4. Mean *Eimeria maxima* lesion scores. A – gross lesions. B – oocyst count scores. C – microscopic lesion scores.

CHAPTER 5

SEQUENCE ANALYSIS OF THE PROPOSED COCCIDIAN SPECIES *EIMERIA MIVATI*[1]

[1]G. A. Albanese, D.-H. Lee, B.J. Jordan. To be submitted to *Poultry Science*

ABSTRACT

Coccidiosis is an economically impactful disease of commercial poultry caused by *Eimeria* infection. There are seven *Eimeria* species known to infect chickens, ranging in disease severity from subclinical infection to death. These species have been identified and characterized based on traits such as oocyst morphology, life cycle characteristics, and pathogenicity. Speciation by traits like morphology are subjective, and one *Eimeria* type described using these classical techniques, *E. mivati*, is currently disputed as a species. Many claim that *E. mivati* is actually a variant of *E. mitis* based on newer molecular techniques used to differentiate *Eimeria* species. This research aimed to further characterize *E. mivati* via sequence analysis of the mitochondrial cytochrome C oxidase subunit I (COI) gene, which is currently considered the most reliable gene for species differentiation. For this research, an *E. mivati* monoculture originating from a single oocyst propagation, a commercially available coccidiosis vaccine containing *E. mivati*, and *Eimeria* DNA from a suspected *E. mivati* field case were all sequenced using Illumina high throughput sequencing. None of the samples analyzed contained COI sequence attributed to *E. mivati*, but instead matched with a COI gene sequence of *E. mitis* found in the publicly available GenBank database. There is currently a lack of reference sequences for *E. mivati* and it is unknown if the COI gene is reliable for species differentiation of this *Eimeria* type, so further genome examination is needed to determine its status.

Key words: broiler, coccidiosis, *Eimeria mivati*

Abbreviations: COI = cytochrome C oxidase subunit I; *E.* = *Eimeria*; PCR = polymerase chain reaction; SPF = specific-pathogen free

INTRODUCTION

Coccidia are a group of parasitic protozoans belonging to the phylum Apicomplexa. These parasites have a wide host range, infecting numerous vertebrate and invertebrate species, and can produce severe disease in both humans and animals, often with strict host and tissue tropism (2, 12). One Apicomplexan genus, *Eimeria*, consists of thousands of species, many capable of infecting only a single host (7). Many new *Eimeria* isolates are still being discovered, leaving researchers to determine if a truly novel species has been identified, or simply if variants of known species are emerging.

The poultry industry worldwide suffers a particularly significant economic burden as an outcome of *Eimeria* infection (5, 29). Currently, there are seven confirmed species of *Eimeria* known to infect chickens: *E. acervulina*, *E. maxima*, *E. tenella*, *E. mitis*, *E. necatrix*, *E. brunetti*, and *E. praecox*. These species differ in traits classically used for identification, including oocyst morphology, region of the chicken intestine and location within the intestinal epithelial cells parasitized, disease pathology, prepatent period, and lack of cross-immunity, to name a few (8, 13, 15, 17, 27). However, these traditional methods for speciation of *Eimeria* are often highly subjective and species may be difficult to discern, especially if morphological traits or infection characteristics are not obviously distinct between species. For these reasons, an additional *Eimeria* of chickens, *E. mivati*, has been posited by some as a new species since it was first isolated the 1970's (10), while others feel classical methods of speciation have not been able to reliably distinguish *E. mivati* from the very similar *E. mitis*, leading to the theory that it is a variant strain (16, 18, 23, 26).

Due to the subjective nature of classical differentiation techniques, molecular identification techniques have been employed as a complement to traditional speciation practices when

characterizing ambiguous *Eimeria* types like *E. mivati*. Enzyme electrophoresis and restriction fragment length polymorphism have been used as speciation tools in the past (24, 25), however, polymerase chain reaction (PCR) amplification and sequencing of various genes conserved among *Eimeria* is the most common assay performed today. Ribosomal DNA, including the 5S (3), 18S (1, 19), and internal transcribed spacer region 1 (22) and 2 (6) genes were the first regions of the genome used to speciate *Eimeria*. However, the ribosomal genes have been shown to vary within a species, particularly in the 18S gene sequence (11, 28), making this region unreliable for speciation. More recently, mitochondrial DNA has been used to separate species, as the cytochrome C oxidase I (COI) gene shows a distinct and consistent sequence for each known species (20).

