INFECTIOUS BRONCHITIS VIRUS DMV/1639 AND FALSE LAYER SYNDROME

by

ADREA ELIZABETH MUELLER

(Under the Direction of Brian J. Jordan)

ABSTRACT

Infectious Bronchitis Virus (IBV), a gammacoronavirus of chickens, is considered a respiratory disease virus. Some strains of IBV cause reproductive disease, such as decreased egg quality and production. IBV has also been associated with False Layer Syndrome, characterized by large, fluid-filled cysts in the oviduct of laying hens that cannot lay eggs. These hens appear otherwise normal, making early diagnosis difficult. Known IBV strains associated with False Layer Syndrome include the QX, Massachusetts, and Australian T strains. In the last decade, IBV variant DMV/1639 has been isolated from field cases of False Layer Syndrome. Our research aimed to characterize the role and prevention of DMV/1639 infection in the development of cystic oviduct and False Layer Syndrome. Groups of SPF pullets were challenged with either DMV/1639 or M41 (positive control) at 3-, 7-, or 14-days of age and monitored for gross and histopathological reproductive lesions. Our results indicated that IBV DMV/1639 can cause cystic oviducts that will lead to False Layer Syndrome and a younger age at challenge yields a higher prevalence and severity of cystic oviduct. Our second study evaluated the role of vaccination on development of cystic oviduct caused by DMV/1639. Based on the gross and histological lesions in vaccinated and unvaccinated SPF birds challenged at 7-days of

age with DMV/1639, there did not appear to be a relationship between vaccination strain or timing of administration and the development of cystic oviduct, indicating that incidence of False Layer Syndrome is not just dependent on age at infection or vaccination status. Because SPF pullets do not have maternal antibodies against IBV, we investigated the role of maternal antibodies in the development of cystic oviducts in a third study. From this study, there appears to be a pattern between maternal antibody-positive birds and lower incidence of cystic oviduct. Overall, our results indicate that the development of False Layer Syndrome caused by IBV DMV/1639 is multifactorial, influenced by age at infection, vaccination status, and maternal antibody status. Prevention of loss due to False Layer Syndrome should include a combination of biosecurity, efficient layer breeder vaccination, and vaccination at hatch for layer pullets.

INDEX WORDS: Cystic oviduct, DMV/1639, False layer syndrome, Infectious bronchitis virus, Laying hens, Maternal antibodies, Reproductive disease, Vaccination

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ADREA E. MUELLER

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by

ADREA E. MUELLER

Major Professor:	Brian J. Jordan
Committee:	K. Paige Carmichael
	Karen Grogan
	Mike Petrik

Electronic Version Approved:

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

In memory of my grandmother, MaryAnn Mueller.

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CHAPTER 1

INTRODUCTION

Infectious Bronchitis Virus (IBV) is an economically significant pathogen in commercial chickens that causes increased mortality, decreased feed conversion, high condemnation, and decreased egg production and quality [Awad et al., 2014]. Though mortality rates are low (less than 5%) unless secondary infections are involved, morbidity rates can reach 100% [Awad et al., 2014; Broadfoot et al., 1956]. IBV is horizontally transmitted in birds in natural infection [Sevoian and Levine, 1957] and chickens of all ages are susceptible to IBV, though most severe disease is typically seen in younger birds.

IBV is an avian coronavirus in the subfamily gamma-coronavirus. Coronaviruses are enveloped RNA viruses and have the largest genomes of all the RNA viruses [Masters, 2006]. As a coronavirus, the RNA genome of IBV is single-stranded and positive-sense. Because it is positive-sense RNA, the host cell can initially transcribe the genome as if it were mRNA [Masters, 2006]. The non-segmented genome of IBV codes for four structural proteins. Of these structural proteins, the “spike” outer membrane protein is important for viral attachment to cells and is the main antigen for host immune response [Jackwood and de Wit, 2020]. The spike protein is cleaved into different domains, S1 and S2. Sequence of the spike S1 protein domain varies considerably even across strains in the same species [Wang et al., 1994]. Antibodies to all four structural proteins have been found in response to both live and inactivated vaccinations, though only S1, S2, and N induced antibodies with cross-reactivity [Ignjatovic and Galli, 1995].

Because IBV is an RNA virus that utilizes a polymerase with poor proof-reading capacity for replication, it has a higher mutation rate than DNA viruses and can also undergo recombination. The accumulation of these mutations, particularly in the spike gene, lead to new serotypes of IBV. A serotype is a viral strain under the Infectious Bronchitis Virus species but is antigenically different from other IBV strains. New serotypes of IBV primarily emerge from genetic drift, where a few of these mutated strains infect susceptible birds and is especially significant when the mutations occur in the antigenic region of the viral genome [Lee and Jackwood, 2001, Jackwood and Jordan, 2021]. There is little cross-protection between serotypes, which is an important consideration in vaccine development [Jackwood et al., 2012].

IBV most commonly causes respiratory disease, though it can also affect kidneys and the reproductive tract. The clinical signs of IBV are generic, including gasping, tracheal rales, and "snicking" [Raj and Jones, 1997]. Additional clinical signs include weakness, depression, huddling, ruffled feathers, and anorexia leading to decreased body weight [Ignjatovic and Sapats, 2000]. Gross lesions for respiratory disease caused by IBV are slight tracheal hyperemia, tracheitis, catarrhal exudate in the trachea, nasal and ocular discharge, and airsacculitis [Benyeda et al., 2010; Cook et al., 2012].

IBV targets upper respiratory ciliated epithelial cells, kidney epithelial cells, and epithelial cells in the female reproductive tract [Cook et al., 2012]. There is also evidence of viral replication in lymphoid tissues, though this does not appear to lead to immunosuppression as a direct result of IBV infection [Raj and Jones, 1996c]. Typically, IBV respiratory disease occurs in young chicks two to six weeks of age, but it can also cause clinical signs and production loss in older chickens. Age, immune status, genetic

line, and other factors can influence disease severity seen with infectious bronchitis virus infection [Nakamura et al., 1991].

Nephropathogenic strains of IBV cause swelling and urates which lead to kidney damage. Kidney damage can be exacerbated by predisposing factors such as genetic lines, diet, and stress. Some research suggests that, when combined with stressors, all IBV strains can induce kidney disease [Cook et al., 2012]. At necropsy, affected kidney lesions can include swelling and enlargement, pallor, white urate accumulation in tubules, and occasional urate deposits resembling visceral gout on pericardium, serosal liver surface, and articular cartilage joint surface [Ziegler et al., 2002]. Renal damage caused by IBV leads to increased mortality, however in some instances the kidneys will recover from infection via regeneration and contain little to no permanent lesions [Chen et al., 1996; Purcell et al., 1976].

Some serotypes of IBV are known to affect the epithelial cells of the oviduct, leading to transient or permanent decreases in egg production and quality. Economic losses in the laying industry are due to not only quantity decreases but decreases in quality such as soft-shelled or misshapen eggs. Considerable economic loss comes from infected hens who pause production and then do not resume normal production levels. Eggs might be smaller or have poorer internal qualities such as watery albumin [Broadfoot et al., 1956; Muneer et al., 1986; Ignjatovic and Sapats, 2000]. In breeder flocks, the hatchability of eggs can be significantly reduced after infection [Broadfoot et al., 1956; Muneer et al., 2000]. IBV has been isolated from the semen and eggs of infected adults, but there is no indication that this causes disease in the progeny via vertical transmission [Cook, 1971].

False Layer Syndrome is described as a reproductive clinical sign and result of early exposure to Infectious Bronchitis Virus that causes fluid-filled cysts to develop in the oviduct [Sevoian and Levine, 1957]. These cystic oviducts lead to permanent damage that will prevent egg production in the affected birds [McMartin and Macleod, 1972; de Witt et al., 2011]. Previously, IBV strains QX, Massachusetts, and the Australian T have been associated with cystic oviduct formation and False Layer Syndrome, especially when birds are infected at a young age [de Wit, et al., 2011; McMartin and Macleod, 1972; Broadfoot et al., 1956]. Other factors that determine the variability of reproductive disease severity are viral strain/serotype, length of infection, and factors specific to individual hosts such as breed [Chousalkar and Roberts, 2007; Dolz et al., 2012].

Recently, a new strain of IBV, DMV/1639/11, has been associated with cystic oviduct development and false layer syndrome in the United States and Canada, though no causal relationship had been shown at the initial time of virus isolation. This serotype appears to have evolved from a variant that caused the 1997-2000 outbreak in Pennsylvania, referred to as NIBV PA/171/99 (nephropathogenic infectious bronchitis virus) [Gelb et al., 2012]. Its pathogenicity seems to have progressed from nephropathogenic to primarily respiratory, though effects on the reproductive tract were not noted initially [Jackwood and Lee, 2017]. DMV/1639 is now present across the United States and Canada [Hassan et al.,2019] and is the most prevalent variant circulating in the United States [Jackwood and Jordan, 2021], particularly in breeder and layer flocks.

In the field, prevention of cystic oviduct formation has been attempted through the addition of IBV vaccination at the hatchery to offer some protection against early DMV/1639 challenge [da Silva et al., 2021]. Vaccination is the most used prevention

method against IBV, though it can be difficult to achieve protection due to differences between the field or variant serotypes and the vaccine serotypes [Gelb Jr et al., 1991]. Vaccination with high-titer vaccines has been shown to produce better serological protection than lower titer vaccinations, though clinical signs caused by vaccination must be considered for vaccines of significant titers [Raggi and Lee, 1965]. Massachusetts-strain vaccines are considered the "industry-standard" for IBV vaccination [Sheng et al., 2020], although vaccination strategies are often serotype specific and can have limited cross-protection [Kulkarni and Resurreccion, 2010]. Instances of vaccination with one serotype vaccine protecting against an antigenically distinct serotype have been recorded, however. To date, there is no commercial vaccine available for variant DMV/1639.

Vaccination programs for chickens are typically evaluated based on efficacy to decrease or eliminate respiratory challenge as opposed to reproductive effects [McMartin, 1993]. Ideally, protection against respiratory challenge will prevent the systemic spread of the virus and eliminate the need for specific reproductive efficacy assays. The case report by Landman [2005] illustrates the importance of using serotype-specific vaccines. In this case, birds were vaccinated in the hatchery for one serotype of IBV but later developed False Layer Syndrome and cystic oviduct. Landman hypothesizes that this development is a result of improper protection at the hatchery and exposure of an unrelated IBV serotype in the field at a young age. Vaccination programs that induce high antibody titers and uniform coverage are most effective at reducing loss due to reproductive lesions [Box et al., 1988].

One key difference between the specific pathogen free (SPF) birds commonly used in research and commercial birds is the presence of maternal antibodies against IBV in

commercial birds. Maternal antibodies are passed from hen to chick to protect the chick in the early stages of the development of its immune system. Passive immunity through maternal IgY antibodies is suspected to protect young chicks from abnormal oviduct development and severe reproductive lesions due to early IBV infection [Broadfoot et al, 1956; Crinion et al., 1971a]. The effects of maternal antibody presence on vaccination are debated. It is possible that the presence of maternal antibodies in chicks will decrease the success of vaccination against infectious bronchitis virus at a young age [de Wit et al., 2011]. Similar results have been seen in Infectious Bursal Disease Virus, where high maternal antibody levels against IBDV decreased the bird's response to both vaccination and to challenge [Naqi et al., 1983].

Vaccination of chicks who have maternal antibodies against IBV has been shown to be less effective than vaccination in maternal antibody negative birds. In addition, vaccination at day-of-hatch can also speed up the decline of maternal antibodies against IBV [Mondal and Naqi, 2001]. Alternatively, de Wit et al found that a combination of high maternal antibody presence and heterologous vaccination at hatch offered protection against the kidneys when birds were challenged at 6 or 10 days of age with the D388 virus. The authors extrapolated this protection to extend to the oviduct [2011]. In addition, Winterfield et al. [1976] describe an increased shed-rate of challenge virus in birds that did not have passive maternal antibodies compared to the group of birds with maternal antibodies when both were vaccinated at one day-of-age.

This dissertation investigates the reproductive effects of IBV variant DMV/1639 challenge at a young age and the impact that the immune status of a flock has on these effects. Specifically, this research aims to (1) evaluate the effect of IBV DMV/1639 on the

reproductive tract of pullets and the effect that age at challenge has on these effects; (2) determine the impact of commercial vaccination on cystic oviduct prevalence in a flock infected at 7 days of age with DMV/1639; and (3) evaluate the effect of maternal IBV antibodies on cystic oviduct prevalence in young vaccinated and unvaccinated pullets challenged with DMV/1639. The results of this research provide valuable insight that will aid clinicians with the identification and prevention of cystic oviduct and subsequent False Layer Syndrome in layer flocks.

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CHAPTER 2

LITERATURE REVIEW

1. Layer Industry

The "layer industry" refers to the production of chicken eggs and the rearing of the pullets and hens to provide those eggs. Eggs are a primary source of animal proteins in both the United States and across the world [Mench et al., 2011]. After China and the European Union, the United States is the third largest egg producer worldwide [Mench et al., 2011]. The quality of eggs and production values are influenced by hen health, nutrition, and housing/environmental factors.

Pullets, or immature female chickens, are kept in "grow-out" houses until 16-18 weeks of age. At this age, they will begin to lay eggs and are moved to layer houses. There are several types of housing systems used in the layer industry: conventional, enriched cages, and non-cage systems. Current research into the advantages of one system over another is conflicting and all have benefits and drawbacks [Dikmen et al., 2016]. Conventional housing, or battery cage system, is the most common laying hen housing system in the US. Conventional battery cages are economically efficient and decrease the incidence of parasitic diseases because hens and eggs are separated from feces. The most prominent concerns with the conventional housing system are animal welfare and public perception due to movement and space restrictions [Mench et al., 2011].

Alternatives to the conventional battery systems are enriched cage systems or non-cage systems. Enriched cage systems [also referred to as furnished or modified cages] aim

to resolve the animal welfare concerns of the conventional systems. Enriched cages can include perches, nesting areas, dust bath material, foraging material, and/or scratch pads to increase the natural behaviors of the animals [Mench et al., 2011]. One example of enriched cages is the Edinburgh Modified Cage System. This system uses cages enriched with perches, nest boxes, and dust baths. Appleby found that the Edinburgh Modified Cage system increased the welfare of the birds without decreasing the hen and egg quality [1998].

Non-cage systems include aviaries and floor systems. Aviaries are multi-level, incorporate nesting boxes in the above the ground level, and can allow for higher stocking densities than floor systems. The nest boxes of floor systems are at ground level and hens are housed solely on the floor [Mench et al., 2011]. Floor systems such as barn systems often require manual egg collection, so collection timing and storage are especially important considerations for egg quality coming from these systems [Ahammed et al., 2014]. Research colony houses are considered floor systems.

Laying hen operations in the 2000s are most often either “in-line” or “off-line” systems. In-line systems are most common, use the same facility for laying, egg collection, and egg-grading and result in improved hen productivity and efficiency. In the 21st century, family-owned off-line system operations are increasing for specialty markets such as organic or free-range eggs. In off-line systems, the eggs are cleaned, graded, and packaged at locations other than where they are laid [Kidd and Anderson, 2019]. Laying hen flocks in the United States are often kept for two or fewer production cycles [Fredrickson, 1987]. Regardless of the changing regulations regarding housing systems, egg production remains an important aspect of US agricultural economy and diet.

2. Production

There are several factors that can affect the productivity of laying hens, both environmental and internal, or combinations of both. Light duration, wavelength, and intensity are known to impact the reproductive activity of hens. In a study by Baxter and Bedecarrats, red light led to stronger ovarian activity than light at other wavelengths [2019]. Length of light exposure can also affect reproductive activity. These factors can be manipulated in housing environments to increase or cease production, as necessary.

Though the layout and size of hen housing is one of the most important factors in hen management, there is conflicting evidence regarding the impact of housing systems on egg quality and production. Some authors found that egg production and hen performance are not significantly impacted by the type of housing system used [Ahammed et al., 2014]. Others have found that housing systems do have an influence, especially on egg quality factors [Englmaierova et al., 2014]. Egg quality factors significantly impacted by housing include shell weight, thickness, density, and strength [Sokolowicz et al., 2018].

When comparing shallow caged systems to deep caged systems, improved productivity can be associated with shallow caged systems even when stocking densities are controlled for. However, it is important to note that the shallow cage systems are likely to be more costly per hen [Adams and Craig, 1985]. High stocking density can contribute to increased disease, which negatively impacts hen productivity [Tauson et al., 1999]. Some studies have determined that caged eggs have higher egg quality and less bacterial contamination that alternative systems, but more research needs to be conducted because study results vary [Enlmaierova et al., 2014].

Outside of housing system, other factors that influence production are both internal and environmental. The genetic line of hens can influence hen productivity, especially when combined with other factors such as housing system [Tauson et al., 1999; Sokolowicz et al., 2018]. Baxter and Bedecarrats stated that metabolic status, age, and body weight appeared to have more of an impact on the initiation of sexual maturity than photoperiod or wavelength of light exposure [2019]. High ambient temperatures and other environmental stressors can lead to decreased feed conversion rates, decreased body weights, and impaired immune system function [Sahin et al., 2009]. When trying to maximize hen productivity, access to and composition of feed need to be taken into consideration. Without easy access to these, the hens will experience elevated stress. Many of the scenarios mentioned above will elevate stress for hens. Stress can lead to decreased feed and water consumption, ovarian regression, and shortened oviducts, all which cause decreased hen productivity [Sevoian and Levine, 1957].

3. Normal Female Reproduction

3.1 Ovary

Eggs originate as follicles in the ovary of hens. The chicken ovary is a collection of follicles that stem from the ovarian cortex, which is found around the vascular medulla. Each follicle is made of an ovum. Around the ovum are the membrane granulosa and two thecae layers [Sevoian and Levine, 1957]. The high rate of egg productivity in chickens in the laying industry is a result of their rapid ovarian follicle development. Laying hens can lay clutches of eggs over consecutive days, up to an egg a day, and ovulate at approximately 24-hour intervals [Johnson, 2015].

At hatch, the hen's ovary is fully formed and contains all the ova a hen could potentially lay. The young ovary is smaller than the ovary of a sexually mature bird and contains undeveloped follicles. Ovulation of the next yolk occurs immediately after the previous egg is oviposited [Warren and Scott, 1935; Nys, 1999]. The largest primary follicle [F1] is the follicle selected for ovulation. The selected follicle/yolk is ovulated from the left ovary into the left oviduct [Johnson, 2015].

3.2 Oviduct

In most healthy chickens, the right oviduct and ovary regress, and the left proceeds with development. In some instances, in normal and abnormal hens, the right atretic oviduct will develop a small fluid-filled cyst. The healthy left oviduct, made up of repeated loops and folds, is attached to the dorsal wall of the abdominal cavity via the dorsal mesentery [Warren and Scott, 1935]. The glandular tissue of the oviduct secretes parts of the egg aside from the yolk and is divided into five regions: infundibulum, magnum, isthmus, uterus, and vagina [Johnson, 2015].

3.3 Infundibulum

The muscular infundibulum captures the ovulated yolk and it is here that the perivitelline layer and the chalazae of the egg are formed. The perivitelline layer surrounds the yolk and separates it from the albumen [egg white]. Egg chalazae are twisted membranes that ultimately keep the yolk in place within the eggshell. Fertilization occurs in the infundibulum in birds who are used for breeding [Johnson, 2015].

3.4 Magnum

The next region of the oviduct, the magnum, is referred to as the albumen-secreting region. It is here that the albumen proteins are added to the developing egg. The albumen

of the egg is made up of two layers, thick and thin albumen. Albumen proteins provide the yolk with protection against physical and bacterial damage. The magnum is made up of mucous membranes that fold longitudinally [Warren and Scott, 1935]. The mucous membranes are composed of lining epithelium of "ciliated cells alternating with granular cells and an underlying layer of tubular glands" [Davidson, 1986]. There is a sharp line of demarcation between the magnum and isthmus, which resembles a transparent band between the folds [Warren and Scott, 1935; Johnson, 2015].

3.5 Isthmus

In the isthmus, the shell membranes [inner and outer] are produced for approximately 3.5 hours after ovulation [Nys, 1999]. The glands and folds in the isthmus are less prominent than those in the magnum. Some studies show that small quantities of proteins are added to the albumen in this region. In the isthmus, proteins are produced that are essential for the shell membrane production. The isthmus-uterine line is distinguished by the abrupt end of the longitudinal folds of the isthmus mucous membrane [Warren and Scott, 1935; Johnson, 2015].

3.6 Uterus [shell gland]

The uterus, also referred to as the shell gland, is where the eggshell is formed. The shell is made predominantly of calcium carbonate from both the bone calcium via bloodstream and the dietary calcium of the hen. Eggshells are composed of six layers, including the inner and outer shell membranes [Nys, 1999]. The cranial section of the shell gland is short in appearance. The developing egg spends the longest amount of time in the distal and pouch-like region of the uterus, sometimes longer than 20 hours [Johnson, 2015].

3.7 Vagina

The vagina is the final section of the oviduct and is separated from the uterus by the vaginal sphincter. Near this sphincter are mucosal folds referred to as sperm storage tubules where hens can store sperm for short periods of time after mating. The muscles of the vagina push the egg out of the vagina and cloaca, a process also referred to as oviposition. The opening of the cloaca is the vent, where the fully formed egg passes through during oviposition [Johnson, 2015].

3.8 Eggs

Egg quality is an important component of the layer industry. Egg quality, both exterior and interior, is evaluated in several ways. Egg-shell quality can be measured by size, specific gravity, color, breaking strength, presence of deformities, weight, thickness, and ultrastructure [Roberts, 2004]. The internal quality of eggs is measured by yolk color, albumen quality, and the integrity of the perivitelline membrane around the yolk [Roberts, 2004]. Albumen index [height of the thick white divided by the width] can also be used as a determinant of egg quality [Heiman and Carver, 1936]. The Haugh unit, another calculation considering albumen height and egg weight, is widely used to assess egg albumen quality. However, Haugh measurements can sometimes overestimate albumen height of small eggs and underestimate for larger eggs, leading to inaccurate quality assessment [Eisen et al., 1962].

4. Normal Development

In the chick embryo, there are two periods of reproductive development. In the first period, the reproductive tissues begin to develop without distinction between genotypic sex. The second period is controlled by steroid hormones and is sex-specific [Gasc and Stumpf, 1981]. Both the reproductive system and the urogenital system develop from the

intermediate mesodermal ridge [Hoar, 1978]. The mesoderm is the layer of cells between the ectodermal and endodermal cell layers in embryonic development. The oviduct tissue specifically develops from the mesonephric [Wolffian] ducts from the mesodermal ridge. The paramesonephric ducts combine, and at their junction, the Müller's tubercle (first evidence of embryonic female reproductive organs) develops [Hoar, 1978]. Beginning on the third day of embryonation, the Müllerian ridge forms, from which the Müllerian ducts later develop once the Wolffian ducts reach the cloaca [Jacob et al., 1999]. The female reproductive tract develops from the Müllerian ducts, which are retained in the hen and involved in further reproductive development. The right gonad and Müllerian duct later regress [Ayers et al., 2015]. The ostium tubae funnels develop from the cranial ends of the Müllerian ducts and the ducts fuse with the mesonephric ducts ventrally [Jacob et al., 1999]. When the chick hatches, the reproductive tract is still developing.

Estrogen stimulates oviduct development in immature chickens, which involves post-embryonic cytodifferentiation [Kohler et al., 1969]. Stem cells differentiate from the immature oviduct tissue into three epithelial cell types: tubular gland cells, ciliated cells, or goblet cells [Kohler et al., 1968]. The tubular gland cells are responsible for the main egg white protein, ovalbumin, production. Ciliated oviduct cells move material through the oviduct, and goblet cells are involved in the synthesis of avidin in the egg white [Kohler et al., 1968]. Of the five regions of the developing oviduct, the magnum develops the fastest due to continuous hyperplasia [Yu and Marquardt, 1972].

In the two months before the onset of lay, the pullet reproductive tract undergoes rapid maturation and is regulated by sex hormones [Hafez and Kamar, 1955]. The most important factor in reproductive maturation is photoperiodicity. A mature hen's body will

recognize an increased photoperiod length and Gonadotropin-releasing hormone [GnRH] will be released from the hypothalamus. This GnRH stimulates the release of pituitary gonadotrophins that initiate ovarian follicle activation. Nutrition and feed consumption also play a role in the sexual maturation of hens. When the reproductive tract matures, the oviduct increases in length and diameter. Oviduct size and age are often but not always directly related. After maturation, the lay period of mature hens lasts approximately twelve months [Yu and Marquardt, 1972]. After the lay period, the hen spends approximately two months in a molting stage where the oviduct regresses. Oviduct regression resembles the reverse stages of oviduct development. [Yu and Marquardt, 1972; Johnson, 2015].

5. Normal Reproductive Histology

The immature oviduct is composed primarily of mucosal epithelium with stroma underneath. The mucosal epithelium is composed of pseudostratified columnar cells that contain sporadic microvilli. The nuclei of the epithelial cells are prominent and oval in shape. Below the mucosal layer is the stromal layer of collagen and compacted polygonal cells. The nuclei of these cells are typically rounded, and mitotic figures are present occasionally in both the mucosa and the stromal layers [Kohler et al., 1969].

Sevoian and Levine [1957] describe seven layers of mature oviduct cross-sections, as written below. The innermost layer is a lining of epithelial elements, surrounded by the tubular gland layer. Next is the lamina propria, a layer of connective tissue with small blood vessels. Circular muscle fibers make up the layer on top of the lamina propria. Around this layer is another layer of connective tissue with large blood vessels. The final two layers are longitudinal muscle fibers that are associated with the muscles of the ventral and dorsal oviduct ligaments and the outermost layer of serous epithelium.

6. Common Diseases of Laying Hens

Caged laying hens in the United States are vulnerable to a number of common ailments of environmental, nutritional, and infectious origin. Rickets, caused by an imbalance in the calcium:phosphorus ratio is often found in adult layers and is sometimes referred to as Cage Layer Fatigue. Birds will exhibit lameness and may lay thin-shelled eggs or stop production. A lack of vitamin D can cause Rickets in young birds [Shivaprasad, 2019]. Fatty Liver Syndrome, a result of too many carbohydrates in feed, is one of the highest causes of death in caged laying hens and causes hemorrhaging of the liver [Shini et al., 2019]. Caged laying hens also face viral diseases such as Marek's, Egg-Drop Syndrome, and Infectious Bronchitis Virus which cause decreases in egg production and flock performance.

7. False Layer Syndrome

In the last decade, the influence of False Layer Syndrome on the levels of production in the layer industry has become more prominent. The presentation of False Layer Syndrome, as defined by Sevoian and Levine in 1957, is "a bird with every appearance of a normal bird" that "is out of production and does not lay any eggs." More specifically, false layer syndrome refers to a decrease in egg production of mature layers that are exhibiting little to no other clinical signs [de Wit et al., 2011]. Some birds affected by False Layer may exhibit waddling or a pendulous abdomen when palpated, but many exhibit normal behaviors including nesting behaviors, vent appearance, and pubic bone characteristics [Broadfoot et al., 1956; Landman, 2005]. Egg production levels in these outwardly normal birds have been shown to drop as low as 30% of normal production

values, as demonstrated in a case report from a Canadian layer flock [Landman, 2005] but commonly cause production drops in the 13-18% range [de Silva et al., 2021].

The ovaries of false layers can appear normal, including empty follicles from recent ovulation. Some cases have included accumulation of yolk in the abdomen, indicating that the oviduct was not accepting ovulated yolk and ovulation was taking place into the body cavity instead [Broadfoot et al., 1956; McMartin and Macleod, 1972]. Cyst development in the oviduct with normal ovary development is hypothesized to cause these instances of False Layer. These cystic oviducts lead to permanent damage that will cease egg production in the affected birds [Crinion et al., 1971b]. In the 1957 description, False Layer Syndrome is described as a clinical sign and result of exposure to Infectious Bronchitis Virus [Sevoian and Levine, 1957].

8. Infectious Bronchitis Virus

Infectious Bronchitis Virus [IBV] is an economically significant pathogen in commercial chickens that causes increased mortality, decreased feed conversion, high condemnation, and decreased egg production and quality [Awad et al., 2014]. Though mortality rates are low [less than 5%] unless secondary infections are involved, morbidity rates of IBV can reach up to 100% [Broadfoot et al., 1956; Awad, et. al 2014]. IBV is horizontally transmitted in birds post-embryonically in natural infection [Sevoian and Levine, 1957]. Birds of all ages are susceptible to IBV, though most severe disease is typically seen in younger birds [de Wit, et al., 2011; McMartin and Macleod, 1972; Broadfoot et al., 1956; Hassan et al., 2021; Mueller Slay et al., 2022]. Disease severity is dependent upon a combination of factors including age at exposure, strain of IBV that birds are exposed to, breed of bird, and route of inoculation. For example, a QX-like strain can

cause more severe disease in birds 30-days old versus a Malaysian strain in birds one-day old at inoculation [Khanh et al., 2018].

IBV is an avian coronavirus in the subfamily gamma-coronavirus. Coronaviruses are enveloped RNA viruses and have the largest genomes of all the RNA viruses. As a coronavirus, the RNA genome of IBV is single-stranded and positive-sense. Because it is positive-sense RNA, the host cell can initially transcribe the genome as if it were mRNA. The non-segmented genome of IBV codes for four structural proteins. The membrane protein [M] provides the shape of the virion and the sequence is relatively well conserved [Masters, 2006]. The M protein is also the most common protein in coronaviruses [Rottier, 1995]. The envelope protein [E] is an integral membrane protein that plays a role in viral assembly [Corse and Machamer, 2000], and the role of the nucleocapsid [N] protein is to bind the viral RNA to the viral nucleocapsid [Laude and Masters, 1995].

The final of these four structural proteins, the "spike" glycoprotein, gives coronaviruses their "sun-like" appearance [Almeida et al. 1968]. This spike protein is important for viral attachment to cells and is the main antigen for host immune response [Jackwood and de Wit, 2020]. The spike protein can be cleaved into different domains, S1 and S2. Sequence of the spike S1 protein domain varies considerably even across strains in the same species and is the major antigenic region for the virus [Wang et al., 1994]. Antibodies to all four structural proteins have been found in response to both live and inactivated vaccinations, though only S1, S2, and N induced antibodies with cross-reactivity [Ignjatovic and Galli, 1995]. In addition to the four structural proteins, the genome of coronaviruses codes for viral replicase [the only protein to be transcribed directly from the genome], non-structural and accessory proteins, and contains multiple

open reading frames [Masters, 2006]. IBV targets epithelial cells throughout the body. It uses sialic acids on these cells' surface for primary attachment for infection and replication [Winter et al., 2006].

Because IBV is an RNA virus, it has a higher mutation rate than DNA viruses and can also undergo recombination. The mutation rate for IBV is estimated to be $1x10^{-4}$ substitutions per nucleotide [Lee and Jackwood, 2001]. Accumulation of these mutations, especially in the spike protein, lead to new serotypes of IBV. A serotype is a viral strain under the Infectious Bronchitis Virus species but has enough difference in the spike glycoprotein sequence to be antigenically separate from other IBV strains. New serotypes of IBV primarily emerge from genetic drift, which is especially significant when the mutations occur in the antigenic region of the viral genome [Lee and Jackwood, 2001, Jackwood and Jordan, 2021]. Virus neutralization tests or hemagglutinin-inhibition tests can be used to differentiate between IBV serotypes [Ingjatovic and Sapats, 2000]. There is little cross-protection between serotypes, which is an important consideration in vaccine development [Jackwood et al., 2012]. The R_0, the average number of birds that a single infected bird will transmit virus to, of IBV is approximately 20 without vaccination. When birds are vaccinated, the R_0 value decreases to 0.69 [de Wit et al., 1998].

9. IBV Respiratory Disease

9.1 General

IBV most commonly causes respiratory disease, though it can also affect kidneys and reproductive tracts, and the effects on the gastrointestinal tract are debated [Jackwood and de Wit, 2020] IBV targets upper respiratory ciliated epithelial cells, kidney epithelial cells, and epithelial cells in the female reproductive tract [Cook et al., 2012]. There is also

evidence of viral replication in lymphoid tissues, though this does not appear to lead to immunosuppression as a direct result of IBV infection [Raj and Jones, 1996c]. Typically, IBV respiratory disease occurs in young chicks two to six weeks of age, but it can also cause clinical signs and production loss in older chickens. Age, immune status, genetic line, and other factors can influence disease severity seen with infectious bronchitis virus infection [Nakamura et al., 1991].

9.2 Clinical signs

The clinical signs of IBV are generic, including gasping, tracheal rales, and "snicking" [Raj and Jones, 1997]. Additional clinical signs include weakness, depression, huddling, ruffled feathers, and anorexia leading to decreased body weight [Ignjatovic and Sapats, 2000; Xu, 2019]. Gross lesions for respiratory disease caused by IBV are slight tracheal hyperemia, tracheitis, catarrhal exudate in the trachea, nasal and ocular discharge, and airsacculitis [Benyeda et al., 2010; Cook et al., 2012].

9.3 Histopathology

Histopathological lesions caused by IBV can be divided into degenerative, hyperplastic, and recovery stages [Nakamura et al., 1991 and Benyeda, et al., 2010]. In a study conducted by Nakamura et al., the following histological changes were noted with IBV infection and characterized into the three stages. The degenerative stage can be characterized by a loss of cilia in tracheal epithelial cells, a decrease in mucus-producing cells such as goblet cells, and an increase in heterophils and desquamated epithelial cells found between ciliated epithelial and basal cells [Nakamura et al., 1991]. In the hyperplasic stage that follows the degenerative stage, non-ciliated cells made up most of the newly formed epithelial cell population and these cells exhibited hypertrophy and hyperplasia

with occasional mitotic figures. Mucus-producing goblet cells were not seen. Purulent exudate in the lumen was noted in some cases. [Nakamura et al., 1991]. Goblet cell populations began to return by the recovery stage and alveolar mucus glands were once again found in the epithelium crypts. The epithelium was determined to be made up by columnar ciliated cells in this stage. IBV antigen was detected in tracheal epithelial cells at highest quantities in the degenerative and hyperplastic phases [Nakamura et al., 1991].

10. IBV Nephropathogenic Disease

In addition to respiratory disease, some strains of IBV cause kidney disease. These effects can be compared to gout in humans [Xu et al., 2019]. Nephropathogenic strains of IBV cause swelling and urates which lead to kidney damage. Kidney damage can be exacerbated by predisposing factors such as genetic lines, diet, and stress. Some research suggests that, when combined with stressors, all IBV strains can induce kidney disease [Cook et al., 2012].

At necropsy, affected kidney lesions can include swelling and enlargement, pallor, white urate accumulation in tubules, and occasional urate deposits resembling visceral goat on pericardium, serosal liver surface, and articular cartilage joint surface [Ziegler et al., 2002]. Histopathological analysis of kidneys infected with IBV show tubular epithelial cell detachment with some necrosis and regeneration, loss of tubular structure, interstitial expansion and inflammatory cell infiltration [primarily lymphoplasmacytic] [Xu et al., 2019; Ziegler et al., 2002]. IBV can also cause cystic tubules in the cortex and medulla, as well as necrotic tubules throughout the kidneys [Purcell et al., 1976]. Renal damage caused by IBV leads to increased mortality, however in some instances the kidneys will recover

from infection via regeneration and contain little to no permanent lesions [Chen et al., 1996; Purcell et al., 1976].

11. IBV Gastrointestinal Disease

The effects of IBV on the gastrointestinal tract of chickens is controversial. Cecal tonsils are known play an important role in IBV persistence and replication. [Alexander et al., 1978]. Ambali and Jones [1990] found some microscopic lesions such as desquamation, congestion, and focal immune cell infiltration in the rectum of IBV-infected birds. These birds were inoculated at 3 weeks of age and the virus persisted for up to 28 days in the GI tissue. No microscopic lesions were noted in other areas of the GI tract. Despite viral persistence and microscopic lesions, no gross lesions or enteritis was noted. These findings suggest that IBV is capable of replicating in the GI epithelium, though it might not cause pathological changes [1990]. Huack et al. recently isolated the IBV strain CalENT from the intestinal tract of birds experiencing Runting-Stunting Syndrome and were able to recreate the disease in SPF chickens and isolate it from the embryonic intestinal tract, though they were unable to recover it from the chorioallantoic membrane [2016].

12. IBV Reproductive Disease

12.1 Overview

Some serotypes of Infectious Bronchitis Virus are known to affect the epithelial cells of the chicken reproductive tract. As in the tracheal epithelium, IBV challenge can cause ciliostasis in oviduct epithelial cells [Raj and Jone, 1996b]. IBV infection can cause decreases in egg production and quality. Economic losses in the laying industry are due to not only quantity decreases but decreases in quality such as soft-shelled or misshapen eggs. Considerable economic loss comes from infected hens who pause production and then do

not resume normal production levels. Eggs might be smaller or have poorer internal qualities such as watery albumin [Broadfoot et al., 1956; Muneer et al., 1986; Ignjatovic and Sapats, 2000]. In breeder flocks, the hatchability of eggs can be significantly reduced after infection [Broadfoot et al., 1956; Muneer et al., 2000]. IBV has been isolated from the semen and eggs of infected adults, but there is no indication that this causes disease in the progeny via vertical transmission [Cook, 1971].

Though numerous factors contribute to the pathogenesis of IBV, gender also appears to play a role. In some cases, cockerels develop more severe tracheal lesions than hens infected with the same serotype. In this same case, hens develop more severe harderian gland lesions than cockerels [Chousalkar et al., 2007]. In males, IBV has a less understood effect on the reproductive system but live attenuated Massachusetts (Mass) vaccination in males has been shown to increase formation of calcium carbonate epididymal stones which decreases sperm production and testosterone levels [Boltz et al., 2004]. Research indicates that IBV can replicate in the testes, which contain ciliated epithelial cells [Villarreal et al., 2007]. Villarreal et al discuss that IBV is likely to replicate in the ciliated epithelium of the testes in roosters and can cause reproductive damage such as destruction of efferent ductules, destruction of the epididymis, and destruction of the seminiferous tubules that are all associated with decreases in male fertility, though the exact pathogenesis is unknown [2007]. Unilateral testis hypertrophy and the presence of infiltration of heterophilic and mononuclear inflammatory cells has also been described [Pereira et al., 2019].

As a result of IBV infection, the presence of cytotoxic cells and proinflammatory cytokines increases. In the oviduct, specifically the magnum and isthmus, this leads to a

loss of collagen and proteins essential to calcium transport and can cause soft or mishappen eggs [Nii et al., 2014]. The effects of IBV on egg production rates/quality can vary in severity per individual based on several factors, including vaccination methods, age, and immune status [Box et al., 1988]. Even in experimental studies of infected birds kept in the same housing/environmental conditions from the same parental flock, a portion of infected birds begin to lay normally and comparably to control uninfected birds despite other birds in the same group being negatively affected by infection [Jones and Jordan, 1972]. Some literature suggests that these negative effects are permanent and production rates will not recover, especially after significant epithelial cell damage [Sevoian and Levine, 1957; Crinion and Hofstad, 1972a].

Other factors that determine the variability of reproductive disease severity are viral strain, length of infection, and factors specific to individual hosts such as breed [Chousalkar and Roberts, 2007; Dolz et al., 2012]. In some markets, brown eggs are preferred over white. IBV can cause pale color in brown eggs, an effect that will lead to economic loss [Cook and Huggins, 1986; Chousalkar and Roberts, 2007]. Additional economic loss due to reproductive effects of IBV is due to the reduced hatchability of eggs and increased chick mortality from affected breeder flocks [Broadfoot et al., 1956; Muneer et al., 2000].

The reproductive effects of IBV infection can also vary across and within serotypes. Chousalkar and Roberts [2007] did not see the widespread reduced egg production with infection of 30-week-old hens that Cook [1971] saw with Massachusetts strain infection. A few of the birds in the Chousalkar and Roberts 2007 study did exhibit production loss or cessation, which they associated with viral particles in the oviduct. Coinfection with other pathogens such as Mycoplasma gallisepticum can increase the severity of the reproductive

effects seen with IBV infection, though the exact relationship is not well described [McMartin, 1968; Cook et al., 2012].

Compared to the respiratory tract, IBV in the reproductive tract is detected later in infection and in a smaller time frame. Some studies detected IBV in the oviduct first at day 5 post infection and in the ovary first at day 7 post infection [Jones and Jordan, 1972]. Birds infected at a younger age experience more severe lesions than older birds [McMartin and Macleod, 1972; Crinion and Hofstad, 1972b], though it is important to note that IBV can cause reproductive disease in hens in full lay that results in decreased egg production and quality [Muneer et al., 2000; Chousalkar et al., 2007]. Previous evidence indicates that the incidence of false layers increases as the age of birds at infection decreases [Broadfoot et al., 1956].

In older hens, egg production losses and egg abnormalities may not appear until after respiratory symptoms decline, sometimes four or more weeks after the respiratory signs disappear. In addition, hens who develop respiratory symptoms might not develop any reproductive symptoms, even if others in the same group do [McMartin, 1968]. Urban and Goodwin [1953] reported that pullets infected right before the onset of lay experienced a more significant decline in production and quality than other age groups. Hens of the same flock infected at older ages but before sexual maturity might exhibit varying degrees of oviduct development at the expected time of sexual maturity, though the organ might appear normal otherwise [Duff et al., 1971]. IBV infection is historically demonstrated to influence egg quality but can also cause decreases in production without effect on egg quality [McMartin and Macleod, 1972]. Amarasinghe et al in 2018 found that there was no statistically significant difference of IBV prevalence in flocks with high incidence of shell-

less egg syndrome versus flocks without it. Factors other than age, virus strain, and vaccination status must play a role in the development of reproductive effects of IBV.

After infection with IBV and initial decline of production, production levels will rarely return to normal and might only resume to a fraction of the pre-infection production level as far out as nine weeks post infection [Sevoian and Levine, 1957]. The method in which birds are challenged [for example, direct application versus contact application] can influence the pathogenicity of the disease [Duff et al., 1971]. Infection with IBV can affect both the ovaries and the oviduct of hens. Zhong et al found ovarian interstitial congestion, hemorrhage, and lymphocytic infiltration with occasional atrophy of follicles and degeneration of granule cells, though no lesions were seen grossly in the ovary. The most ovarian histological lesions were seen day 4-15 post infection [2016]. The QX strain of IBV has been demonstrated to cause abnormal development of the ovarian follicle in addition to follicle atrophy [Shao et al., 2020]. Effects of IBV on the oviduct are described in significantly more detail. IBV replicates in all regions of the avian oviduct but replicates in highest quantities in the shell gland at the distal end of the oviduct [Cook et al., 2012].