Typically, molecular speciation work for *Eimeria* is performed using nested PCR reactions and Sanger sequencing. Nested PCR amplifies a specific region of an initial target gene universal to any *Eimeria* species present in the sample. Then, a species-specific PCR has to be performed using the initial product as template material. This has drawbacks for an unknown or novel species such as *E. mivati* that may not have a validated species-specific PCR primer set for the target gene. In addition, Sanger sequencing has limitations for *Eimeria* species distinction. If a sample containing multiple genetic populations is sequenced using Sanger, such as the initial "generic" PCR product in the nested reaction, only a single, predominant population will be represented in the sequencing product. For these reasons, next-generation Illumina sequencing was chosen for this project to sequence the "generic" COI PCR product of *E. mivati* samples, rendering the species-specific portion of the reaction unnecessary. The Illumina platform sequences every piece of genetic material in a sample, allowing for detection of novel variants, and shows not only all of the *Eimeria* genomic populations present, but also the proportion of each within a sample (14, 21).

The objective of this study was to use next-generation sequencing technology to analyze the COI gene of *E. mivati* to provide further evidence as to its status as a species. Amplified COI genes from three samples represented as containing *E. mivati* were sequenced: an *E. mivati* seed stock from a vaccine company, a commercially available coccidiosis vaccine licensed to contain *E. mivati*, and a suspected *E. mivati*-infected chicken intestine from a field case.

MATERIALS AND METHODS

Maintenance of an E. mivati monoculture. An *E. mivati* vaccine seed stock was obtained in the form of sporozoites frozen in liquid nitrogen ampoules. Upon receipt, the ampoules were thawed and administered to two-week-old specific pathogen free (SPF) chickens via the oral gavage method. The chickens were housed in Horsfall isolation units, and feces were collected from days 5-8 post-infection for oocyst isolation and sporulation based on the method specified by Conway and McKenzie (9).

Upon initial inspection under a microscope, the oocysts produced from infection with the *E. mivati* sporozoite seed stock did not appear to be of a single population and contained two slightly different morphologies. Single oocyst inoculations were again performed to obtain a pure sample. The sporulated oocyst stock was enumerated using the McMaster counting method (9) and diluted to a concentration of 1 oocyst per 5 µl of deionized water. 5 µl droplets were pipetted onto a hydrophobic printed well microscope slide and each droplet was examined at 100X magnification for the presence of an *E. mivati* oocyst. If the desired oocyst was seen with no other contaminants present, the droplet was pipetted into a microcentrifuge tube containing 500 µl of deionized water. Each microcentrifuge tube was made to contain 4 oocysts, and was used to inoculate a single one-week-old SPF chicken via oral gavage. Five chickens were inoculated and housed in Horsfall isolation units for 7 days, at which point they were humanely euthanized and their intestines were collected for sporulation in potassium dichromate. Sporulated *E. mivati* oocysts obtained from the intestinal material were stored at 4C and routine passage in SPF chickens was performed to maintain the viability of the oocysts.

Commercial vaccine. The commercial coccidiosis vaccine provided was licensed as containing sporulated oocysts of *E. maxima, E. acervulina, E. tenella,* and *E. mivati*.

Isolation of DNA from the E. mivati field case. Chicken intestines that were suspected to be infected with *E. mivati* were collected by a veterinarian and sent to the University of Georgia Poultry and Diagnostic Research Center Diagnostic Laboratory (Athens, GA). *Eimeria*-specific lesions were identified in the duodenal loop, from which portions were cut parallel to the longitudinal axis and placed into coded cassettes for processing through graded ethanols and xylene and embedding in paraffin. DNA was extracted from the formalin-fixed, paraffin-embedded intestinal tissues using the QIAmp DNA FFPE Tissue Kit (Qiagen). Isolated DNA concentration and purity was measured using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific Inc.).

Isolation of genomic Eimeria DNA from oocysts. Genomic DNA was extracted from the commercial vaccine oocysts and from the *E. mivati* monoculture based on a method by Blake et al (4). One million oocysts stored at 4C in potassium dichromate were pelleted by centrifugation for 2 minutes at 13,000 x *g*. The oocyst pellet was washed three times in TE buffer and resuspended in 0.5 ml of TE and placed into a Lysing Matrix D tube (MP Biomedicals, LLC). Homogenization was performed using a FastPrep-24™ 5G Homogenizer (MP Biomedicals, LLC) at a speed of 6.0 m/s for 20 s and was repeated 3x until oocyst rupture was observed at 10X magnification. Following homogenization, the sample was centrifuged at 1500 x *g* for 1 minute to pellet debris, and the supernatant was collected and combined with 0.33 volumes of 10% sodium dodecyl sulfate. The sample was incubated at 37C for 2 hours with 50 µl proteinase K (20 mg/ml). RNase