Another factor that determines the pathogenesis of reproductive disease associated with Infectious Bronchitis infection is the serotype of IBV involved. The serotype of IBV might also play a part in which segment of the reproductive tract is affected by the infection. In their 2007 study, Chousalkar et al found that the T strain had an affinity for the tubular shell gland and shell gland pouch and the N1/88 strain had more pathogenic effects in the magnum of the oviduct. They note that not only can IBV affect the developing oviduct, but it also can affect the mature functional oviduct. Not every serotype of IBV has been shown to cause pathological changes in the oviduct and serotypes vary in their ability

to induce reproductive disease [McMartin, 1968]. The Massachusetts strain, QX strain, and Australian T strains of IBV have been demonstrated to cause negative reproductive effects in hens [Crinion and Hofstad, 1972a; de Witt et al., 2011].

12.2 Reproductive Clinical Signs and Gross Lesions

Because there is variation in reproductive effects of IBV on individual birds, some birds may even have normal and functional ovaries and oviducts post infection with IBV [McMartin, 1968]. The percentage of affected birds appears to decrease as the age of birds at exposure increases [Broadfoot et al., 1956]. Though the birds may appear outwardly normal, numerous gross lesions may be present throughout the abdominal cavity and reproductive tract. McMartin in 1968 noted that affected eggs were distorted at identical points and hypothesize that damage to the reproductive tract due to IBV infection occurs in specific localized areas.

IBV appears to affect the oviduct of hens more severely than the ovary. Normal ovary development [follicular hierarchy formation and empty follicles] has been noted in combination with abnormal oviducts and can lead to internal lay or false layers [Broadfoot et al., 1956]. These abnormal oviducts can range from underdeveloped but patent to simply a blind sac projecting near the cloaca [Jones and Jordan, 1972]. Additional oviduct lesions, especially in false layers, include oviducts considerably less than normal length and non-patent oviducts [Broadfoot et al., 1956]. The oviducts of infected birds might be normal in appearance but the degree of development across an affected flock might not be uniform [Duff et al., 1971]. In a study by Crinion in 1972, 17% of the non-layers exhibited morphologically normal but quiescent oviducts. Narrowing or complete absence of parts of the oviduct may be seen [McMartin and Macleod, 1972].

Regions of the oviduct affected varies based on the challenge serotype, though almost all regions of the oviduct have had lesions described in previous work. Chousalkar et al saw the most damage to the tubular shell gland and shell gland pouch for birds infected with the T strain, but the N1/88 strain affected the magnum more than other areas of the oviduct [2007]. In the infected uterus, the major components that play a role in shell deposition might be present, but the damage associated with infection appears to inhibit normal shell deposition [Crinion, 1972]. IBV can cause patchy or complete ciliary loss in the two upper regions of the oviduct, the infundibulum and the magnum, when hens are in lay during infection. The function of the cilia may return during recovery [Chousalkar and Roberts, 2007]. Further down the oviduct, the translucent demarcation ring between the magnum and the isthmus of the oviduct can become less apparent, [Broadfoot et al., 1956]. This is especially common in birds experiencing false layer syndrome or cystic oviduct.

Because IBV damage affects reproductive tissues, the normal egg development process is disrupted. Damage to the anterior aspect of the oviduct either prevents the infundibulum from properly enveloping the yolk or causes the infundibulum to eject the yolk via reverse peristalsis [Broadfoot el al., 1956]. In many cases, authors have described an accumulation of yolk fluid in the abdominal cavity of infected birds. This accumulation indicates that ovulation is occurring directly into the body cavity rather than into the infundibulum [Broadfoot et al., 1956]. The abdominal yolk material can be thin or sometimes congealed [Jones and Jordan, 1972]. In addition, increased visceral fat may be seen and can be mottled in color that resembles pigmentation affects from the free yolk in the abdominal cavity [Broadfoot et al., 1954].

Egg internal and external quality can also be negatively impacted and have a positive correlation with each other [Crinion, 1972]. Affected eggs have increased calcareous deposits, yolk separation from the albumen, meat spots in the albumen, and watery albumens [Crinion, 1972; Muneer et al., 2000]. When IBV affects the magnum [albumen-secreting region] of the oviduct, incidence of water albumen increases. The IBV-associated drop in glycoproteins and ovimucin secretion causes the thick layer of the albumen to thin and appear watery [Butler et al., 1972].

Research conclusions on ovarian involvement from Infectious Bronchitis infection varies. Some previous research indicates that no lesions or histological changes in the ovary are seen with IBV infection [Benyada et al., 2010]. Other research, such as the 2012 study by Hong et al, saw significant inhibition of ovarian follicle hierarchy formation in 80% of birds challenged at 16 weeks of age. Ovarian involvement of IBV infection likely is dependent upon age, viral strain, and the other characteristics mentioned previously. Ovarian and oviduct regression due to infectious bronchitis infection has also been noted and grossly looks similar to regression caused by stress. However, reproductive tissue from stressed birds appeared histologically similar to control birds who were not exposed to stress or IBV. Regression due to IBV is often associated with histological lesions [Sevoian and Levine, 1957].

12.3 Reproductive Histopathology

Histologically, oviduct lesions include significant epithelial cell involvement as these cells are the primary cells targeted by the virus [Crinion and Hofstad, 1972a]. These lesions resemble the respiratory epithelial cell lesions, including desquamination, necrosis, congestion, and hemorrhage, though it should be noted that when examining tissue

histologically, method of euthanasia should be considered. Birds that are euthanized via CO2 are more likely to exhibit hyperemia in tissue on histological analysis than birds that are euthanized via cervical dislocation under normal conditions. After IBV infection, inflammatory cell infiltrates may be present in addition to edema of the lamina propria or submucosa [Zhong et al., 2016; Landman, 2005]. The muscular layers of the oviduct, the lamina propria, intertubular stroma, and especially glandular layers, are subject to heavy infiltration of inflammatory cells. Plasma cells, mononuclear cells, and lymphocytes make up most of the infiltrates. The lamina propria and intertubular stroma sections might also be affected by fibroplasia and/or edema [Sevoian and Levine, 1957]. The effects of IBV on the epithelial cells of the testes of the rooster are less defined. Villarreal et al discuss that IBV can cause damage in the reproductive tissue of the rooster such as destruction of efferent ductules, destruction of the epididymis, and destruction of the seminiferous tubules. These are all associated with decreases in male fertility, though the exact pathogenesis is unknown [2007].

Lymphoid cell infiltration into the oviduct occurs as early as the third day post inoculation, which leads to the development of lymphoid nodules in the oviduct wall especially near blood vessels. The infiltration lesions are less likely to lead to permanent damage than the epithelial cell lesions [Crinion and Hofstad, 1972a] and the degree of infiltration in the oviduct of birds producing poor quality eggs is approximately the same as the infiltration in the oviduct of birds producing normal eggs [Crinion et al., 1971b]. Age does not seem to play a factor in the development of infiltrative lesions in the oviduct [Crinion and Hofstad, 1972a]. Lymphoid nodules are more likely to be found in birds

infected with IBV than in uninfected birds and the nodules are often found in glandular layers of the oviduct [Sevoian and Levine, 1957].

Sloughing of the epithelial cells lining the lumen of oviduct has been noted in Mass-type IBV infection and severe epithelial damage can be permanent. Necrosis and degeneration of these cells is also possible [Crinion and Hofstad, 1972a]. The oviduct epithelial cells of hens in the peak of IBV infection decrease in height, become cuboidal in shape, and exhibit partial or complete loss of cilia. The loss of height and shape change are especially seen in goblet cells [Sevoian and Levine, 1957]. Cilia-stasis is seen in the oviduct epithelium of infected hens compared to the normal cilia activity in vaccinated or non-challenged hens [Raj and Jones, 1996b]. Oviduct epithelial cell recovery from IBV infection is slow, if it happens at all [Sevoian and Levine, 1957].

Crinion et al. theorize that IBV might inhibit the differentiation of epithelial cells in the lumen into glandular cells, as indicated by the hypoplasia of glandular cells in the oviducts of infected birds. It is also possible that the virus destroys the already differentiated glandular cells [1971a]. In addition to hypoplasia, IBV infection cause severe tubular gland dilation and loss of mucopolysaccharides in these epithelial cells [Sevoian and Levine, 1957; Chousalkar et al., 2007].

Hypoglandular tissue in localized areas of the oviduct of affected birds is associated with an increase in poor quality eggs and is seen in non-layers with patent oviducts [Broadfoot et al., 1956, Crinion, 1972]. Crinion et al describe hypoplastic lesions in the caudal magnum and the cranial isthmus of pullets infected at one day of age. Above the hypoplastic area, glandular development is normal [1971a]. In the magnum region specifically, hypoplasia of glandular tissue is commonly found with the presence of secreta

in the glandular cells. In cases of cystic oviduct, the glandular tissue was not atrophied and was still productive and the uterus muscular wall was thick [Landman, 2005]. Hypoplastic tissue contains little to no epithelial lining cells and is made up of connective-tissue elements [Crinion et al., 1971a].

13. IBV Immunohistochemistry

Viral antigen localizes to the epithelial cells in the oviduct and is often confined to the cytoplasm, making early diagnosis possible with immunohistochemistry [Crinion and Hofstad, 1972b; Braune and Gentry, 1965]. In their 1972 experiment by Crinion and Hofstad, viral antigen quantities in the chick oviduct were highest at 6 days post-inoculation and tapered off 10 days post-inoculation. As hen age-at-inoculation increases, viral antigen becomes more difficult to detect in reproductive tissue. Chickens inoculated at a younger age retain the viral antigen in the oviduct longer than older inoculated chickens [Crinion and Hofstad, 1972a]. Jones and Jordan [1972] hypothesize that the immunological naivety of young birds leads to increased viral persistence in the blood, which in turn leads to increased viral presence in the tissue over a longer period of time than birds inoculated at older ages. The best time to test for viral antigen presence appears to be 5-7 days post inoculation based on previous literature [Crinion and Hofstad, 1972a; Jones and Jordan, 1972], However it is important to note that instances of cystic oviduct without the presence of IBV viral antigen in the oviduct have been recorded [Benyeda et al., 2010]. It is important to consider the potential for blue autofluorescence in the oviduct tissue when choosing an immunohistochemical stain or fluorescent antibody to use [Crinion and Hofstad, 1972a].

14. Cystic Oviduct and False Layer Syndrome Caused by IBV

Infectious bronchitis virus infection can lead to development of cysts in oviducts, which are strongly associated with False Layer Syndrome. Oviduct cyst formation with colorless serous fluid, shorter oviduct lengths, and thin or transparent oviduct walls are common gross lesions of IBV infection in the oviduct [Duff et al., 1971; Zhong et al., 2016; Landman, 2005; Benyada et al., 2010; de Witt et al., 2011]. Broadfoot et al. [1956] describe the fluid in cysts to be clear but containing floating secretions of mucin fibers resembling chalazae. Birds experiencing False Layer Syndrome exhibit little to no clinical signs different from unaffected birds. Affected birds will have bright combs, depigmentation, widened hips, and normal temperament [Broadfoot et al., 1956; de Silva et al 2021]. Affected birds may stand more upright or waddle in a "penguin" fashion with pendulous abdomens when compared to normal hens [Crinion, et al., 1971b]. Though they may not be exhibiting clinical signs, these affected birds do seroconvert after bronchitis infection [Muneer et al., 2000]. Da Silva et al. [2021] describe a case where hens at 50 weeks of age began showing clinical signs of False Layer Syndrome and had an increase in mortality.

Yolk material in the abdomen in various quantities is a common finding in birds with False Layer. Broadfoot et al. [1956] suggest that this accumulation is either a result of improper function of the anterior portions of the oviduct where the infundibulum does not properly engulf the yolk, or the expulsion of the yolk from the oviduct after successfully passing through some portion.

Infection with the QX strain [among others] of IBV can cause fluid-filled cystic oviduct formation, especially in younger birds. These cystic oviducts lead to permanent damage that will decrease egg production in the affected birds. Other strains with known

association between cystic oviduct and IBV include Mass strains, such as M41, and the Australian T strains [McMartin and Macleod, 1972; de Witt et al., 2011]. While most birds experiencing False Layer Syndrome will have occluded oviducts, some might have normal oviducts that are not functioning [Crinion, 1972]. Cysts in the atretic right oviduct can occur in healthy birds, but an increased incidence of right oviduct cysts has been identified as a result of IBV infection at a young age [de Wit, et al., 2011; McMartin and Macleod, 1972; Broadfoot et al., 1956; Hassan et al., 2021; Mueller Slay et al., 2022].

Bozorgmehri-Fard et al observed that cystic oviduct and False Layer will develop in a flock when birds are infected with Infectious Bronchitis Virus before ten days of age [2013]. Cysts can generally be detected as early as 20-30 days post-inoculation with IBV [Crinion and Hofstad, 1972a], but Benyeda et al identified cystic oviduct at 14 days when chicks were infected at one day of age with a QX strain of IBV [2010]. Cysts are often located caudal to areas of oviduct hypoplasia, such as near the suspensory ligament of the oviduct or the lower portion of the immature oviduct, and can become larger as the hen gets older [Jones and Jordan, 1972; Duff et al., 1971; Crinion et al., 1971a]. In some birds, the magnum of the oviduct may be partially or mostly developed with the cyst located in the posterior portion of the oviduct [Broadfoot et al., 1956]. The percentage of cystic oviducts in a flock of 6-10 weeks old is used in the Netherlands and other countries to estimate the percentage of false layers that will develop in a flock during production [de Wit et al., 2011].

The cause for the development of oviduct cysts with IBV infection is not clear. IBV virus replication is known to damage epithelial cells and might led to cyst formation when oviduct epithelium is damaged. Crinion and Hofstad note an increased presence of oviduct

epithelial cells degenerated and sloughed into the oviduct lumen. The connective tissue fills in for the damaged epithelial tissue and can result in lumen closure. This blockage, combined with the presence of the occluding plate between the vagina and cloaca, is suggested to lead to fluid accumulation and cyst formation [Crinion and Hofstad, 1972a]. Benyeda et al [2010] describe few histopathological changes associated with cystic oviducts to explain their formation. They did not find epithelial damage, excessive secretion, or excessive inflammation in cystic oviducts of birds infected with the QX strain at 1 day of age. Some hypothesize that the cyst formation is not directly caused by virus replication, but by the inflammatory response to virus infection. The connection between the cloaca and vagina occurs when the Mullerian duct opens during post-embryonic development by the seventh day post-hatch. Inflammation in the developing oviduct before the Mullerian duct has opened might lead to obstruction of the posterior oviduct or improper formation of the Mullerian duct into the cloaca and subsequent fluid accumulation develops [Benyeda et al., 2009; Benyeda et al., 2010].

15. IBV DMV/1639

In the last decade, an increase in False Layer Cases has been reported across the US and Canada. IBV variant DMV/1639 is a serotype of Infectious Bronchitis Virus that was first described in a case from Pennsylvania in 2010 that spread to the Delmarva region, though the exact epidemiology is unknown [Gelb et al.,2011]. This serotype appears to have evolved from a variant that caused the 1997-2000 outbreak in Pennsylvania, referred to as NIBV PA/171/99 [nephropathogenic infectious bronchitis virus] [Gelb et al., 2011]. Studies by Jackwood and Lee found that DMV/1639 is evolving under negative selection

pressure and suggest that it can even recombine with live attenuated Arkansas IBV vaccination strains [2017].

DMV/1639 isolates from Georgia, first seen in 2019 in a commercial broiler farm, were sequenced and determined to be from the IBV GI-17 lineage with relation to the Cal56b/1991 strain [Goraichuk et al., 2019]. Hassan et al also found DMV/1639 isolates from Ontario to contain genetic material similar to Connecticut live vaccine strains, 4/91 live vaccine strains, and other unknown IBV sequences [2019]. DMV/1639 is now present across the United States and Canada [Hassan et al., 2019] and is the most prevalent variant circulating in the United States [Jackwood and Jordan, 2021], particularly in breeder and layer flocks. Its pathogenicity appears to have progressed from nephropathogenic to primarily respiratory, though the effects on the reproductive tract were not initially noted [Jackwood and Lee, 2017]. DMV/1639 has been isolated from some field cases of False Layer Syndrome, and the Canadian strain of DMV/1639 has been previously demonstrated to be pathogenic to the oviduct and ovary of laying chickens [Hassan et al., 2021].

16. IBV Diagnostics

The standard diagnostic tool for infectious bronchitis virus is virus isolation via passage in embryos or tracheal organ culture. Virus isolation is conducted in chicken embryos at 9-11days of embryonation. The VI process is considered time consuming and expensive. It might take several passages in embryos before virus can be detected [citation?]. Immunoassays such as ELISA can be used for IBV diagnostics and commercial kits are available. Other serological assessment of IBV infection include virus neutralization assays [VN] and hemagglutinin-inhibition assays [HI] and can be used to differentiate between IBV serotypes [Ingjatovic and Sapats, 2000; Gough and Alexander,

1978]. Hemagglutinin-inhibition tests have less specificity than the virus neutralization test but might show considerable diversity [Cook et al., 1987; Gough and Alexander, 1978]. Because there is little cross-protection between serotypes, and there is notable diversity of IBV serotypes, serological assays for IBV may not be specific or sensitive enough to accurately detect the strain being investigated [Jackwood et al., 2012].

Molecular diagnostics are becoming the more prominent method for identification of IBV. Reverse-transcriptase-polymerase chain reactions can amplify the spike protein S1 sequence. Analysis of the S1 sequence of the spike glycoprotein can differentiate between IBV serotypes. Standard PCR combined with gel electrophoresis or sequencing can detect these S1 sequences. Real-time qualitative PCR can be used to detected viral presence and quantity. Trachea samples [swabs or tracheal tissue] are best to use to detect active infection, choanal swabs can also be used, and cecal tonsil or kidney tissue is best to use to detect viral persistence [Villarreal, 2010].

17. IBV Prevention

Vaccination is the most used prevention method against IBV, though it can be difficult due to the differences between the field or variant serotypes and the vaccine serotypes [Gelb Jr et al., 1991]. Vaccination of high titers has been shown to produce better serological protection than lower titer vaccinations, though clinical signs caused by vaccination must be considered for vaccines of significant titers [Raggi and Lee, 1965]. The average vaccine dose for IBV is approximately 1 x 10^4 EID50 but can vary [McKinley et al., 2008]. Mass-strain vaccines are considered the “industry-standard” for IBV vaccination [Li et al., 2019].

Live vaccination for IBV is used in broilers and as the initial vaccination of breeders and egg-laying pullets. Live vaccines can either be administered through eye/nasal drops, in drinking water, or with course/aerosol spray methods [Sulaiman and Roberts, 2011]. Inactivated vaccines are used as "boosters" for breeders and pullets between the ages of 10 and 18 weeks. Inactivated vaccines can either be administered via subcutaneous or intramuscular injection before hens come into lay [Jackwood and de Wit, 2020].

The most important times in a hen's life for IBV vaccination is at or around lay and soon after hatching [Cook et al, 2012]. In order to create a protective local immune response in the trachea, priming vaccination at day of hatch is given followed by a booster vaccination around two weeks of age [Cavanagh, 2007]. Pullets and hens may have less of a serological response to killed IBV antigen when it is administered after recent exposure to live IBV [Box et al., 1988]. Successful vaccination relies on multiple factors. Vaccines must be properly prepared and administered to be most effective. They should also be administered at the recommended dosage. The route of challenge also influences the success of vaccination, particularly with an inter-tracheal challenge when vaccination is received via eyedrop [Davelaar and Kouwenhoven, 1981]. Even in the most ideal conditions with excellent application, some birds will not be protected with vaccination [Winterfield et al., 1976].

Successful vaccination relies on the adaptive immunity factors that depend on antigen-specific interactions, such as B-cell activation, T-cell activation, antibody production, and other memory-based immune responses. Initial nonspecific immune responses important to fight IBV challenge are heterophils, macrophages, natural killer cells, the complement system, and pattern recognition receptors that work together to target

infected cells [Chhabra et al., 2015]. Both these innate and humoral immune reactions are important to protection against IBV. Innate reactions are especially important in protection against respiratory effects. Humoral response and the presence of some passive immunity and maternal antibodies protect birds from systemic response. MacDonald et al. describe these protective effects in the kidneys [1981]. Though the authors did not investigate the protection of the oviduct, since the oviduct is also a systemic target of IBV replication, similar protection of the oviduct might be found.

Early vaccination of birds in the hatchery induces a poorer systemic and mucosal immune response, caused by both a delay in antibody production and a decrease in the amount of antibodies produced, than later vaccinations at 7, 14, 21, and 28 days of age [van Ginkel et al., 2015]. Saiada et al. [2019] found that a booster vaccination after vaccination at hatch overcame the effects of the poor initial immune response and that increasing the age at vaccination significantly increased both systemic antibodies and mucosal antibodies, though it is important to consider that study by Saiada et al. was conducted in leghorns without maternal antibodies against IBV. An increase in IgA antibodies, the antibodies that protect the mucosa, after vaccination or challenge leads to faster clearance of the viral infection [Toro et al., 2012].

Although vaccination strategies are often serotype specific and can have limited cross-protection [Kulkarni and Resurreccion, 2010], instances of vaccination against one serotype vaccine protecting against an antigenically distinct serotype have been recorded. The Massachusetts-type vaccination is one that shows evidence of cross-protection. Terregino et al worked with a Mass strain vaccine that offered protection against QX challenge [2008]. In 2009, Wood et al worked with a Mass strain vaccine that offered

protection against DMV/5642/06 variant challenge. They go on to state that in their experiment, strains with higher genetic similarity exhibit stronger cross-protection. However, it is important to note that there is also evidence that Mass-strain vaccines will not protect against some other variants [Li et al., 2020]. To date, no vaccination is available that offers complete cross-protection of heterologous IBV challenge.

Vaccine strains such as the Arkansas strain have been documented to persist in a flock after the expected clearance frame and might contribute to clinical signs caused by IBV cycling in the flock after modified live vaccination [Jackwood et al., 2009]. These birds can shed vaccine virus that can infect birds that did not receive proper vaccination and the subsequent virus replication can lead to mutations and subpopulations of better-fit IBV adapted to the live host [McKinley et al., 2008, Jackwood and Lee, 2017]. Vaccines that are highly specific but low in titer will suppress the replication of subpopulations but will allow the predominate IBV strain to replicate [Toro et al., 2012]. In addition, improper vaccine application or storage can cause immune pressures that result in the emergence of disease-causing variants [Gelb et al., 1991], especially after recombination events [Kulkarni and Reynaldo, 2010; Li et al., 2020]. It is important to note, however, that to date there has not been evidence of recombination between a vaccine strain virus and a field strain virus that has resulted in the emergence of a new variant virus in the field [Jackwood and Jordan, 2021].

The environment and rearing methods for laying hens are especially conducive to the emergence of variant strains. Layers are housed in high numbers in close proximity which increases the contact between individuals, many flocks are multi-age which contributes to cycling in the flocks, and layers have a longer lifespan than broilers and

require additional preventative considerations [Gelb Jr et al., 1991]. However, Naqi et al found that recombination events and the tendency of IBV to frequently change genetically are lower than the incidence of frequent genetic change in mammalian coronaviruses [2003]. Vaccination, especially poor vaccination, is considered a selection pressure that influences IBV evolution [Toro et al., 2012].

Another vaccination strategy to protect against IBV is to vaccinate against multiple serotypes with combination vaccines. A bivalent vaccine of Mass/Arkansas strains has shown promising potential results against some variant challenges [Gelb Jr et al., 1991]. In the initial DMV/1639 investigation, vaccination with a Mass-Ark combination vaccine was less protective against DMV/1639 respiratory and kidney lesions than the M41 challenge virus, though a reduction in kidney lesions was noted [Gelb et al., 2011]. This relationship and others should be investigated as a means for protection against the reproductive effects of DMV/1639 and other reproductive disease-causing variants. De Wit et al conducted an experiment with Mass/D274 in combination with either Mass/Ark or 793/B and examined the protection capability on the oviduct after challenge. Birds the Mass/D274+Mass/Ark combo group experience more severe vaccine reaction, but birds in both combo groups were protected from oviduct lesions associated with D388 challenge virus [2011]. Massachusetts Ma5 vaccination at hatch followed by a 4/91 vaccination at 14 days of age was shown to be more productive in SPF chickens than a homologous vaccination at 14 days of age or from 4/91 at hatch followed by Ma5 at 14 days, indicating that order of vaccination also plays a role in protective response [Cook et al, 1999].

The younger chicks are vaccinated, the less likely cross-protection will be effective. For example, Karimi et al. found that there was no significant difference between

protection in birds vaccinated at 1-day of age with a 793/B vaccine combined with a H120 vaccine compared to the 793/B vaccination alone [2019]. Chicks at hatching have maternally derived IgG antibodies that offer systemic immune protection against diseases the hen had been exposed to or vaccinated against, including Infectious Bronchitis Virus [Raj and Jones, 1997]. In a study conducted by Darbyshire et al., it was determined that chicks are protected against IBV Massachusetts strain [M41] challenge via their maternal antibodies for two to three weeks post-hatch and optimal vaccination age is two weeks for highest protection against IBV respiratory disease [1985]. Initial vaccination at 14 days of age is a common strategy that can result in strong systemic antibody response [van Ginkel et al., 2015; Saiada et al., 2019].

18. IBV Vaccination and Reproductive Effects

Vaccination programs for chickens are typically evaluated based on efficacy to decrease or eliminate respiratory challenge as opposed to reproductive effects [McMartin, 1993]. Raj and Jones [1996b] developed an in-vitro assay for evaluating the ciliary activity of oviduct epithelium after vaccination and challenge that can be used in place of long-term studies on the reproductive effects of vaccines. Ideally, protection against respiratory challenge will prevent the systemic spread of the virus and eliminate the need for specific reproductive efficacy assays, as shown by the work of Karimi et al. [2019] where vaccination of 1-day old chicks with higher antibody titer against IBV provided increased protection of the internal organs. Additionally, da Silva et al. [2021] describe a field incidence where vaccination at the hatchery with a Massachusetts strain vaccine decreased the incidence of cystic oviduct at the farm compared to previous flocks.

Vaccination in the hatchery is often used to combat the development of False Layer Syndrome. The case report by Landman [2005] illustrates the importance of using serotype-specific vaccines. In this case, birds were vaccinated in the hatchery for one serotype of IBV but later developed False Layer Syndrome and cystic oviduct. Landman hypothesizes that this development is a result of improper protection at the hatchery and exposure of an unrelated IBV serotype in the field at a young age.

Stimulation of local immunity is important to the success of vaccination. IgY, associated with local immunity, has been identified in the oviduct washes of hens infected with IBV. IgY [the chicken equivalent of IgG] found in these washes indicates that the chicken oviduct may play a role in the secretory immune system [Raj and Jones, 1996a]. The consensus in literature on the ability of vaccines to cause reproductive disease in hens is debated. Broadfoot et al state that a commercial attenuated IBV vaccine did not cause abnormal oviducts [1956]. Hong et al found that IBV vaccination prevented chickens from reproductive lesions by up to 70% when compared with unvaccinated controls after challenge at 16 weeks of age [2012]. Some low passage live vaccines have produced cysts in chicks exposed in the first week of age [Crinion, 1972]. Birds challenged with an embryo-passage 55 Mass strain had reproductive lesions develop when challenge occurred at 1 or 8 days of age while the embryo-passage 7 Mass strain caused oviduct lesions in birds challenged at 1, 8, 15, 22, and 29 days of age [Crinion and Hofstad, 1972a].

Economic losses due to egg production and hatchability decreases can be prevented by efficient and effective vaccination programs [Muneer et al., 2000]. Inactivated IBV vaccine administration has been shown to be protective against the reproductive effects of IBV infection [Berry, 1965]. De Wit et al found that vaccination at either day 0 or day 0

combined with day 14 was effective in protecting birds against damage to the oviduct caused by the D388 IBV strain [related to QX strain]. Significantly lower D388 antigen levels were found in the oviducts of vaccinated chicks versus unvaccinated chicks [2011].

In a study by Box et al, birds with high IBV antibody titers had less severe egg production losses than birds with low antibody titers when challenged at 38 weeks of age. These findings suggest that vaccination programs that result in the highest antibody titers with the most uniform coverage will be most effective at preventing egg production losses associated with IBV infection. The Box et al experiment had the most protection in groups vaccinated with an initial live attenuated vaccine followed by an oil emulsion killed booster [1988]. Local immunity is important for the success of vaccination and seems to be more protective against ciliary damage/replication in the trachea than in the oviduct [Raj and Jones, 1996b].

It is important to consider that the use of live vaccination for revaccination can cause a drop in egg production as birds are reacting to the live virus, but the same group states that birds should be vaccinated during lay to ensure protection in the face of challenge [Sulaiman and Roberts, 2011] Revaccination for IBV in laying hens outside of the standard live initial vaccination and inactivated boosters does not appear to have any benefit on protection when compared to the standard vaccination protocol. In fact, in birds that were regularly revaccinated, a decrease in egg quality was seen and attributed to reoccurring exposure to the virus even as a vaccine [Sulaiman and Roberts, 2011]. In ovo vaccinations for IBV are not frequently used. However, results from Chew et al indicate that in ovo vaccination against IBV might be an option to decrease the reproductive effects of IBV when hatched birds are challenged. This in-ovo vaccine was passed in cell culture

repeatedly and was not determined to cause negative effects in the oviducts of maternal-antibody positive embryos [1997].

To date, there is no commercial vaccine available for variant DMV/1639. Because IBV vaccination is often serotype-specific, additional work should be completed in this area and the timing of vaccination should be evaluated to prevent development of cystic oviduct formation.

19. IBV Maternal Antibodies

Maternal antibodies are passed from hen to chick to protect the chick in the early stages of the development of its immune system. Passive immunity through maternal IgY antibodies is suspected to protect young chicks from abnormal oviduct development due to early IBV infection [Broadfoot et al, 1956]. Crinion et al. noted that birds with maternal antibodies are less likely to exhibit the severe reproductive lesions seen in SPF birds without maternal antibodies [1971a]. These results were supported by work by de Wit et al in 2011 investigating the effects of vaccination and maternal antibodies on the development of reproductive lesions after early infection with D388 strain IBV.

The effect of maternal antibody presence of vaccination is debated. It is possible that the presence of maternal antibodies in chicks will decrease the success of vaccination against infectious bronchitis virus at a young age [de Wit et al., 2011]. Similar results have been seen in Infectious Bursal Disease Virus, where high maternal antibody levels against IBDV decreased the bird's response to both vaccination and to challenge [Naqi et al., 1983]. Vaccination of chicks who have maternal antibodies against IBV has been shown to be less effective than vaccination in maternal antibody negative birds. In addition,

vaccination at day-of-hatch can also speed up the decline of maternal antibodies against IBV [Mondal and Naqi, 2001].

Some studies have been conducted that support the theory that maternal antibodies do not influence vaccination. Van Ginkel et al. used maternal antibody negative SPF birds and found that these birds vaccinated at 1-day or 7-days of age were not fully protected and cite the importance of pathogen-specific maternal antibody presence to proper vaccine response against IBV [2015]. De Wit et al found that the combination of high maternal antibody presence and heterologous vaccination at hatch offered protection against the kidneys when birds were challenged at 6 or 10 days of age with the D388 virus. The authors extrapolate this protection to extend to the oviduct as well [2011]. In addition, Winterfield et al. [1976] describe an increased shed-rate of challenge virus in birds that did not have passive maternal antibodies compared to the group of birds with maternal antibodies when both were vaccinated at one day-of-age. Davelaar and Kouwenhoven [1977] state that vaccination in the presence of maternal antibodies against IBV can still be successful.

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CHAPTER 3

INFECTION WITH IBV DMV/1639 AT A YOUNG AGE LEADS TO INCREASED INCIDENCE OF CYSTIC OVIDUCT FORMATION ASSOCIATED WITH FALSE LAYER SYNDROME[1]

[1]Adrea Mueller Slay, Monique Franca, Mark Jackwood, and Brian Jordan
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ABSTRACT

Infectious bronchitis virus (IBV) is an avian coronavirus that causes respiratory disease but can affect the reproductive tract of laying-type chickens. If infection occurs in pullets, false layer syndrome, which is characterized by the development of large, fluid-filled cystic oviducts, can occur. Recently, IBV strain DMV/1639 has been detected in parts of Canada and the U.S., where false layer syndrome has occurred, though it is not clear if IBV is the sole cause or if age at infection is an influencing variable. Our study investigates the role and timing of IBV infection on the development of false layer syndrome, using the IBV types DMV/1639 and Massachusetts (Mass). Six groups of 120 SPF chickens were challenged at either three, seven, or fourteen days of age, using either DMV/1639 or Mass IBV. Cystic oviducts were seen in all the challenged groups, and the pullets challenged at 14 days of age had fewer cystic oviducts than pullets challenged at 3 or 7 days of age. The highest percentage of severe histology lesion scores were seen in the 3-day challenge groups. The data collected in this experiment confirm that IBV DMV/1639 causes cystic oviducts and indicate that age at infection plays a role in the pathogenesis of false layer syndrome.

1. Introduction

Infectious bronchitis virus (IBV) is an economically significant pathogen in commercial chickens that causes increased mortality, decreased feed conversion, high condemnation, and decreased egg production and quality [Awad et al., 2014]. Though mortality rates are low (less than 5%), unless secondary infections are involved, morbidity rates can reach 100% [Awad et al., 2014 and Broadfoot et al., 1956]. IBV is horizontally transmitted in birds in natural infection [Sevoian and Levine 1957], and chickens of all ages are susceptible to IBV, though most the severe disease is typically seen in younger birds. Disease severity is dependent upon a combination of factors, including age at exposure, the strain of IBV that the birds are exposed to, and the route of inoculation.

IBV is primarily a respiratory disease; however, some serotypes of the virus are known to affect the epithelial cells of the chicken's reproductive tract, leading to decreases in egg production and quality. Considerable economic loss can occur in infected hens who pause production and then do not resume normal production levels. IBV also causes poor egg-quality characteristics, such as soft-shelled eggs, misshapen eggs, and eggs with watery albumen [Sevoian and Levine, 1957; ,Muneer at al., 1986; Igniatovic and Sapats, 2000]. In addition, False Layer Syndrome is often described as a clinical sign and the result of exposure to infectious bronchitis virus [Sevoian and Levine, 1957].

The presentation of false layer syndrome, as defined by Sevoian and Levine in 1957, is "a bird with every appearance of a normal bird" that "is out of production and does not lay any eggs". More specifically, false layer syndrome refers to an absence of egg production of mature layers that are exhibiting little to no other clinical signs [de Wit et al., 1972]. Some birds affected by false layer syndrome exhibit waddling and/or a pendulous

abdomen, but many exhibit normal behaviors, including nesting behaviors, vent appearance, and pubic bone characteristics [Broadfoot et al., 1956; Landman, 2005]. The ovaries of false layers can also appear normal, including empty follicles from recent ovulation. Cyst development in the oviduct with normal ovary development is hypothesized as a cause of these instances of false layers. These cystic oviducts lead to permanent damage that will prevent egg production in the affected birds [Crinion et al., 1971b].

Infectious bronchitis virus infection can lead to the development of cystic oviducts, which are strongly associated with false layer syndrome. Cystic oviduct formations with a colorless serous fluid, shorter oviduct lengths, and thin or transparent oviduct walls are common gross lesions of IBV infection [de Wit et al., 1972; Landman, 2005; Duff et al., 1971; Zhong et al., 2016; Benyeda et al.,2010]. Infection with the QX strain (among others) of IBV has been shown to cause fluid-filled cystic oviduct formation, especially in younger birds. These cystic oviducts lead to permanent damage that will prevent egg production in the affected birds. Other IBV strains with a known ability to cause cystic oviducts include the Massachusetts and the Australian T strains [de Wit et al, 1972; McMartin and Macleod, 1972]. Recently, a new strain of IBV, DMV/1639/11, has been associated with cystic oviduct development and false layer syndrome in the United States and Canada, though no causal relationship was shown at the initial time of virus isolation. Therefore, in this work, we describe the ability of the DMV/1639 IBV strain to cause cystic oviducts and false layer syndrome in comparison to a Mass strain known to cause these lesions. We also aimed to determine the effect of the timing of infection on disease severity, both grossly and histologically.

2. Materials and Methods

2.1. Viruses

The IBV strains used in this study were of the Massachusetts (Mass/41/41) and DMV/1639 (DMV/1639/11) serotypes. The Mass-type IBV challenge is a pathogenic laboratory strain and has been previously described [Roh et al., 2014]. The DMV-type IBV was isolated from a clinical case of respiratory disease in chickens submitted to the Poultry Diagnostic and Research Center (PDRC) diagnostic laboratory in 2019 and has been previously described [Jordan, 2019]. The fourth passage in embryonated chicken eggs was used for the challenge.

2.2. Experimental Design

Specific pathogen-free (SPF) chicks were hatched at the PDRC, University of Georgia, and vent-sexed. The female chicks were randomly separated into six groups of 120 birds each. The birds were reared in colony-type houses on fresh pine shavings and given food and water ad libitum. Each colony house was contained and separate from the others. Chicks in select groups were challenged with either the pathogenic Mass (M41) or the DMV/1639/11 IBV strain via the intraocular/intranasal route. Groups 1–3 were given the M41 challenge, and groups 4–6 were given the DMV challenge. Groups 1 and 4 were challenged at 3 days of age; groups 2 and 5 were challenged at 7 days of age; and groups 3 and 6 were challenged at 14 days of age (Table 3.1).

Table 3.1: Experimental Design and Summary of Groups

Group	Challenge	Age at Challenge
1	M41	3 days
2	M41	7 days
3	M41	14 days
4	DMV	3 days
5	DMV	7 days
6	DMV	14 days

At seven days post-challenge, choanal swabs were collected from every bird and stored in PBS solution at −80 °C. These swabs were used in quantitative reverse-transcriptase real-time polymerase chain reaction (qRT-PCR) to confirm a successful challenge. Necropsies were conducted seven days post-challenge, five weeks post-challenge, and every following 4 weeks until the end of the experiment at 19 weeks. Ten birds were selected from the groups of interest at the necropsy. Samples of trachea and oviduct were collected and stored in 5% neutral buffered formalin for histopathology submission. Additional samples of trachea and cecal tonsils were taken and stored at −80 °C for additional qRT-PCR if needed. At 19 weeks, all the birds were necropsied. Observations of affected oviducts were noted for each bird in every group. At the final necropsy, five cystic/affected oviducts per challenge group and five unaffected/grossly normal oviducts were chosen for histopathology submission. Tracheas from those ten birds were also submitted for histopathology. The gross and histological lesions for the challenged groups were compared to the established literature for normal oviduct development and histology. The groups were compared to each other to evaluate the challenge timepoint that resulted in the most severe lesions.

2.3. RNA Extraction and qRT-PCR

Viral RNA was extracted from 50 µL of swab fluid or tissue homogenate using the MagMax96 total RNA isolation kit (Ambion, Austin, TX, USA) and the KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA, USA) according to the manufacturer's protocols. The AgPath-ID™ One-step RT-PCR kit (Ambion Inc., Austin, TX, USA) was used to perform qRT-PCR assays according

to the manufacturer's instructions. The 25 µL qRT-PCR reaction mixture included 12.5 µL 2× RT-PCR buffer, 10 µM of each primer, 4 µM of each probe, 1 µL of 25× RT-PCR enzyme mix, and 5 µL of viral RNA. The primers and probes corresponding to the Mass- and DMV/1639/11-type IBV were previously published [Mo et al., 2020]. The qRT-PCR reactions were performed on the Applied Biosystems® 7500 Fast Realtime PCR system (Life Technologies Ltd., Carlsbad, CA, USA) under the following conditions: one cycle of 50 °C for 30 min and 95 °C for 15 min, followed by 40 cycles of 94 °C for 1sec and 60 °C for 60 s (Mass-P), or 40 cycles of 94 °C for 1 s and 53 °C for 60 s (DMV/1639/11-P). Each assay included positive and negative controls for the qRT-PCR reaction and RNA extraction.

2.4. Histopathology

The trachea and oviduct tissue collected at the necropsy for histopathological examination were stored in 10% neutral buffered formalin at room temperature for a minimum of 24 h. The tracheal tissue was cut into two pieces for submission: one transverse and the other sagittal. The undeveloped oviduct tissue of birds 7 days post-challenge was transferred directly into cassettes for histological processing. The immature oviduct tissue of the older birds was trimmed as needed to fit into the cassette. The developed oviduct tissue was cut transversely in various sections. The tissue was embedded in paraffin wax and cut into 4 µm slide sections. The slides were stained with hematoxylin and eosin and evaluated with light microscopy. The slides were scored for lesion severity using the following criteria: inflammation (none, mild, moderate, or severe), presence/absence of exudate in the lumen, and presence/absence of lymphoid nodules. The total scores consist of the sum of each lesion score.

2.5. Riboprobe RNAScope Analysis

Twenty samples (including a positive and negative control) were selected for in situ hybridization. The RNAScope® Assay from Wang et al. [Wang et al., 2012] was performed with either a DMV/1639-specific riboprobe or a Mass-specific riboprobe and followed the Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment procedure, as outlined in the Advanced Cell Diagnostics document 322452.

3. Results

3.1. qRT-PCR

qRT-PCR was used to measure the viral load per group seven days post-challenge, and is expressed as a CT value, where a lower CT value indicates a higher viral load. The mean CT value for the M41-challenged groups was 26.14, while the mean CT value for the DMV-challenged groups was 24.18. Ninety-nine to 100% of the chicks were successfully challenged in all groups (Table 3.2).