A was added to a concentration of 20 µg/ml and further incubation was performed at room temperature for 1 hour. The solution was centrifuged at 10,000 x *g* for 1 minute, and DNA was recovered from the supernatant using TRIzol™ Reagent (Thermo Fisher Scientific Inc.). Purified DNA was resuspended in 100 µl of deionized water and stored at 4C overnight, after which contaminants were removed from the DNA sample using the PowerClean® Pro DNA Clean-Up Kit (MO BIO Laboratories, Inc.).

COI gene amplification by PCR. PCR was performed to amplify the *Eimeria* COI gene for all DNA samples. Generic COI primers COI-400F (5'-GGDTCAGGTRTTGGTT GGAC-3') and COI-1202R (5'-CCAAKRAYHGCACCAAGAGATA3') used were from El-Sherry et al (11) to amplify the approximately 800 bp portion of the COI gene. The PCR reaction was conducted using the Thermo Scientific ™ Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Inc.) and an Applied Biosystems Thermal Cycler (Life Technologies). The protocol consisted of initial denaturation at 98C for 30s, followed by 35 cycles of 98C denaturation for 10s, annealing at 48C for 30s, and 72C extension for 30s. A final elongation step was performed at 72C for 10 minutes. Agarose gel electrophoresis was used to confirm the proper band size of the PCR products, and COI DNA was extracted from the gel using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Inc.).

Sequence analysis of the COI gene. Eimeria COI DNA was isolated as described and treated with DNase I (New England Biolabs). Complementary DNA (cDNA) was synthesized using SuperScript IV (Invitrogen/Thermo Scientific). Double stranded cDNA (dsDNA) was generated from cDNA templates using the Second Strand cDNA Synthesis Kit (Applied Biological

Materials Inc.). COI gene sequencing was conducted using the Nextera XT DNA Sample Preparation Kit (Illumina) and MiSeq sequencer (Illumina) according to manufacturer's instructions. De Novo and directed assembly of gene sequences were carried out using the MIRA3 sequence assembler and Geneious r8 program (www.geneious.com). Non-synonymous substitutions in the assembled sequence reads were compared to consensus sequence at 5% of minimum variant frequency using Geneious r8 program. Following assembly, the COI sequences were compared using the DNASTAR suite of programs (DNASTAR, Inc.).

RESULTS

Single oocyst propagation. Microscopic oocyst examination after initial infection of SPF chickens with the liquid nitrogen frozen *E. mivati* sporocysts clearly showed two different oocysts sizes and morphologies at 10X magnification. It was suspected that a contaminating species was present, necessitating a second single oocyst propagation to obtain a pure sample. After propagation, oocysts of two morphologies were again observed, one spherical and one slightly more ellipsoid. Further propagation in SPF chickens with oocyst selection for a single morphology continued to result in the production of oocysts of varied sizes and shapes (Figure 5-1).

Illumina sequencing of the PCR amplified COI gene for each sample. The mitochondrial COI DNA population from the *E. mivati* monoculture, the duodenal loop tissue from a suspected *E. mivati* field case, and from a vaccine containing *E. mivati* oocysts was compared. For all of the samples analyzed, 100% of the *Eimeria* reads assembled corresponded to reference sequences. None of the samples were found to contain a genetic population that matched with *E. mivati* reference sequences. Both the intestinal field sample and the vaccine contained COI DNA that matched with four species of *Eimeria*: *E. maxima, E. tenella, E. acervulina,* and *E. mitis*. The *E. mivati* monoculture contained only one genetic population that was a match with a GenBank reference sequence for *E. mitis*. All sequences detected from every sample matched greater than 99% with the corresponding reference sequences (Table 5-1). The isolated *E. mitis* sequences were translated and compared with a reference amino acid sequence for *E. mitis*. Any nucleotide mutation seen in the gene was silent and did not alter the translated amino acid sequence.

Using reference sequences from GenBank for *E. maxima, E. tenella,* and *E. acervulina*, in addition to a reference COI sequence for *T. gondii*, a phylogenetic tree was constructed from the

isolated *Eimeria* sequences from all samples and all *E. mivati* and *E. mitis* sequences available in the database (Figure 5-2). All *E. mivati* and *E. mitis* sequences grouped into a separate clade from the other species, and within that clade, the vaccine, field, and *E. mivati* monoculture *E. mitis* sequences were more closely related to reference *E. mitis* sequences than *E. mivati*, although all sequences were highly related.