Table 3.2: Post-Challenge Viral Load as Measured by qRT-PCR

Group/Challenge	**Age at Challenge**	**Mean CT Value ± SEM 7 Days Post-Challenge**	**% Positive Challenged**
1/M41	3 days	25.19 ± 2.23	100%
2/M41	7 days	25.85 ± 1.73	99.1%
3/M41	14 days	27.38 ± 2.39	100%
4/DMV	3 days	24.171 ± 2.83	100%
5/DMV	7 days	22.47 ± 2.47	100%
6/DMV	14 Days	25.90 ± 2.08	100%

3.2. Gross Lesions

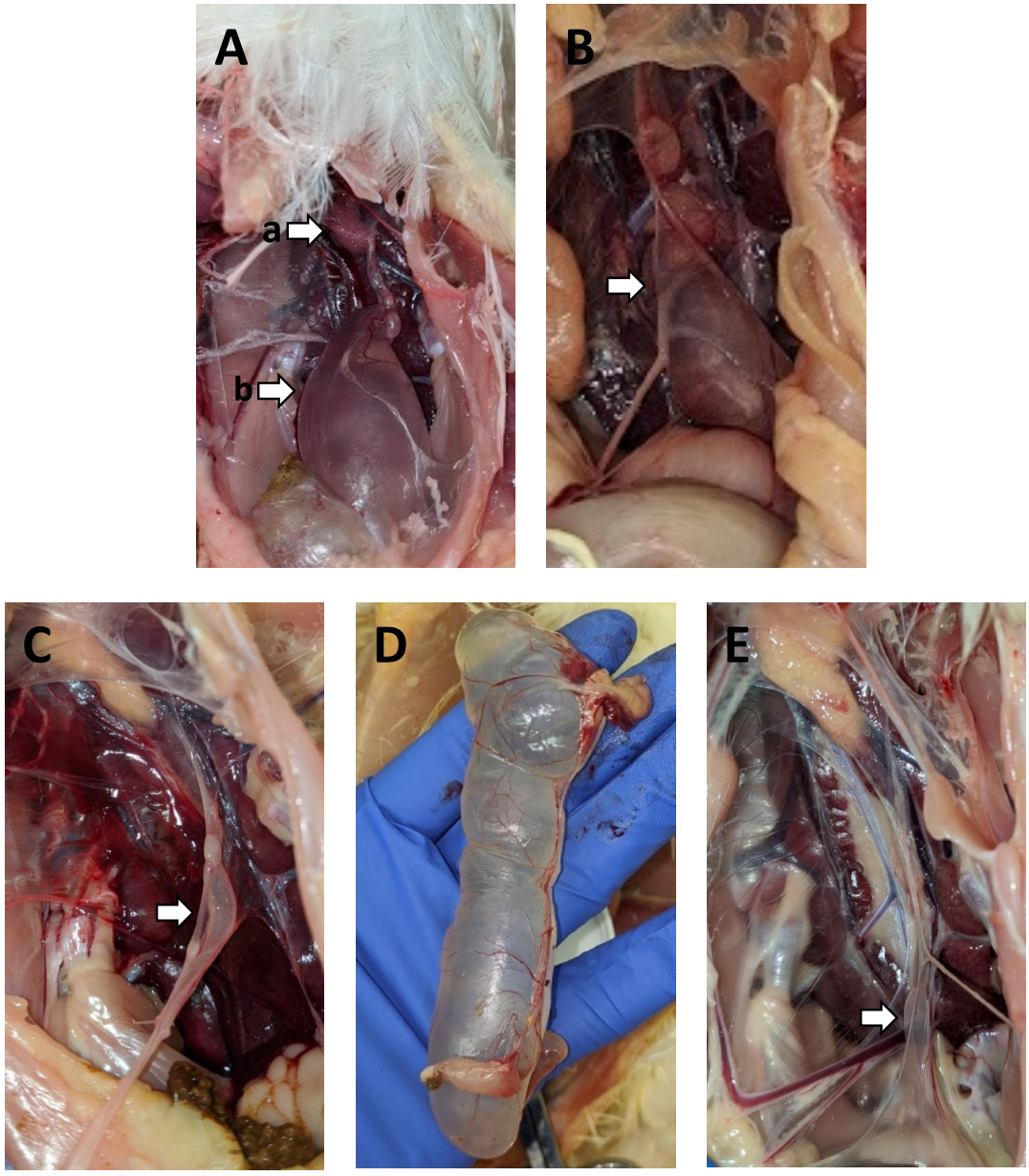

Figure 3.1: **Lesions in the M41 Challenge Group** (Aa) Ovary and (Ab) Cyst in the caudal end of the oviduct from a 5-week-old bird challenged at 7-DOA (B) Cystic oviduct from an 8-week-old bird challenged at 3-DOA (C) Abnormal fluid-filled oviduct from a 12-week-old bird challenged at 3-DOA (D) Large cystic oviduct of a 13-week-old bird challenged at 7-DOA (E) Small cyst at the caudal end of the oviduct of a 13-week-old bird challenged at 7-DOA

Total cystic and affected oviduct prevalence from all necropsy times is summarized in Table 3.3. The highest total percentage of cystic oviduct was seen in Group 2 with M41 challenge at 7 days of age with 32% cystic oviduct total. The highest percentage of cystic oviduct for DMV challenge was seen in group 4 challenged at 3 days of age at 24.56% cystic oviduct total. The highest percentages of affected oviducts follow the same trends.

For the M41-challenged groups, cystic oviducts were observed at the 1-month (2/10 birds) and the 3-month (2/10 birds) post-challenge necropsy for the chickens challenged at 7 days of age (Figure 3.1A). The cysts were most prominent in the caudal end of the oviduct (Figure 3.1A) and were filled with clear fluid. Cystic oviducts were also observed at the 2-month post-challenge (4/10 birds) and the 3-month post-challenge (1/10 birds) necropsy for the chicks challenged at 3 days of age (Figure 3.1B). Some oviducts were not fluid-filled but were considered abnormal as they were especially thin or appeared to be in the early stages of potential cystic oviduct formation. When these abnormal oviducts also appeared to contain fluid, they were considered cystic (Figure 3.1C). Some cystic oviducts in these groups were large and the cystic dilatation spanned the entire oviduct (Figure 1D), while other oviducts had milder cystic dilatation isolated to the caudal ends (Figure 1E). Additional lesions seen included free yolk in the coelomic cavity (yolk coelomitis) and two instances of red-tinged fluid in the cystic oviducts.

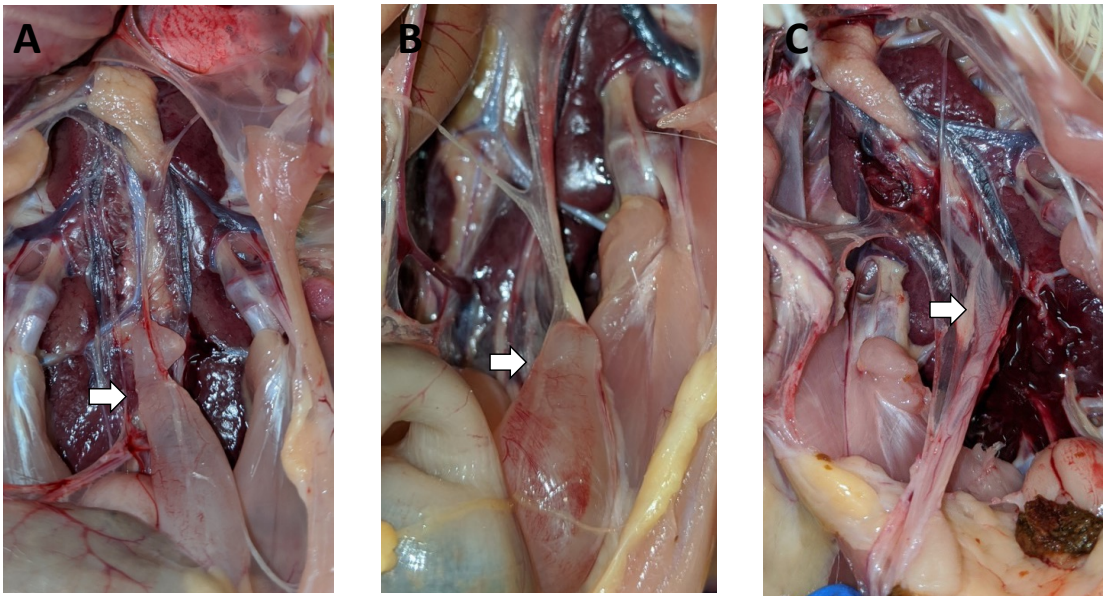

Figure 3.2. Oviduct Lesions in the DMV/1639 Challenge Group (A) The first cystic oviduct detected in the birds challenged with DMV, seen in a 9-week-old bird challenged at 7-DOA (B) Cyst in the caudal end of an oviduct from a 12-week-old bird challenged at 3-DOA (C) Small fluid-filled oviduct from a 13-week-old bird challenged at 7-DOA

For the DMV-challenged groups, cystic oviducts were first observed at the 2-month post-challenge (1/10 birds) necropsy in the 7-days-of-age challenge group (Figure 3.2A). Cystic oviducts were also observed at the 3-month post-challenge necropsy in the 3-days-of-age challenge group (1/10 birds) (Figure 3.2B) and the 14-days-of-age challenge group (3/10 birds). Affected oviducts without distinct cystic dilatation were present in the 3-month necropsy of both the 7- (1/10 birds) and the 14- (1/10 birds) days-of-age challenge groups. These affected oviducts were thin and translucent but were not dilated and fluid-filled like typical cystic oviducts.

Table 3.3: Percentage of Cystic and Affected Oviduct per Group

Group/Challenge	Age at Challenge	Total % Cystic Oviduct	Total % Affected (Including Cystic Oviduct)
1/M41	3 days	14.78%	20.87%
2/M41	7 days	32%	34%
3/M41	14 days	9.91%	9.91%
4/DMV	3 days	24.56%	28.07%
5/DMV	7 days	12.28%	13.16%
6/DMV	14 days	8.55%	9.40%

3.3. Histopathology

The most prominent histological lesion observed was inflammatory cell infiltration, primarily lymphocytes, plasma cells, and heterophils, in both the tissue mucosa and the lamina propria. Many oviducts also had edema and aggregates of lymphoid nodules. Exudate consisting of cell debris or eosinophilic material was found in the lumen of oviducts across all the groups and in some instances could be correlated with cystic oviducts grossly (Figure 3.3 and Figure 3.4). Additional histology findings in a few oviducts include mucosal atrophy, epithelial hyperplasia, and epithelial sloughing. There does not appear to be a relationship between the age at the challenge and the histology lesion score. The lesion scores were similar across all the groups, with some variation. The groups with the highest percentage of scored oviducts with all lesions were challenged at 3 days of age (Table 3.4 and Table 3.5).

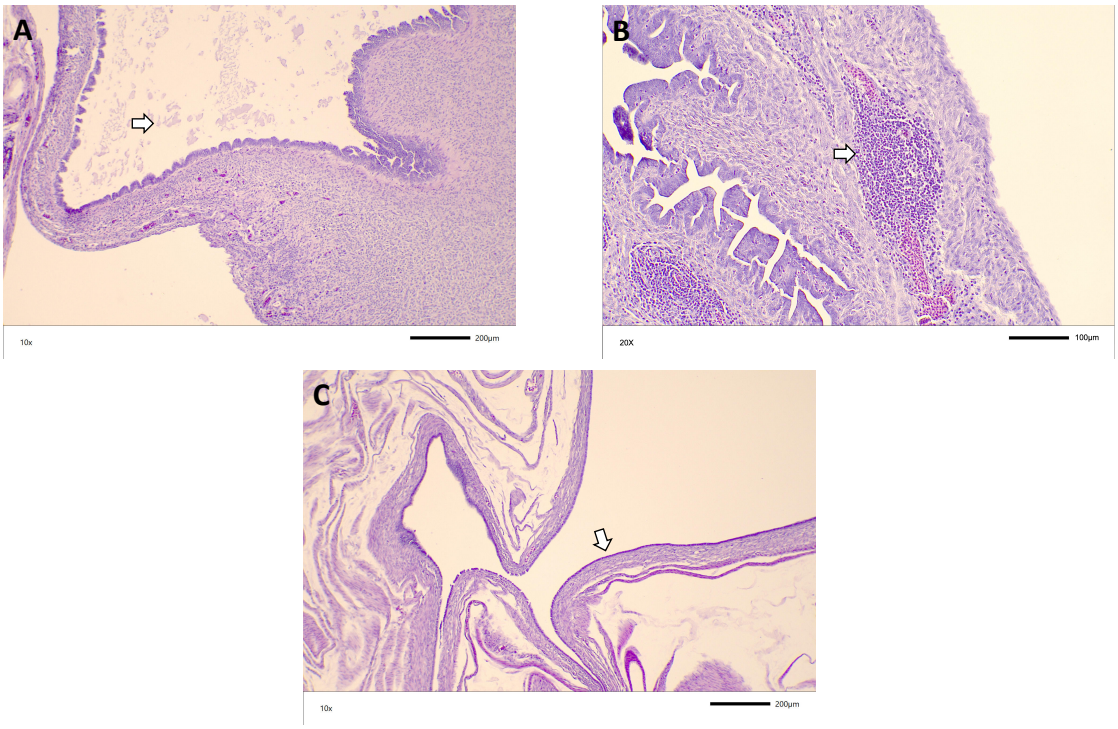

Figure 3.3: Representative Histological Findings (A) Exudate in the lumen of the oviduct (arrow) of a 13-week-old bird challenged with M41 at 7-DOA (B) Lymphoid nodules (arrow) in the oviduct of a 19-week-old bird challenged with DMV at 3-DOA (C) Dilated oviduct with flattened mucosa (arrow) from a 5-week-old bird challenged with M41 at 7-DOA

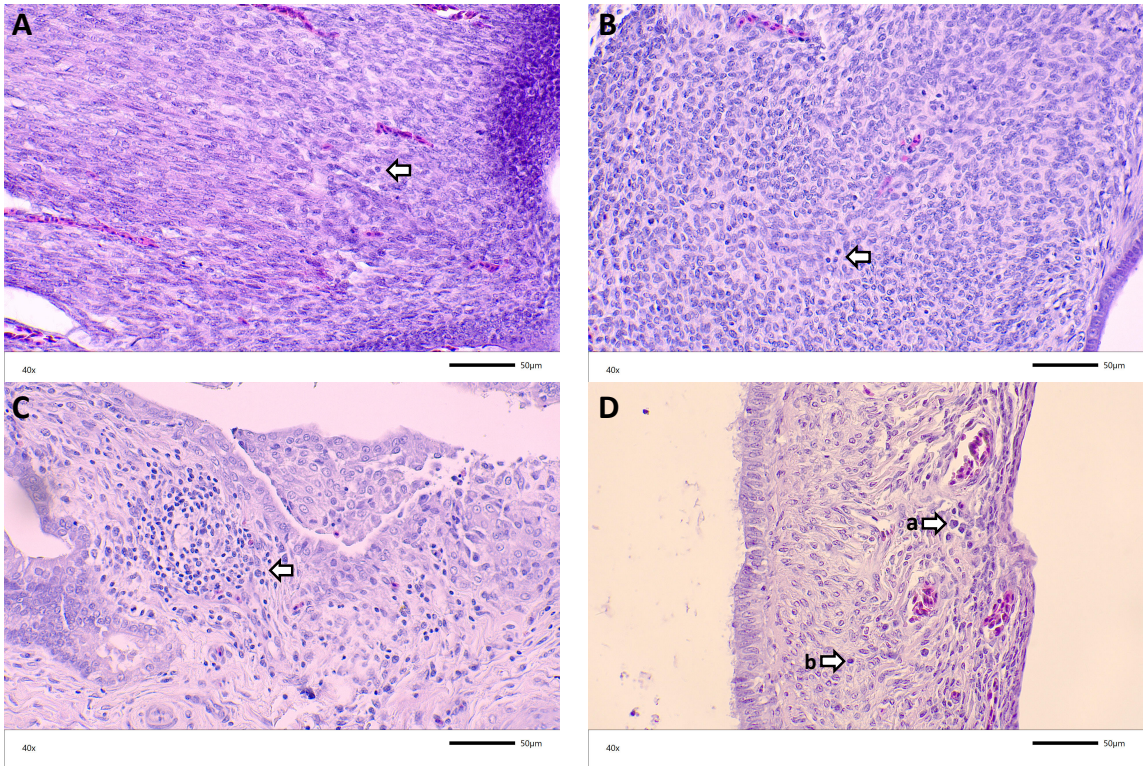

Figure 3.4: Inflammatory Histological Findings (A) Minimal inflammatory infiltrates in oviduct of a 2-week-old bird challenged with DMV at 7-DOA (B) Lymphocytes in the oviduct of a 5-week-old bird challenged with M41 at 7-DOA (C) Lymphoplasmacytic inflammation in the oviduct of a 12-week-old bird challenged with DMV at 3-DOA (Da) Plasma cells and (Db) lymphocytes in the oviduct of a 13-week-old bird challenged with M41 at 7-DOA

Table 3.4: Histopathology Score Breakdown

Score[1]	Inflammation -	Inflammation +	Exudate -	Exudate +	Nodule -	Nodule +
Group 1	43.48%	56.52 %	52.17%	47.83%	71.74%	28.26%
Group 2	37.5%	62.50%	68.75%	31.25%	64.58%	35.42%
Group 3	38.78%	61.22%	53.06%	46.94%	71.43%	28.57%
Group 4	51.02%	48.98%	46.94%	53.06%	65.31%	34.69%
Group 5	50%	50%	70.45%	29.55%	68.18%	31.82%
Group 6	31.91%	68.09%	65.96%	34.04%	48.94%	51.06%

Table 3.5: Total Histopathology Scores

Score[1]	Total -	Total +	Total ++	Total +++
Group 1	23.91%	39.13%	17.39%	19.57%
Group 2	16.67%	39.58%	41.67%	2.08%
Group 3	16.33%	42.86%	28.57%	12.24%
Group 4	12.24%	34.69%	30.61%	22.45%
Group 5	34.09%	29.55%	27.27%	9.09%
Group 6	14.89%	40.43%	27.66%	17.02%

[1]Histological scores of slides were combined into total scores. (-) = no lesions seen, (+) one lesion present, (++) two lesions present, (+++) all scored lesions present

3.4. RNAScope

Using the IBV riboprobe (both Mass and DMV) and the RNAScope, the virus was detected in multiple tissues. Most notably, the virus was detected in the epithelium of the oviduct tissue in a ten-day-old chick challenged with DMV/1639 at 3 days of age. Of the twenty samples submitted for in situ hybridization analysis, only one oviduct was positive for DMV/1639 in the oviduct epithelium. Positive results were also obtained in the trachea, salivary gland, kidney, ureter, and blood monocytes in the oviduct across the twenty tested samples challenged with either M41 or DMV/1639 (Figure 3.5).

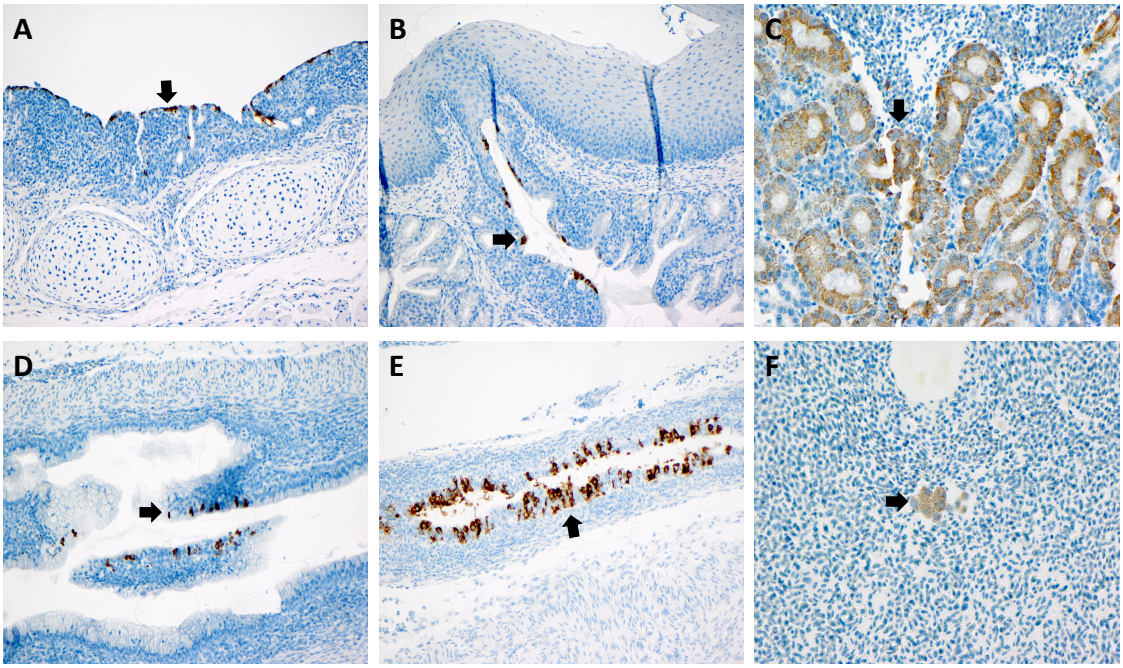

Figure 3.5: RNAScope Findings (A) IBV in the tracheal epithelium (arrow) of a 10-day-old bird challenged with DMV at 3-DOA (B) IBV in the salivary gland epithelium (arrow) of a 10-day-old bird challenged with DMV at 3-DOA (C) IBV in the renal tubular epithelium (arrow) of a 10-day-old bird challenged with DMV at 3-DOA (D) IBV in the ureter epithelium of a 10-day-old bird challenged with DMV at 3-DOA (E) IBV in the oviduct epithelium (arrow) of a 10-day-old bird challenged with DMV at 3-DOA (F) IBV in blood monocytes (arrow) within the oviduct of a 2-week-old bird challenged with M41 at 3DOA.