DISCUSSION

The purpose of this study was to examine the COI genes of *E. mivati* oocyst samples to provide further evidence as to the species identity of this *Eimeria* type. Previous literature has shown that the ribosomal RNA sequence that was once attributed to *E. mivati* corresponds with a second 18S gene sequence of *E. mitis*, which is not unusual for this gene. The COI gene is now widely recognized as the most reliable gene for species differentiation of *Eimeria*, and it was hoped that sequencing of this region of the *E. mivati* genome would provide insight as to its taxonomic identity. The three samples analyzed were used as representatives of *E. mivati* to isolate any potential sequence variability or detection of a novel *E. mivati* COI gene product.

In this trial, no specific *E. mivati* sequence was identified, and all of the samples that were posited as containing *E. mivati* and lacking *E. mitis* were found to have *E. mitis* sequence. Furthermore, all of the *E. mitis* sequences obtained in the samples were identical to each other and 99.9% similar to the *E. mitis* database reference at the nucleotide level and 100% identical in the translated amino acid sequence. This sequence homology, especially within the *E. mivati* monoculture that was obtained from selection of single oocysts for propagation, contradicts the mixed morphology that is seen upon microscopic examination. If *E. mivati* is truly a separate species, it may be possible that the COI gene region is not a sensitive enough marker to distinguish between the two closely genetically related *E. mivati* and *E. mitis* species. If *E. mivati* is not a separate species and is, in fact, a variant of *E. mitis* or is the same species entirely, it remains unknown what accounts for the morphological disparity in the *E. mivati* monoculture sample. If these two types are variants, perhaps the multiple morphologies give indication to the predominant phenotypical traits that are attributed to each type, and may account for the original assumption of a separate species of *E. mivati*.

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TABLES AND FIGURES

Table 5-1. Illumina sequencing results for the COI genes of *Eimeria* species present in the *E. mivati* monoculture, the suspected *E. mivati* field case, and a vaccine containing *E. mivati*. The species detected during sequencing were *E. acervulina*, *E. maxima*, *E. tenella*, and *E. mitis*.

Sample sequenced	*Eimeria* species detected based on sequencing of the COI gene		Percentage similarity of the detected sequence to a known reference sequence for each species
E. mivati monoculture	*E. acervulina*	No	N/A
	E. maxima	No	N/A
	E. tenella	No	N/A
	E. mitis	Yes	99.9%
Intestinal sample from a suspected *E. mivati* field case	*E. acervulina*	Yes	100%
	E. maxima	Yes	100%
	E. tenella	Yes	100%
	E. mitis	Yes	99.9%
Vaccine containing *E. mivati*	*E. acervulina*	Yes	99.6%
	E. maxima	Yes	100%
	E. tenella	Yes	100%
	E. mitis	Yes	99.9%