4. Discussion

From this work, we have shown that both DMV/1639 and M41 can cause cystic oviduct development, which is the primary cause of false layer syndrome. The Massachusetts strain M41 has previously been associated with cystic oviduct development and reproductive lesions [McMartin and Macleod, 1972], while the DMV/1639 strain has only recently been associated with the cystic oviduct in both the field and the laboratory challenge [Hassan et al., 2021]. In previous studies, cystic oviducts could be detected as early as 14 days post-challenge, when chicks were infected at one day of age with a QX strain of IBV [Benyeda et al., 2010], but more commonly 20–30 days post-inoculation with IBV [18]. Our results support this timeline, where the earliest cysts were seen 28 days post-challenge in the M41 group, challenged at 7 days of age, and 56 days post-challenge in the DMV group, challenged at 7 days of age. It should be noted that because we only selected ten birds per necropsy before the final necropsy, it is possible that cystic oviducts were present earlier in the chicks that were not selected for necropsy. Our results also demonstrate that young birds are particularly vulnerable to the reproductive effects of IBV. The groups that were challenged prior to two weeks of age had higher percentages of grossly affected oviducts than the chicks challenged at two weeks of age. This supports many of the previous investigations into false layer syndrome and cystic oviduct development, where younger birds were affected in greater numbers and experienced more severe gross lesions than birds infected at older ages [Broadfoot et al., 1957; McMartin and Maclead, 1972; Crinion and Hofstad, 1972a; Bozorgmehri-Fard et al., 2013].

Cystic dilatation was most commonly found at the shell gland of the oviducts, though this lesion was present in some magnum and isthmus or continuously throughout the length of the oviduct. The cystic dilatation noted in previous research was often located caudal to the areas of oviduct hypoplasia, such as the level of the suspensory ligament or in the lower portion of the immature oviduct and became larger as the hen got older [Crinion et al., 1971a; Duff et al., 1971; Jones and Jordan 1972]. In addition to cystic oviducts filled with clear fluid that had a definitive shape, some fluid-filled but shapeless and undeveloped oviducts were also noted. This also supports previous work demonstrating that infection with IBV causes a range of reproductive lesions and abnormal oviducts, not just cystic oviduct development. These abnormal oviducts can range from underdeveloped, but patent, to a blind sac projecting near the cloaca [20]. Additional oviduct lesions, especially in false layers, include oviducts considerably shorter than normal-length and non-patent oviducts [Broadfoot et al., 1956]. While most birds experiencing false layer syndrome will have occluded oviducts, some might have normal oviducts that are not functioning [Crinion, 1972]. The oviducts of infected birds might be normal in appearance, but the degree of development across an affected flock might not be uniform [Duff et al., 1971].

In a few cases, IBV-infected birds in this study had free yolk in the coelomic cavity at final necropsy. Because IBV damage affects reproductive tissues, the normal egg-development process is disrupted. Damage to the anterior aspect of the oviduct either prevents the infundibulum from properly enveloping the yolk or causes the infundibulum to eject the yolk via reverse peristalsis [Broadfoot et al., 1956]. In many cases, authors have described an accumulation of yolk fluid in the coelomic cavity of infected birds. This

accumulation indicates that ovulation is occurring directly into the body cavity rather than into the infundibulum [Broadfoot et al., 1956]. This presentation has also been frequently noted in field cases of false layer syndrome [Petrik, 2021].

Historically, microscopic oviduct lesions include significant epithelial cell involvement, as these cells are the primary cells targeted by the virus [Crinion and Hofstad, 1972a]. These lesions resemble respiratory epithelial cell lesions, including epithelial sloughing, necrosis, congestion, and hemorrhage. Inflammatory cell infiltrates can be present, and there may be edema of the lamina propria or submucosa [Landman, 20015; Zhong et al., 2016]. The muscular layers of the oviduct, the lamina propria, and the mucosal glands are subject to an infiltration of inflammatory cells. Plasma cells, mononuclear cells, and lymphocytes make up most of the infiltrates. The lamina propria might also be affected by fibroplasia and/or edema [Sevioan and Levine, 1957]. The primary lesion seen on slides of infected birds in this study was inflammatory cell infiltration, specifically lymphocytes, plasma cells, and heterophils, which is consistent with IBV infection. Edema was present in many oviducts of all the challenge groups and ages and reflects common post-infection findings after IBV challenge [Hoerr, 2021].

Lymphoid nodule development varied from a few localized nodules to the severe multifocal presence of nodules. Previous studies demonstrate that lymphoid cell infiltration into the oviduct occurs as early as the third day post-inoculation, which leads to the development of lymphoid nodules in the oviduct wall, especially near blood vessels. The infiltration lesions are less likely to lead to permanent damage than the epithelial cell lesions [Crinion and Hofstad, 1972a], and the degree of infiltration in the oviducts of birds producing poor-quality eggs is approximately the same as the infiltration in the oviducts of

birds producing normal eggs [Crinion et al., 1971b]. There did not appear to be a relationship between the presence of inflammatory infiltrates and the age at the challenge, which supports the work of Crinion and Hofstad, who found that age did not play a role in the development of infiltrative lesions in the oviduct [Crinion and Hofstad, 1972b]. Aside from the presence of inflammation, edema, and dilated lumens, there was little to no indication of the cause of the cyst formation. Benyeda et al. [Benyeda et al., 2010] describe few histopathological changes associated with cystic oviducts to explain their formation, which was true for this study as well. In that study, they did not find epithelial damage, excessive secretion, or excessive inflammation in the cystic oviducts of birds infected with the QX strain at 1 day of age.

DMV/1639 was detected in oviduct epithelium using riboprobe and RNAScope analysis. IBV has been detected in the lung and cloacal bursa via in situ hybridization with a riboprobe [Kapczynski et al., 2002], though to our knowledge no previous study has used in situ hybridization with a riboprobe to locate IBV in oviduct tissue. The detection of IBV in the salivary gland via Riboprobe is further supported by the results of Franca et al. [Franca et al., 2011], who detected IBV antigen in the salivary gland via immunohistochemistry. Similar to riboprobe analysis, immunohistochemistry has been conducted on oviduct epithelium to target the IBV antigen. The viral antigen localizes to the epithelial cells in the oviduct and is often confined to the cytoplasm, making early diagnosis possible with immunohistochemistry [Hassan et al., 2021; Braune, 1965]. In their 1972 experiment, Crinion and Hofstad showed that viral antigen quantities in the chick oviduct were highest at 6 days post-inoculation. As hen age-at-inoculation increases, the viral antigen becomes more difficult to detect in reproductive tissue. Chickens inoculated

at a younger age retain the viral antigen in the oviduct longer than older inoculated chickens [Crinion and Hofstad, 1972a]. Jones and Jordan [Jones and Jordan, 1972] hypothesize that the immunological naivety of young birds leads to increased viral persistence in the blood, which in turn leads to increased viral presence in the tissue over a longer period of time than birds inoculated at older ages. The best time to test for viral antigen presence appears to be 5–7 days post-inoculation, based on previous literature [Crinion and Hofstad, 1972a; Jones and Jordan, 1972]; however, it is important to note that instances of cystic oviducts without the presence of the IBV viral antigen in the oviduct have been recorded in birds 6 weeks of age [Benyeda et al., 2010], and the results of riboprobe analysis may be similar.

5. Conclusions

IBV DMV/1639 infection at a young age causes reproductive lesions and cystic oviducts in laying hens, particularly when pullets are infected prior to two weeks of age. IBV DMV/1639 and M41 were detected with the DMV-specific or Mass-specific riboprobe RNAscope technique in the oviduct epithelium, confirming IBV infection as the cause of oviduct damage.

Author Contributions

Conceptualization, B.J. and M.J.; Methodology, B.J., M.J. and M.F.; Investigation, B.J. and A.M.S.; Data Curation, B.J. and A.M.S.; Writing—Original Draft Preparation, A.M.S.; Writing—Review and Editing, B.J., M.J. and M.F.; Funding Acquisition, B.J. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of the University of Georgia Animal Care and Use Committee (UGA IACUC) under the approved animal use protocol (AUP) A2019 02-016-Y3-A1.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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CHAPTER 4

EVALUATING THE EFFECTS OF IBV VACCINATION ON THE DEVELOPMENT OF FALSE LAYER SYNDROME[2]

[2]Adrea Mueller Slay, Mark Jackwood, Brian Jordan *(to be submitted to Avian Diseases)*

ABSTRACT

Infectious Bronchitis virus (IBV) is an avian coronavirus that primarily causes respiratory disease but can also affect the reproductive tract of laying type chickens. If infection occurs in young pullets, False Layer Syndrome can develop. False Layer Syndrome is characterized by changes in oviduct development and the formation of large, fluid-filled cysts. Vaccination is used to control disease caused by IBV, but False Layer Syndrome is still seen in vaccinated hens. We hypothesize that the timing of vaccination in relation to infection with pathogenic IB virus influences the development of cystic oviducts and False Layer Syndrome regardless of vaccine serotype used. To test this, five groups of 150 SPF pullets each were placed into separate colony type houses at day of hatch. Groups 1 and 5 were not vaccinated at hatch, while Groups 2 and 4 were given Mass vaccine at hatch and Group 3 was given GA08 vaccine at hatch. IBV variant DMV/1639 challenge was administered oculonasally to groups 1-3 and 5 at 7 days of age as this virus has been proven to cause False Layer Syndrome. Groups 1-4 were also given a Mass/Conn live attenuated IBV vaccine at 2-weeks of age. All vaccinated groups received subsequent Mass/Conn live-attenuated IBV vaccines at 6 and 12-weeks. Necropsies were conducted every four weeks post-challenge, and at 20 weeks of age, to evaluate reproductive effects and development of cysts. Trachea and oviduct tissue were submitted for histology to assess microscopic changes. It was determined from gross lesion accounts and histological scores that vaccination status had little to no effect on cystic oviduct formation and

histological lesions associated with early DMV/1639 challenge in maternal antibody negative SPF pullets, further indicating that the prevalence and severity of false layer syndrome development in a flock is multifactorial.

1. Introduction

Infectious Bronchitis Virus (IBV) is an economically significant pathogen in commercial chickens that causes increased mortality, decreased feed conversion, high condemnation, and decreased egg production and quality [Awad et al., 2014]. Though mortality rates are low (less than 5%) unless secondary infections are involved, morbidity rates can reach 100% [Awad et al., 2014; Broadfoot et al., 1956]. IBV is horizontally transmitted in birds in natural infection [Sevoian and Levine, 1957] and chickens of all ages are susceptible to IBV, though most severe disease is typically seen in younger birds. Disease severity is dependent upon a combination of factors including age at exposure, strain of IBV that birds are exposed to, and route of inoculation.

IBV is primarily a respiratory disease, however some serotypes of the virus are known to affect the epithelial cells of the chicken reproductive tract leading to permanent decreases in egg production and quality. Considerable economic loss can occur in infected hens who pause production and then do not resume normal production levels. IBV also causes poor egg quality characteristics such as soft-shelled eggs, misshapen eggs, and eggs with watery albumen [Broadfoot et al, 1956; Muneer et al., 1986; Ignjatovic and Sapats, 2000]. In addition, False Layer Syndrome is described as a clinical sign and result of exposure to Infectious Bronchitis Virus that causes fluid-filled cysts to develop in the oviduct [Sevoian and Levine, 1957]. These cystic oviducts lead to permanent damage that will decrease egg production in the affected birds [McMartin and Macleod, 1972; de Witt et al., 2011]. IBV strains QX, Massachusetts, Australian T, and variant DMV/1639 have been associated with cystic oviduct formation and False Layer Syndrome, especially when

birds are infected at a young age [de Wit, et al., 2011; McMartin and Macleod, 1972; Broadfoot et al., 1956; Hassan et al., 2021; Mueller Slay et al., 2022].

In the field, prevention of cystic oviduct formation has been attempted through the addition of IBV vaccination at the hatchery to offer some protection against early DMV/1639 challenge [da Silva et al., 2021]. Vaccination is the most used prevention method against IBV, though it can be difficult due to differences between the field or variant serotypes and the vaccine serotypes [Gelb Jr et al., 1991]. Vaccination of high titers has been shown to produce better serological protection than lower titer vaccinations, though clinical signs caused by vaccination must be considered for vaccines of significant titers [Raggi and Lee, 1965]. Massachusetts-strain vaccines are considered the "industry-standard" for IBV vaccination [Li et al., 2020]. Although vaccination strategies are often serotype specific and can have limited cross-protection [Kulkarni and Reynaldo, 2010], instances of vaccination against one serotype vaccine protecting against an antigenically distinct serotype have been recorded. The Massachusetts-type vaccine is one that shows evidence of cross-protection [Terregino et al., 2008]. Vaccination programs that induce high antibody titers and uniform coverage are most effective at reducing loss due to reproductive lesions [Box et al., 1988]. To date, there is no commercial vaccine available for variant DMV/1639.

In the current disease situation with False Layer Syndrome and cystic oviduct formation in the US and Canada caused by infection with variant IBV DMV/1639, most producers introduced a Mass type vaccine in the hatchery for day of hatch vaccination. Other producers decided to use a GA08 type vaccine in the hatchery based on cross-protection claims by vaccine manufacturers. Both vaccines were successful at limiting the

incidence of false layer syndrome, even though neither vaccine was serotype matched to the DMV/1639 field virus. Because of this, we set out to determine if the vaccine type was the predicating factor for protection of the oviduct, or if vaccination alone with a live attenuated IBV vaccine was enough to stem infection from a DMV/1639 challenge.

2. Materials and Methods

Specific pathogen free (SPF) chicks were hatched at the Poultry Diagnostic and Research Center (PDRC), University of Georgia, and vent-sexed. All female chicks were combined into one cohort, then randomly separated into five groups of 150 birds each. A summary of the vaccination and challenge schedule per group is shown in Table 4.1. Groups 2 and 4 were vaccinated with a Massachusetts strain vaccine at hatch via commercial spray cabinet and Mass/Conn strain vaccine at 14 days of age via oculonasal drop. Group 3 was vaccinated with a Ga08 strain at hatch via commercial spray cabinet and a Mass/Conn strain vaccine at 14 days of age via oculonasal drop. Group 1 received a Mass/Conn vaccination at 14 days of age via oculonasal drop and no vaccine at hatch. Groups 1-4 also received additional Mass/Conn vaccinations at 6 and 12 weeks of age via backpack sprayer. Group 5 did not receive any vaccination. Birds were reared in colony type houses on fresh pine shavings and given food and water ad libitum. Chicks in all groups except Group 4 were challenged at 7 days of age with 1x10^4.2 EID50 IBV DMV/1639/11 strain via the intraocular/intranasal route [Mueller Slay et al., 2022].

Seven days post-vaccination and post-challenge, choanal swabs were collected from every bird in groups 2, 3, and 4 and stored in PBS solution at -80^{oC}. Twenty-five random birds were also swabbed from Groups 1 and 5 at 7-days post hatch to confirm that no vaccine virus was present. All birds in Group 4 were sampled 7-days post challenge to

confirm that no challenge virus was present. Twenty-five random birds from Group 5 were swabbed 7 days post vaccination (14-days of age) to confirm that no vaccine virus was present. These swab samples were used in quantitative reverse-transcriptase real-time polymerase chain reaction (qRT-PCR) to confirm successful vaccination and challenge.

Necropsies were conducted approximately every 4 weeks following challenge until the end of the experiment at 20 weeks. Ten random birds were selected from the groups of interest at necropsy. Samples of trachea and oviduct were collected and stored in 5% neutral buffered formalin for histopathology submission. Additional samples of trachea and cecal tonsils were taken and stored at -80C for additional qRT-PCR if needed. At 20 weeks, all remaining birds were necropsied. Observations of affected oviducts were noted for each bird in every group. At the final necropsy, five cystic/affected oviducts per challenge group and five unaffected/grossly normal oviducts were chosen for histopathology submission. Tracheas from those ten birds were also submitted for histopathology. Gross and histological results were compared among each group to evaluate the effect of vaccination(s) on the development of reproductive lesions after IBV DMV/1639 challenge.

Viral RNA was extracted from 50 µl of swab fluid or tissue homogenate using the MagMax96 total RNA isolation kit (Ambion, Austin, TX) and the KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA) according to the manufacturer's protocols. The AgPath-ID™ One-step RT-PCR kit (Ambion Inc., Austin, TX) was used to perform qRT-PCR assays according to the manufacturer's instructions. The 25µl qRT-PCR reaction mixture included 12.5µl 2X RT-PCR buffer, 10µM of each primer, 4 µM of each probe, 1µl of 25X RT-PCR enzyme mix

and 5 µl of viral RNA. Primers and probes corresponding to the Mass, Ga08, and DMV/1639/11 type IBV are previously published (Mo et al., 2020). The qRT-PCR reactions were performed on the Applied Biosystems® 7500 Fast Realtime PCR system (Life Technologies Ltd., Carlsbad, CA) under the following conditions: one cycle of 50°C for 30min and 95°C for 15min followed by 40 cycles of 94°C for 1sec and 60°C for 60sec (Mass-P, GA08-P), or 40 cycles of 94°C for 1sec and 53°C for 60sec (DMV/1639/11-P). Each assay included positive and negative controls for qRT-PCR reaction and RNA extraction.

Table 4.1: Experimental Design

Group	**Challenge**	Vaccine at Hatch	Vaccine at 14 DOA
1	DMV	-	Mass/Conn (eyedrop)
2	DMV	Mass (spray)	Mass/Conn (eyedrop)
3	DMV	Ga08 (spray)	Mass/Conn (eyedrop)
4	-	Mass (spray)	Mass/Conn (eyedrop)
5	DMV	-	-

Trachea and oviduct tissue collected at necropsy for histopathological examination were stored in 10% neutral buffered formalin at room temperature for a minimum of 24 hours. Tracheal tissue was cut into two sections for submission: one transverse and the other sagittal. Undeveloped oviduct tissue of birds 7 days post challenge was transferred directly into cassettes for histological processing. Immature oviduct tissue of older birds was trimmed as needed to fit into the cassette. Developed oviduct tissue was cut transversely in various sections. Tissue was embedded in paraffin wax and cut into 4um slide sections. The slides were stained with hematoxylin and eosin and evaluated with light microscopy. Slides were scored only for oviduct lesion severity using the following

criteria: presence/absence of inflammation, presence/absence of exudate in the lumen, presence/absence of lymphoid nodules, and presence/absence of oviduct dilation. Total scores consist of the sum of each lesion score.

3. Results

3.1 PCR

qRT-PCR was used to measure the viral load per group seven days after each vaccination and seven days post challenge. This viral load is expressed as a CT value, where a lower CT value indicates higher viral load. The mean CT value for the at-hatch vaccinations was 28.58 with 98.65-100% of the birds positive for vaccine virus. The mean DMV/1639 CT value for the groups that did not receive vaccination before challenge was 28.44. The mean DMV/1639 CT value post-challenge for the groups that were vaccinated at hatch was 30.28. 96.56-100% of chicks in challenged groups were successfully challenged (Table 4.2).

Table 4.2: Post-Vaccination and Post-Challenge Results as Measured by qRT-PCR

Group	CT Post @Hatch Vax	% Vaccinated	CT Post Challenge	% Challenged
1	-	-	29.39±2.43	100%
2	Mass: 28.07±2.20	100%	29.61±2.28	98.36%
3	Ga08: 29.53±2.05	98.65%	30.94±2.69	96.56%
4	Mass: 28.14±2.17	100%	-	-
5	-	-	27.49±1.81	100%

3.2 Gross Findings

Gross lesion findings from all groups are summarized in Table 4.3. Cysts, filled with clear fluid, were most prominent in the caudal end of the oviduct but were also found along the entire length of the oviduct in some birds (Figure 4.1:C and 4.1:D). The highest total percentage of cystic oviduct (23.61%) was seen in Group 1, challenged with DMV/1639 at 7 days of age and vaccinated with a Mass/Conn vaccine at 14 days of age. In addition to cyst formation, a second lesion, referred to here as "fluid-filled" oviducts, was identified (Figure 4.1:B). Of the challenged groups, the groups that received vaccination at hatch had a higher percentage of fluid-filled oviducts than the groups that did not receive vaccination at hatch (Table 4.4).

Some oviducts were "abnormal" but not cystic or fluid filled. These abnormal oviducts were shorter in length than expected, had thin oviduct walls, or included birds with ruptured yolks in the coelomic cavity. Interestingly, the group vaccinated at hatch with Mass and at 14 days of age with Mass/Conn but not challenged with DMV/1639 also had affected oviducts (Figure 4.1:B and 4.1:C). This group was negative for DMV/1639 when choanal cleft swabs were tested via qRT-PCR. This group had the lowest percentage of cystic (6.96%) and abnormal oviducts (0.87%), but the highest percentage of fluid-filled oviducts (13.91%).

Table 4.3: Lesion Prevalence per Group

Group	No Lesion	Fluid	Cyst	Abnormal
1	70.14%	4.17%	23.61%	2.08%
2	69.60%	12.00%	14.40%	4.00%
3	68.97%	11.72%	15.86%	3.45%
4	78.26%	13.91%	6.96%	0.87%
5	69.06%	8.63%	16.55%	5.76%

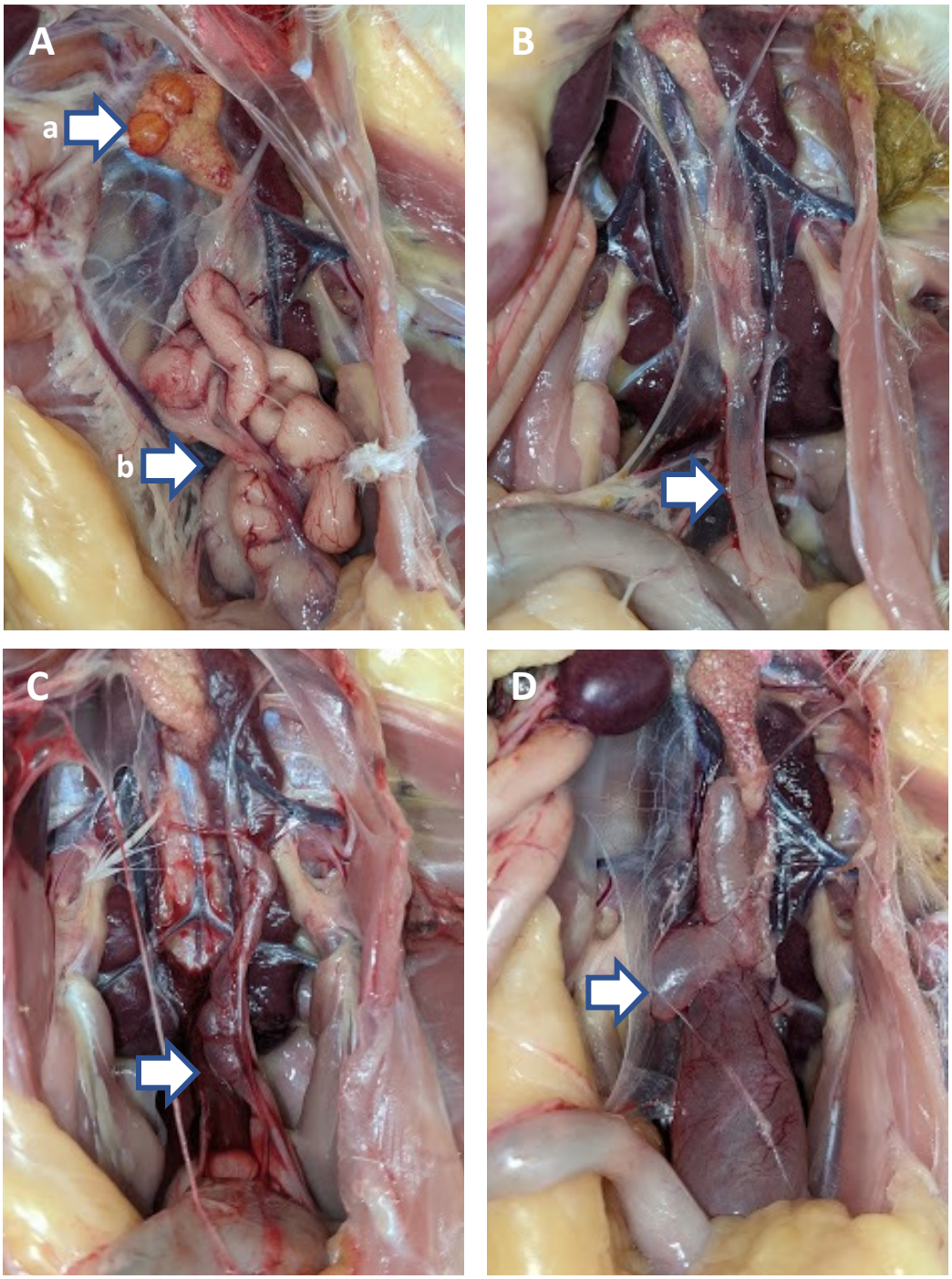

Figure 4.1: **Gross Oviduct Lesions-** (A)Normal developing ovary (a) and oviduct (b) in a 16-week old bird; (B) Fluid-filled oviduct in a 16-week old bird vaccinated with Mass at hatch and Mass/Conn at 14-days; (C) Cystic oviduct in a 16-week old bird vaccinated with Mass at hatch and Mass/Conn at 14-days; (D) Cystic oviduct in a 16 week old bird challenged with DMV/1639 at & days of age, vaccinated with Ga08 at hatch, and vaccinated with Mass/Conn at 14 days of age.

3.3 Histopathology

The most prevalent histological lesion (≥ 58.97%) observed was the presence of exudate composed of cellular debris or eosinophilic material in lumen (Figure 4.2:F). Oviducts that were grossly cystic were more likely to contain exudate though many oviducts that were not grossly cystic contained exudate in the lumen when examined histologically. Additionally, many oviducts contained inflammatory cell infiltration composed primarily of lymphocytes, plasma cells, and heterophils in both the tissue mucosa and lamina propria (Figure 4.2:E). Edema and aggregates of lymphoid nodules (Figure 4.2:C) were also present (Table 4.4).

Table 4.4: Prevalence of Histological Lesions per Group

Group	Inflammation	Exudate	Lymphoid Nodules	Dilated
1	56.00%	72.00%	54.00%	40.00%
2	48.98%	65.31%	44.90%	26.53%
3*	58.97%	58.97%	41.03%	28.21%
4	46.00%	64.00%	34.00%	16.00%
5	54.00%	70.00%	40.00%	18.00%

*Group 3 Necropsy #3 samples were not properly fixed in formalin and were too autolyzed for histological analysis. Percentages reflect the total available slides for scoring

Histological scoring was evaluated as previously described (Mueller Slay et al., 2022), with the addition of a fourth lesion category. These categories include presence/absence (+/-) of Inflammation, presence/absence (+/-) of exudate in the lumen, presence/absence (+/-) of lymphoid nodules in oviduct tissue, and presence/absence (+/-) of dilation evident in the oviduct (Figure 4.2:B). Lesion scores were combined for a total histological score with a maximum of ++++.

Lesion scores were similar across all groups with some variation (Table 4.5). Birds in Group 1 that received DMV/1639 challenge and a Mass/Conn vaccination at 14 days of age had the highest average histology score of 2.16±1.35. The group with the highest percentage of "++++" scored oviducts was also Group 1. The group with the highest percentage of no lesion score "-" was Group 4 which received Mass vaccination at hatch, Mass/Conn vaccination at 14-days, but no challenge virus. There does not appear to be a relationship between vaccination status and histology lesion score.

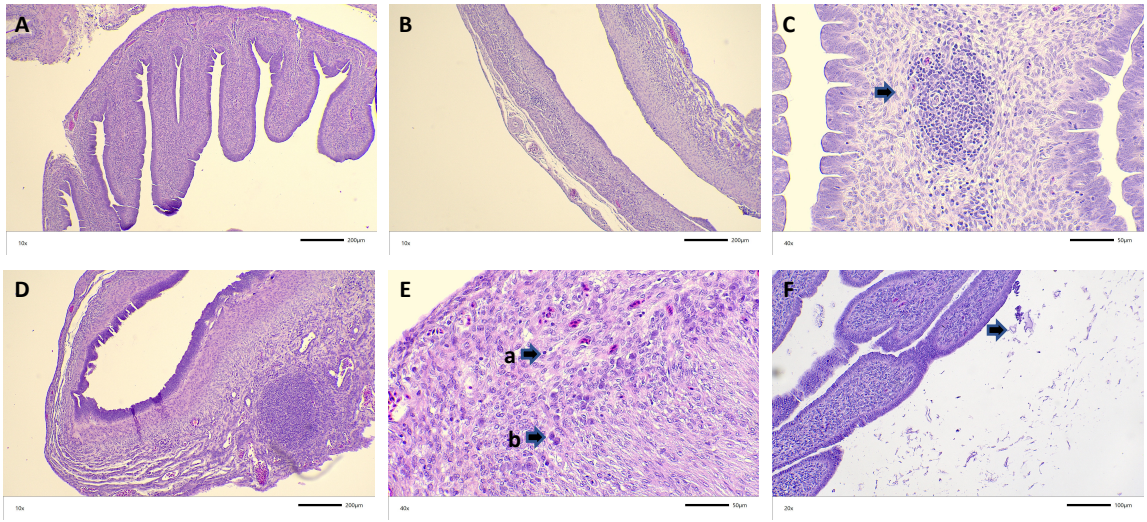

Figure 4.2: Histological Lesions and Scoring- (A) Oviduct with a score of 0 from a 12-week-old bird in Group 2; (B) Dilation and mucosal flattening of an oviduct from a 20-week-old bird in Group 3; (C) Lymphoid nodules in the oviduct of a 9-week-old bird in Group 2; (D) Oviduct with a score of 4 from a 12-week-old bird in Group 1; (E) Inflammatory infiltrate composed of lymphocytes (a) and plasma cells (b) in the oviduct of a 9-week-old chicken in Group 3; (F) Exudate in the lumen of the oviduct from a 6-week-old chicken in Group 2

Table 4.5: Lesion Score Prevalence Per Group

Group	-	+	++	+++	++++
1	8.00%	34.00%	16%	18%	24%
2	18.37%	28.57%	12.24%	32.65%	8.16%
3*	15.38%	28.21%	17.95%	23.08%	15.38%
4	20.00%	28.00%	26.00%	18.00%	8.00%
5	6.00%	38.00%	26.00%	24.00%	6.00%

*Group 3 Necropsy #3 samples were not properly fixed in formalin and were too autolyzed for histological analysis. Percentages reflect the total available slides for scoring

4. Discussion

In this work, SPF pullets were challenged with DMV/1639 at 7 days of age to induce severe reproductive lesions since younger birds are affected in greater numbers and experience more severe gross lesions than birds infected at older ages [Broadfoot et al., 1956; McMartin and Macleod, 1972; Crinion and Hofstad, 1972b; Bozorgmehri-Fard et al., 2013; Mueller Slay et al., 2022]. Similar gross lesions were identified in this study as a result of early challenge when compared to the previous [Mueller Slay, 2022], and fluid-filled lesions were detected in all groups. Vaccinated groups had a higher percentage of fluid-filled lesions compared to unvaccinated groups, which might suggest that the fluid-filled oviduct is a lesser lesion or that it is cyst development delayed by vaccination. Due to the terminal nature of oviduct analysis, it is not known whether these oviducts will continue into functional oviducts or will continue into cyst development. The work by Parent et al. [2020] explores the use of ultrasonography to detect cystic oviducts in live birds, and it would be interesting to further investigate the use of ultrasonography in these fluid-filled lesions.

Cystic dilatation was most often at the shell gland of the oviducts, though this lesion was present in some magnum and isthmus or continuously throughout the length of the oviduct. In some samples, cysts were separated into distinct segments and the fluid was not free moving throughout the entire oviduct. Previous work has demonstrated a range of oviduct lesions as a result of IBV infection, ranging from underdeveloped but patent to a blind sac projecting near the cloaca [Jones and Jordan, 1972]. It has been recorded that segmental cyst development is a result of the collapse and fibrosis of the oviduct during acute infection, causing segmented accumulation of oviduct secretions [Hoerr, 2021].

No appreciative difference in cystic oviduct presence was identified in groups vaccinated compared to groups that were not vaccinated. This seems to contradict the observation that adding a vaccination at hatch can decrease the incidence of false layer syndrome in a flock in the field [da Silva et al., 2021]. The challenge load given to birds in this experiment is known to be strong. Such a strong challenge load might not accurately reflect the amount of challenge virus birds are exposed to in the field and as a result the challenge virus may overcome any potential effects of vaccination.

Additionally, the SPF birds used in this experiment are maternal antibody negative whereas commercial pullets contain maternal antibodies against IBV. Maternal antibodies are passed from hen to chick to protect the chick in the early stages of the development of its immune system. Passive immunity through maternal IgY antibodies is suspected to protect young chicks from abnormal oviduct development due to early IBV infection [Broadfoot et al, 1956]. Crinion et al. noted that birds with maternal antibodies are less likely to exhibit the severe reproductive lesions seen in SPF birds without maternal antibodies [1971a]. These results were supported by work by de Wit et al in 2011

investigating the effects of vaccination and maternal antibodies on the development of reproductive lesions after early infection with D388 strain IBV and might explain why vaccination alone did not offer protection against reproductive lesions in this experiment.

After infection with IBV, the muscular layers of the oviduct, the lamina propria, and the mucosal glands are subject to infiltration of inflammatory cells. Plasma cells, mononuclear cells, and lymphocytes make up most of the infiltrates. The lamina propria might also be affected by fibroplasia and/or edema [Sevoian and Levine, 1957]. Lymphoplasmacytic infiltration in the mucosa, lamina propria, and muscular layers was identified in every treatment group and is consistent with known effects of IBV infection. Occasionally, heterophils and macrophages were also seen. Edema was present in many oviducts of all challenge groups and ages and reflects common post-infection findings after IBV challenge [Hoerr, 2021].

Lymphoid nodule development and dilation of the oviduct lumen were found in every group and varied from a few localized nodules to severe multifocal nodules. The most prevalent lesion across all groups was the presence of exudate in the lumen. The exudate, composed of eosinophilic material and/or cellular debris, was found in most oviducts that were grossly cystic. Cellular debris build up at the vaginal-cloacal orifice is thought to lead to the development of cystic oviducts and the retention of exudate composed of oviduct secretions [Hoerr, 2021].

There did not appear to be a relationship between histological lesion scores and vaccination status. Previous studies demonstrate that infiltration lesions and nodule formation are less likely to lead to permanent damage than the epithelial cell lesions [Crinion and Hofstad, 1972a] and the degree of infiltration in the oviduct of birds producing

poor quality eggs is approximately the same as the infiltration in the oviduct of birds producing normal eggs [Crinion et al., 1971b]. Benyeda et al [2010] did not find epithelial damage, excessive secretion, or excessive inflammation histologically in cystic oviducts of birds infected with the QX strain at 1 day of age to explain the formation of cystic oviduct, which supports the little correlation between histological lesion score and treatment group of this study. de Wit et al. found that a combination heterologous live vaccination at 1 and 14 days of age followed by an inactivated vaccination at 12 weeks of age prevented egg production and quality problems associated with D1466 challenge at 24.5 weeks of age, though it's important to note that the birds in that experiment were challenged as adults and would have more developed immune systems than young pullets [2020]. Homologous vaccination is likely still the best option for protection against the reproductive effects of IBV and should be used when available [Shao et al., 2020].

In this experiment, groups that received a Mass vaccination at hatch and a subsequent Mass/Conn vaccination at 14-days of age developed gross and histological lesions in the oviduct despite never being exposed to challenge virus. The literature on the ability of vaccines to cause reproductive disease in hens is debated. Broadfoot et al state that a commercial attenuated IBV vaccine did not cause abnormal oviducts [1956], but some low passage live vaccines have produced cysts in chicks exposed in the first week of age [Crinion, 1972]. An early live vaccination in chicks without maternal antibodies may explain the increase incidence of affected oviducts seen in this experiment.

4. Conclusion

Vaccination status had little to no effect on cystic oviduct formation and histological lesions associated with early DMV/1639 challenge in maternal antibody negative SPF pullets. Vaccinated birds had an increased incidence of fluid-filled lesions compared to unvaccinated groups, but it is unclear whether these fluid-filled oviducts will progress to cysts or will become functional developed oviducts. Early vaccination in maternal antibody negative SPF pullets led to few instances of both fluid-filled and cystic oviduct formation, though fewer affected oviducts were noted for the vaccinated group than for groups that received challenge virus.

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Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Institutional Review Board Statement:

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the University of Georgia Animal Care and Use Committee (UGA IACUC) under the approved animal use protocol (AUP) A2019 02-016-Y3-A1.

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CHAPTER 5

EVALUATING THE EFFECTS OF IBV MATERNAL ANTIBODIES ON THE DEVELOPMENT OF FALSE LAYER SYNDROME[3]

[3]Adrea Mueller Slay, Rachel Jude, Ana P. de Silva, Rodrigo Gallardo, Brian Jordan *(to be submitted to Avian Diseases)*

ABSTRACT

Infectious Bronchitis virus (IBV) is an avian coronavirus that primarily causes respiratory disease but can also affect the reproductive tract of laying type chickens. If infection occurs in young pullets, False Layer Syndrome can develop. False Layer Syndrome is characterized by changes in oviduct development and the formation of large, fluid-filled cysts. Vaccination is used to control disease caused by IBV, but False Layer Syndrome is still seen in vaccinated hens. We hypothesize that the presence of maternal antibodies, when combined with vaccination, will offer the most protection against cystic oviduct formation as a result of DMV/1639 challenge at three days of age. To test this, four groups of 30 maternal antibody negative specific pathogen free pullets and four groups of 30 maternal antibody positive commercial layer pullets were each were placed into separate colony type houses at day of hatch. For each bird type, there was a negative control group, a group that was only challenged, a group that was only vaccinated with live attenuated Mass-type IBV vaccine oculonasally at day of hatch, and a group that was vaccinated with Mass-type IBV vaccine at hatch and challenged. Serum samples, choanal cleft swabs, and cloacal swabs were collected at day of hatch and 5-, 10-, and 15-days post challenge. Necropsy was performed and oviducts were evaluated grossly and collected for histological analysis at 30 weeks of age. Birds that had maternal antibodies against IBV at hatch had lower percentages of affected oviducts than SPF birds without maternal antibodies.

Vaccinated, maternal-antibody positive birds had the lowest percentages of cystic oviducts. The age of birds at infection, challenge load, vaccination status, and maternal antibody presence influence development of cystic oviduct related to False Layer Syndrome caused by early IBV challenge.

1. Introduction

Infectious Bronchitis Virus (IBV), a gammacoronavirus that affects chickens, is primarily a respiratory disease and can cause low mortality, decreased feed conversion, high condemnation, and decreased egg production and quality [Awad et al., 2014]. Some serotypes of IBV are known to affect the epithelial cells of the chicken reproductive tract leading to permanent decreases in egg production and quality. In addition, the formation of large, fluid-filled cysts as a result of early IBV exposure leads to False Layer Syndrome, where hens appear clinically normal but cannot lay eggs. IBV strains QX, Massachusetts, Australian T, and variant DMV/1639 have been associated with cystic oviduct formation and False Layer Syndrome, especially when birds are infected at a young age [de Wit, et al., 2011; McMartin and Macleod, 1972; Broadfoot et al., 1956; Hassan et al., 2021; Mueller Slay et al., 2022].

In commercial production, prevention of cystic oviduct formation has been attempted through the addition of IBV vaccination at the hatchery to offer some protection against early DMV/1639 challenge [da Silva et al., 2021]. Massachusetts-strain vaccines are considered the "industry-standard" for IBV vaccination [Li et al., 2019]. Although vaccination strategies are often serotype specific and can have limited cross-protection [Kulkarni and Resurreccion, 2010], instances of vaccination against one serotype vaccine protecting against an antigenically distinct serotype have been recorded. The Massachusetts-type vaccination is one that shows evidence of cross-protection. To date, there is no commercial vaccine available for variant DMV/1639. In previous work, vaccination at hatch did not offer increased protection against reproductive lesions associated with DMV/1639 challenge at a young age in specific pathogen free (SPF) pullets

(see previous chapter). One key difference between SPF birds and commercial birds is the presence of maternal antibodies against IBV in commercial birds.

Passive immunity through maternal antibodies is suspected to protect young chicks from abnormal oviduct development due to early IBV infection [Broadfoot et al, 1956]. Maternal antibodies are passed from hen to chick to protect the chick in the early stages of the development of its immune system. Crinion et al. noted that birds with maternal antibodies are less likely to exhibit the severe reproductive lesions seen in SPF birds without maternal antibodies [1971a]. These results were supported by work by de Wit et al in 2011, who found that a combination of high maternal antibody presence and heterologous vaccination at hatch offered protection against the kidneys when birds were challenged at 6 or 10 days of age with the D388 virus. The authors extrapolate this protection to extend to the oviduct [2011]. In addition, Winterfield et al. [1976] describe an increased shed-rate of challenge virus in birds that did not have passive maternal antibodies compared to the group of birds with maternal antibodies when both were vaccinated at one day-of-age.

In previous studies of cystic oviduct formation in SPF birds, vaccination did not appear to offer protection against the development of cystic oviduct after early IBV DMV/1639 challenge, despite record of instances of vaccine-induced protection in the field [(see previous chapter) and da Silva et al., 2021]. SPF birds used in previous experiments are maternal antibody negative for IBV. This study aims to investigate the effects of maternal antibody presence on cystic oviduct prevalence in vaccinated and unvaccinated chicks.

2. Materials and Methods

2.1 Birds

SPF chicks were vent-sexed at hatching and female chicks were randomly separated into four groups of 30 birds per group. Commercial chicks were also vent-sexed at hatching and female chicks were randomly separated into four groups of 30 birds per group. Groups of birds were reared separately in colony houses and given food and water ad libitum.