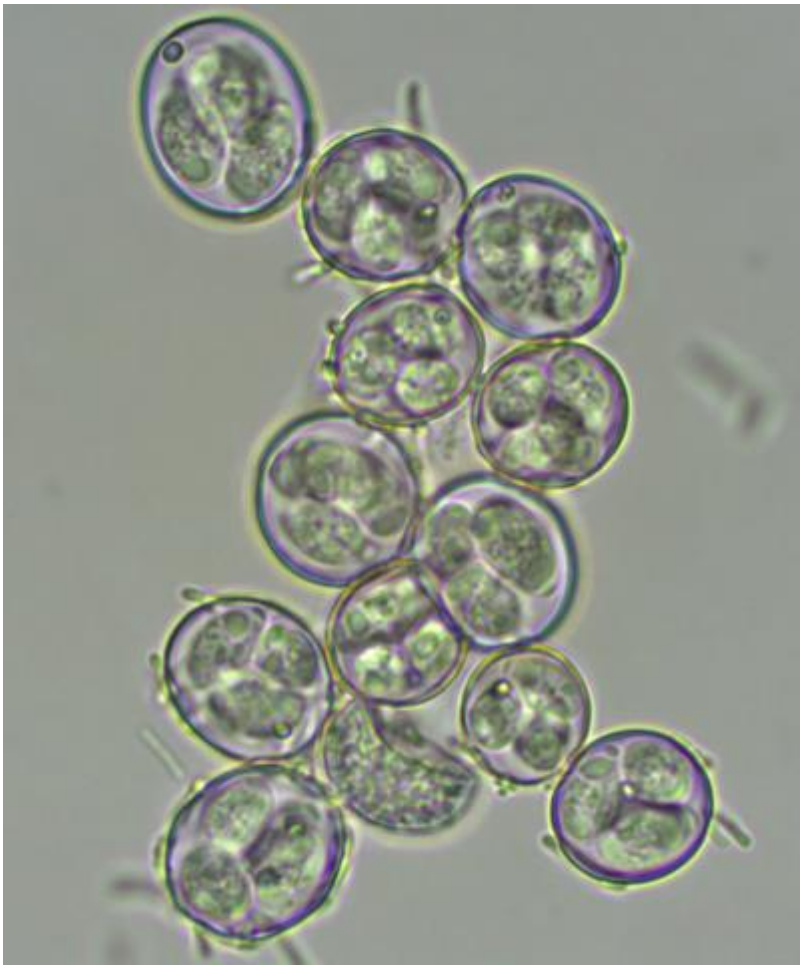

Figure 5-1. Oocyst image taken using a 100x oil immersion lens showing the various sizes and morphologies present after repeated passage of the *E. mivati* monoculture. Measurements taken of oocyst cross sections using the LAS X software (Leica Microsystems Inc., IL) showed a minimum oocyst size of 13.07 µm long x 12.30 µm wide, and a maximum oocyst size of 18.55 µm long x 15.50 µm wide.

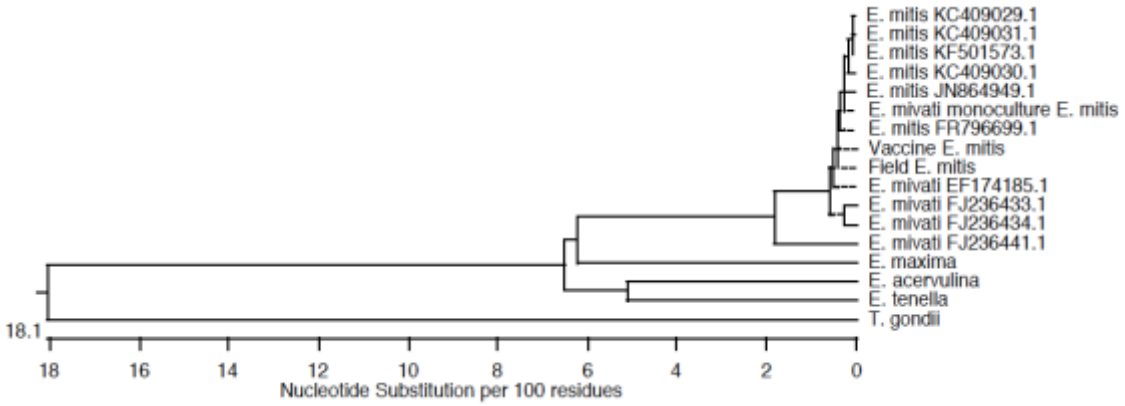

Figure 5-2. Phylogenetic tree of COI sequence alignments created using MegAlign (DNASTAR Inc.). Included are the *E. mitis* sequences detected in the field, monoculture, and vaccine samples, along with reference sequences for the COI gene of *E. mitis*, *E. mivati*, *E. maxima*, *E. acervulina*, *E. tenella*, and *T. gondii* obtained from GenBank (NCBI, MD).

CHAPTER 6

SUMMARY AND CONCLUSIONS

Coccidia and infectious bronchitis virus (IBV) cause two of the most economically significant diseases of commercial chickens: infectious bronchitis and coccidiosis (3, 10). Control of these pathogens and the resulting diseases is essential for the continued success of the poultry industry. Both IBV and coccidia are commonly vaccinated against at day of hatch in the hatchery with the use of live vaccines (1). IBV, as a respiratory pathogen, is typically vaccinated for using a spray cabinet with a serotype-specific vaccine that will ideally reach the mucosal membranes of the chickens through the eyes and nares to infect and stimulate immunity (14). IBV serotypes do not provide cross-immunity, so vaccine serotype selection is essential to prevent a disease outbreak in the field (2). Coccidia vaccines are often applied using a spray cabinet as well, although the application parameters are still a matter of debate. Like IBV, coccidia species do not cross-protect, so vaccines contain live, sporulated oocysts of the species that are most likely to cause a challenge in the environment (21). The goal of this research was to improve vaccination of day-old chicks in the hatchery and increase the level of protection against field challenge for both of these highly impactful diseases. This research aimed to (1) develop a new IBV vaccine of the Arkansas serotype, (2) compare coccidia vaccine application methods, and (3) investigate the genome of one of the contested *Eimeria* species, *E. mivati*.

Recovery of a previously used infectious bronchitis virus Arkansas 99 vaccine. The first aim of this dissertation research was to develop a novel Ark-type IBV vaccine. The Arkansas serotype of IBV is frequently isolated in the field, and the commercially available ArkDPI vaccine

has noted issues regarding efficacy and protection from challenge (9, 11, 12, 18, 23). This lack in infective ability has been attributed to mutations in the spike glycoprotein in the primary vaccine virus population that alter the cell tropism of ArkDPI and render it less capable of infecting and replicating in chicken tissues (16, 17). A previously discontinued Ark99 vaccine was protective against field challenge, however, it caused a severe vaccine reaction in chicks, deeming it unsuitable for continued use (7, 13).

For this project, an Ark99 vaccine seed stock was passaged in embryonating chicken eggs to further attenuate the virus and produce a safe and protective alternative to the ArkDPI vaccine. The new vaccine candidate, deemed ArkGA after passage in embryos, was attenuated over 60 passes in eggs. Every 20 passages, the vaccine was evaluated for efficacy and reaction, and the genome was sequenced using next-generation whole genome sequencing technology to examine the changes occurring over the attenuation process. The ArkGA P60 vaccine was shown to be non-reactive when spray vaccinating broilers, and protective against virulent Arkansas challenge. Furthermore, when examining the genomes of ArkGA P1, P20, P40, and P60, changes in the genome occurred in regions associated with viral pathogenicity, replication, and cell tropism, and the viral SNP population was seen to stabilize over time to form a highly homogeneous vaccine virus population. The genomic data and the infection characteristics in chickens indicate that the ArkGA vaccine candidate is a stable, safe, highly attenuated, and protective alternative to the current ArkDPI vaccine.

Evaluation of coccidia vaccines using traditional and new vaccine application methods. The second aim of this dissertation research was to compare coccidia vaccine application methods. Coccidia vaccines contain live, sporulated oocysts that are ingested by the chickens and stimulate a protective immune response in the intestines (4, 5). The traditional method for coccidia

vaccination is with the use of a water based diluent that is applied by coarse spray in a 21ml volume. However, technology using gel diluents and gel applicator bars for coccidia vaccination is becoming more widely used (22). The purpose of this experiment was to compare the efficacy of the same commercially available coccidia vaccine applied using spray and gel technologies. Fecal oocyst shedding post-vaccination in the first cycle was correlated to the doses applied by each method. In the second cycle, a decrease in shedding was seen for the chickens vaccinated using a more viscous gel. Although there was a slight difference in oocyst shedding data, all vaccinated chickens appeared to be protected from *E. maxima* challenge based on post-challenge body weight gain and gross lesion scores. This experiment demonstrated that all of the current vaccine application methods evaluated are equally effective at protecting chickens from *E. maxima* challenge.

Sequence analysis of the proposed coccidian species Eimeria mivati. As stated previously, *Eimeria* species do not provide cross-immunity in the chicken, so coccidia vaccines need to contain a mixture of oocysts of species that are present in the production environment (26). One commonly used coccidia vaccine for commercial chickens contains *E. mivati* oocysts, which is an *Eimeria* type whose status as a species has been hotly contested since its discovery in the 1970's (6, 8, 15, 19, 24, 25). The aim of this final dissertation project was to investigate the mitochondrial cytochrome C oxidase subunit I (COI) gene of *E. mivati* for comparison to database sequences of other *Eimeria* species. This region of the genome has been cited as the most reliable gene for *Eimeria* species differentiation (20), so the goal was to provide insight into the genetic relatedness of *E. mivati* to other known species that infect chickens. Illumina high throughpout sequencing was used to sequence the COI gene from an *E. mivati* monoculture, a commercially available coccidiosis vaccine containing *E. mivati*, and *Eimeria* DNA from *E. mivati*-infected chicken

intestinal tissue. The Illumina sequencing technology provided COI sequences for all of the species present in each sample, and it was found that none of the samples analyzed contained *E. mivati* DNA. However, all samples contained *E. mitis* DNA, which is the species that many speculate *E. mivati* is a variant strain of. Although this data seems to provide further genomic evidence that *E. mivati* is not a distinct *Eimeria* species, further analysis of other regions of the genome is needed to fully characterize this *Eimeria* type.

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