2.2 Vaccination

At hatch, groups 1, 2, 5, and 6 were vaccinated with a Mass-type vaccine via spray cabinet and manufacturers recommendations. The vaccination and challenge layout are arranged per group in Table 5.1.

2.3 Challenge

At 3 days of age, birds in groups 1, 3, 5, and 7 were challenged oculonasally with 100uL of $1x10^{3.5}$EID50 DMV/1639 virus. It was later detected that the challenge virus was contaminated with a Mass strain of vaccine origin. Because a Mass strain vaccination was used in this study, we do not believe this impacted the results of the study. The IBV strain used for challenge was a DMV type IBV was isolated from a clinical case of respiratory disease in chickens submitted to the Poultry Diagnostic and Research Center (PDRC) diagnostic laboratory in 2019 and has been previously described (Jordan, 2019). The fifth passage in embryonated chicken eggs was used for the challenge.

Five-, ten-, and fifteen-days post-challenge, choanal and cloacal swabs were collected from every bird and stored in PBS solution at -80°C. These swab samples were used in quantitative reverse-transcriptase real-time polymerase chain reaction (qRT-PCR) to confirm successful vaccination and challenge. Viral RNA was extracted from 50 µl of

swab fluid or tissue homogenate using the MagMax96 total RNA isolation kit (Ambion, Austin, TX) and the KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA) according to the manufacturer's protocols. The AgPath-ID™ One-step RT-PCR kit (Ambion Inc., Austin, TX) was used to perform qRT-PCR assays according to the manufacturer's instructions. The 25µl qRT-PCR reaction mixture included 12.5µl 2X RT-PCR buffer, 10µM of each primer, 4 µM of each probe, 1µl of 25X RT-PCR enzyme mix and 5 µl of viral RNA. Primers and probes corresponding to the Mass and DMV/1639/11 type IBV are previously published (Mo et al., 2020). The qRT-PCR reactions were performed on the Applied Biosystems® 7500 Fast Realtime PCR system (Life Technologies Ltd., Carlsbad, CA) under the following conditions: one cycle of 50°C for 30min and 95°C for 15min followed by 40 cycles of 94°C for 1sec and 60°C for 60sec (Mass-P), or 40 cycles of 94°C for 1sec and 53°C for 60sec (DMV/1639/11-P). Each assay included positive and negative controls for qRT-PCR reaction and RNA extraction.

2.4 Serology

Blood samples were collected at hatch from ten birds in each group and at five-, ten-, and fifteen-days post-challenge for fifteen birds in each group. Additional blood samples were collected at 20 weeks of age from fifteen birds in each group. Blood samples were stored in refrigeration at 4°C overnight, and then samples were centrifuged at 8,000 rpm for 10 minutes and serum supernatant was collected from each tube. ELISA assays were performed with the IDEXX IBV Antibody Test Kit (IDEXX, Colombia, MO) using manufacturer instructions and were measured on a BioTek Epoch plate reader (BioTek, Winooski,VT).

Table 5.1: Experimental Design

Group	**Breed**	Vaccination	**Challenge**
1	SPF	Mass	DMV
2	SPF	Mass	-
3	SPF	-	DMV
4	SPF	-	-
5	Commercial	Mass	DMV
6	Commercial	Mass	-
7	Commercial	-	DMV
8	Commercial	-	-

2.5 Necropsy

At 30 weeks of age, all hens were necropsied. Observations of affected oviducts were noted for each bird in every group. At the final necropsy, all cystic/affected oviducts per group and ten unaffected/grossly normal oviducts were chosen for histopathology submission. Cross-sections of magnum, isthmus, and shell gland were collected and stored in 5% neutral buffered formalin before histopathology submission.

2.6 Histopathology

Oviduct tissue was collected at necropsy for histopathological examination. Tissues were stored in 10% neutral buffered formalin at room temperature for a minimum of 24 hours. Oviduct tissue was cut into cross-sections. Tissue was embedded in paraffin wax and cut into 4um slide sections. The slides were stained with hematoxylin and eosin and evaluated with light microscopy.

3. Results

3.1 PCR

qRT-PCR was used to measure the viral load per group five-, ten-, fifteen-, and twenty-days post challenge, and is expressed as a CT value, where a lower CT value indicates higher viral load. Five days post challenge, the mean choanal CT value of DMV/1639 for the SPF challenged groups was 25.33 while the mean choanal CT value for the commercial challenged groups was 26.78 (Table 5.2). The mean cloacal CT value at 5DPC was 23.65 for the SPF challenged groups and 21.60 for the commercial challenged groups (Table 5.3). The average choanal viral load in the SPF challenged groups decreased over time, but there does not appear to be a pattern in viral load in either the choanal or cloacal swabs for the commercial groups, nor is there one in the cloacal swabs for the SPF groups.

Table 5.2: Choanal Cleft Swab PCR Results- DMV/1639

	Group 1	**Group 2**	**Group 3**	**Group 4**
5DPC	27.90±4.94	-	22.75±2.49	-
10DPC	30.34±2.11	-	30.09±3.59	-
15DPC	31.31±3.57	-	33.48±2.63	-
	Group 5	**Group 6**	**Group 7**	**Group 8**
5DPC	27.20±3.99	-	26.35±2.17	-
10DPC	32.33±2.91	-	29.71±3.10	-
15DPC	29.71±3.61	-	32.72±3.39	-

Table 5.3: Cloacal Cleft Swab PCR Results: DMV/1639

	Group 1	Group 2	Group 3	Group 4
5DPC	25.19±6.39	-	22.18±3.56	-
10DPC	21.98±3.26	-	21.81±2.47	-
15DPC	23.62±2.64	-	24.89±2.38	-
	Group 5	**Group 6**	**Group 7**	**Group 8**
5DPC	23.50±6.87	-	19.7±1.79	-
10DPC	22.23±4.38	-	20.15±1.66	-
15DPC	20.82±2.13	-	24.30±2.28	-

3.2 ELISA

Maternal antibodies against IBV at hatch in the SPF chicks were not present. IBV maternal antibodies at hatch in the commercial chicks were present with an average ELISA titer of 1061. Five days post challenge, none of the sampled SPF pullets had detectable antibodies to IBV. All groups of the commercial pullets had detectable titers of IBV antibodies, though not every sample was positive. On average at this timepoint, Group 5 (vaccinated at hatch and challenged at 3-days of age) had the highest average titer (1016.5) and percent positive samples (57.14%) (Table 5.4).

At ten days post challenge, the only SPF-origin group to have detectable antibodies was Group 3 (challenged at 3 days of age), though only 1 of the 15 chicks sampled was positive. Both the average titer and percent positive samples of detectable antibodies in the commercial pullets decreased at this timepoint. Group 6 (vaccinated at hatch but not challenged) did not have detectable antibodies in the birds sampled. Group 8 (not

vaccinated and not challenged) now had the highest percent positive samples (22.22%) but Group 5 continued to have the highest average titer (742.5).

Fifteen days post challenge, the only group to be negative for detectable antibody levels was Group 4 (unvaccinated and unchallenged SPF pullets). The sister group, Group 8, had a decreasing average maternal antibody titer and percentage positive compared to the previous sampling timepoints. Average titer and percent positive samples increased in the other three commercial pullet groups at this timepoint.

Serum samples taken at 21 weeks of age in the SPF pullets and the commercial pullets followed similar trends. The unvaccinated, unchallenged groups were negative for IBV antibodies. The vaccinated, unchallenged groups had the lowest average titers and percent positive samples for their respective groups. The unvaccinated, challenged groups had the highest average titers and percent positive for their respective groups. Throughout the study, there did not appear to be a pattern between ELISA titer and gross lesions at necropsy per bird.

Table 5.4: Average ELISA Titers and Percent Positive of Sampled Birds per Group

Group	5 DPC	10 DPC	15 DPC	21 WOA
1 SPF Vax+/DMV +	-	-	1013.2 (35.71%)	2320.29 (46.67%)
2 SPF Vax+	-	-	776 (7.69%)	721 (33.33%)
3 SPF DMV +	-	441 (7.69%)	723.83 (42.86%)	2538.60 (66.67%)
4 SPF Vax-/DMV -	-	-	-	-
5 Commercial Vax+/DMV +	1016.5 (57.14%)	742.5 (13.33%)	1502.67 (20.00%)	1417.33 (80.00%)
6 Commercial Vax +	593 (26.67%)	-	451 (6.67%)	511 (13.33%)
7 Commercial DMV +	748 (20.00%)	627 (15.38%)	518 (26.67%)	2979 (86.67%)
8 Commercial Vax-/DMV -	780.50 (25.00%)	588 (22.22%)	547 (6.25%)	-

3.3 Gross Findings

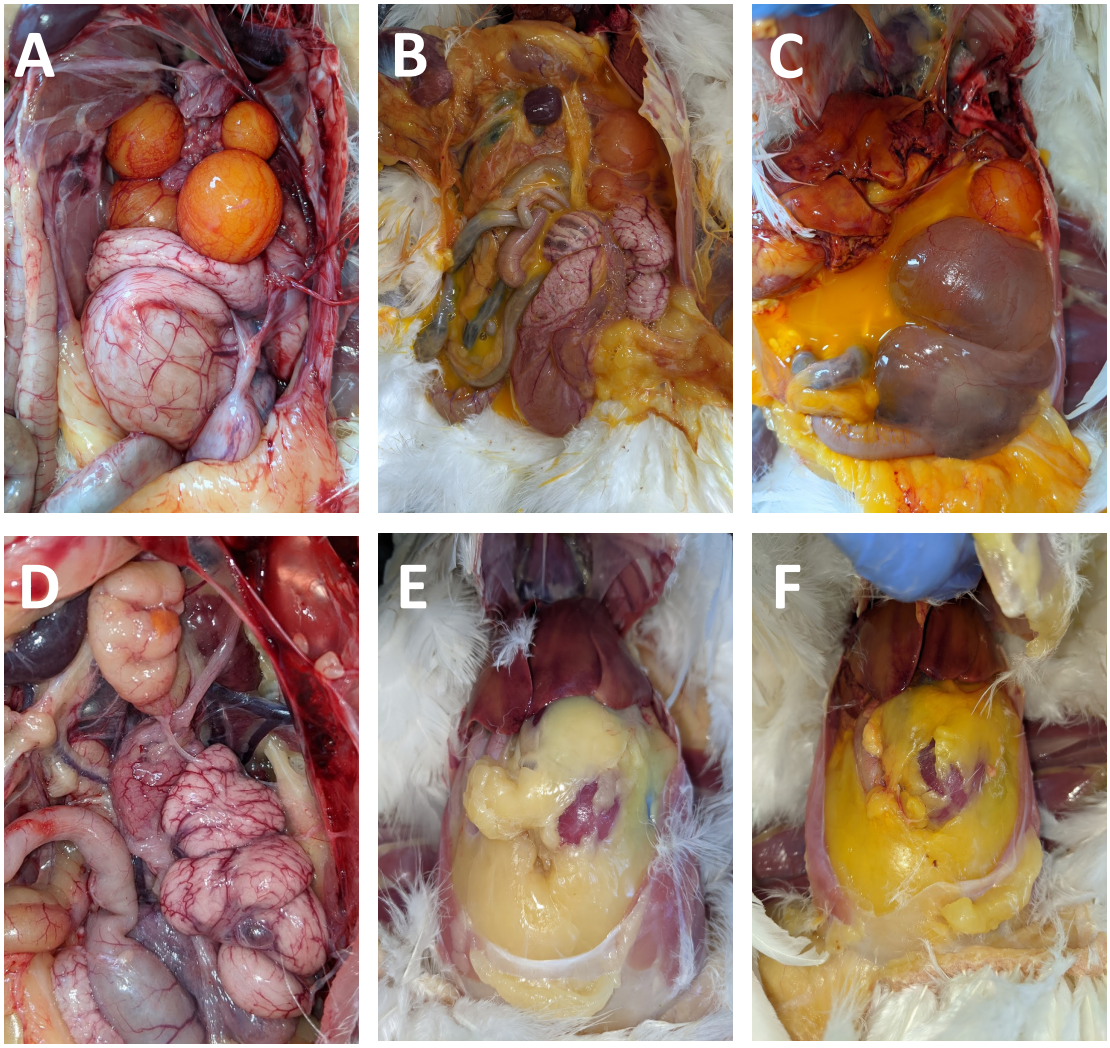

Figure 5.1: (A) Normal ovary and oviduct of a 30-week-old hen; (B) Egg yolk coelomitis and a cystic oviduct in a 30-week-old hen; (C) Cystic oviduct in a 30-week-old hen; (D) Abnormal ovary and oviduct in a vaccinated, unchallenged hen at 30 weeks; (E) Normal fat pad of a 30-week-old hen; (F) Bright yellow fat pad of a 30-week-old hen

Table 5.5: Gross lesions per Group

Group	Vaccination	Challenge	Breed	% Cystic Oviduct	% Abnormal Oviduct
1	+	+	SPF	9.50%	9.50%
2	+	-	SPF	0.00%	4.30%
3	-	+	SPF	19.20%	19.20%
4	-	-	SPF	0.00%	0.00%
5	+	+	Commercial	3.60%	3.60%
6	+	-	Commercial	0.00%	0.00%
7	-	+	Commercial	10.30%	13.80%
8	-	-	Commercial	0.00%	0.00%

Total cystic and abnormal oviduct prevalence from all necropsy groups is summarized in Table 5.5. The highest total percentage of cystic oviduct was seen in Group 3, the unvaccinated, challenged SPF group, with 19.20% cystic oviduct. The highest percentage of cystic oviducts from the commercial groups was also the unvaccinated group (Group 7) at 10.30%. Some oviducts observed were considered abnormal but not cystic (Figure 5.1:D). These abnormal oviducts were shorter and considerably less developed, but lacked cystic dilation. When considering these abnormal oviducts, the percentage in Group 7 increased to 13.80%.

The vaccinated, challenged groups had lower incidence of cystic and affected oviducts than the unvaccinated, challenged groups for both SPF and commercial birds (9.50% and 3.60% respectively). The commercial birds overall had lower percentages of cystic and affected oviducts than their counterparts in the SPF groups. No lesions were

found in the vaccinated, unchallenged commercial group, but 4.30% of the vaccinated, unchallenged SPF group had abnormal oviduct lesions. Groups 4 and 8 did not have any gross lesions present at necropsy. Additional lesions seen included free yolk in the coelomic cavity, yolk coelomitis, internal lay, and bright yellow fat pads in each bird with cystic oviduct (Figure 5.1:F), though one bird without cystic oviduct had a fat pad that was mildly darker than normal. In multiple cystic oviducts, rather than being clear, the fluid appeared milky and contained floating white material. Some birds had fluid filled cysts present on the mesentery but grossly normal reproductive organs. In addition, most of the SPF birds had retained and/or cystic right oviducts compared to few in the commercial groups.

4. Discussion

The goal of this study was to evaluate the potential role that maternal antibodies play in the protection against cystic oviduct formation in birds challenged with IBV at a young age. In this work, pullets were challenged with DMV/1639 combined with Mass strain at 3 days of age to induce severe reproductive lesions [Broadfoot et al., 1956; McMartin and Macleod, 1972; Crinion and Hofstad, 1972b; Bozorgmehri-Fard et al., 2013; Mueller Slay et al., 2022]. As expected, the SPF birds did not have maternal antibodies against IBV. The commercial birds did have maternal antibodies against IBV at hatch, though the average ELISA titer at hatch was lower than expected.

Cystic oviducts were detected in all challenged groups and vaccinated groups had a lower percentage of oviduct lesions compared to unvaccinated groups. This supports previous work and the commercial situation, where a lower incidence of cystic oviducts is seen when birds are vaccinated at hatch [da Silva et al., 2021]. Additionally, the challenge

titer used in this study was lower than in previous studies and may more closely reflect what birds experience in the field, though it is hard to be certain since the challenge strain used in this study was contaminated with a Mass type IBV during propagation. Commercial birds had lower percentages of cystic oviducts compared to SPF birds in similar treatments. These commercial birds were maternal antibody positive at hatch and these results demonstrate a potential pattern between maternal antibody status and prevalence of cystic oviduct in adulthood. This is supported by the work of Broadfoot et al 1956 who describe the protective effects of maternal antibodies against reproductive lesions caused by early IBV challenge.

Similar to previous studies, cystic dilatation was most often at the shell gland of the oviducts, though this lesion was present in some magnum and isthmus or continuously throughout the length of the oviduct. The floating white material seen in cyst fluid of some affected oviducts has been previously reported to be mucin fibers that resemble chalazae [Broadfoot et al., 1956]. Incomplete shell fragments were detected in some birds with cystic oviduct, indicating that a portion of the oviducts may be functioning to some degree. It's possible that the smaller cystic oviducts with some degree of differentiated tissue, as opposed to the large ballooning cystic oviducts with thin oviduct walls, retain some function of shell deposit and albumin protein creation. The bright yellow fat pads seen in each hen with cystic oviduct may be related to the hens age and ovary development by the time of final necropsy. Historically, birds with false layer syndrome have increased visceral fat pigmented the same color as the free yolk in the abdominal cavity [Broadfoot et al., 1956].

In this experiment, the SPF group that received a Mass vaccination at hatch developed gross lesions in the oviduct despite never being exposed to challenge virus. This is consistent with a similar group from the previous study, where vaccinated, unchallenged SPF birds developed oviduct lesions [see previous chapter]. Some low passage live vaccines have been known to produce cysts in chicks exposed in the first week of age [Crinion, 1972]. In both this study and the previous, the vaccine-related oviduct lesions occurred in maternal antibody negative birds. It is interesting to note that the commercial maternal antibody positive birds that received the same vaccination did not develop any oviduct lesions, further supporting the thought that maternal antibodies play a role in the protection of the immature oviduct when birds are vaccinated at hatch. De Wit et al found that the combination of high maternal antibody presence and heterologous vaccination at hatch offered protection against the kidneys when birds were challenged at 6 or 10 days of age with the D388 virus. The authors extrapolate this protection to extend to the oviduct as well [2011].

At 21 weeks of age, the titers against IBV increase in all vaccinated and/or challenged groups. In times of stress, such as onset of lay, birds may experience repeat infection of IBV from virus in the environment or shed from previously infected birds. This is supported by the negative serum samples in the unvaccinated, unchallenged commercial group at 21 weeks of age. There does not appear to be a pattern between sampling timepoint post-challenge and antibody titer levels. Serological assays for IBV such as ELISAs may not be specific or sensitive enough to accurately detect the strain being investigated, especially considering ELISA assays cannot differentiate between serotypes [Jackwood et al., 2012]. In general, birds that were not vaccinated at hatch had higher antibody titers post

challenge than birds that received Mass vaccine at hatch. Birds that had maternal antibodies at hatch had higher percentage positive samples at the 21-week sampling timepoint, indicating that the commercial birds had a stronger immune response and were likely better protected compared to the SPF birds upon repeat infection.

5. Conclusions

Birds that had maternal antibodies against IBV at hatch had lower percentages of affected oviducts than SPF birds without maternal antibodies. Vaccinated, maternal-antibody positive birds had the lowest percentages of cystic oviducts. Vaccination at hatch led to the development of reproductive lesions in some unchallenged SPF birds, but birds with maternal antibodies at hatch did not display similar reproductive effects after early vaccination. The age of birds at infection, challenge load, vaccination status, and maternal antibody presence influence development of cystic oviduct related to False Layer Syndrome caused by early IBV challenge.

Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Institutional Review Board Statement:

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the University of Georgia Animal Care and Use Committee (UGA IACUC) under the approved animal use protocol (AUP) A2019 02-016-Y3-A1.

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CHAPTER 6

CONCLUSION

Infectious Bronchitis virus (IBV) primarily causes respiratory disease but can affect the reproductive tract of laying type chickens. Pullets are especially susceptible to the formation of large, fluid-filled oviduct cysts due to IBV infection at a young age. These cystic oviducts lead to False Layer Syndrome, which causes significant economic impacts in a flock by preventing hens from ever laying eggs. A better understanding of the causes and pathogenesis of False Layer Syndrome will lead to more effective preventative strategies to reduce economic losses.

In 2011, IBV variant DMV/1639 emerged and began as a nephropathogenic strain in broilers. For the last decade, DMV/1639 was detected in parts of Canada and the U.S. in layer flocks where False Layer Syndrome has occurred, though it was not clear if IBV is the sole cause or if there were other influencing variables. The first chapter of this study investigates the role and timing of IBV infection on development of False Layer Syndrome using IBV strains DMV/1639 and Massachusetts (Mass/M41). The M41 strain was considered the positive control, as this strain is known to cause cystic oviduct in pullets [McMartin and Macleod,1972]). Grossly, both the M41- and DMV/1639-infected groups developed cystic oviducts. For both strains, a higher percentage of gross reproductive lesions occurred in groups challenged before two weeks of age than in the groups challenged at two weeks of age, indicating that age at infection impacts the prevalence of birds in a flock with False Layer Syndrome. This supports many of the previous

investigations into False Layer Syndrome and cystic oviduct development, where younger birds were affected in greater numbers and experienced more severe gross lesions than birds infected at older ages [de Wit, et al., 2011; McMartin and Macleod, 1972; Broadfoot et al., 1956; Hassan et al., 2021; Mueller Slay et al., 2022].

In some birds in the final necropsy, the oviducts were considered abnormal but did not have the large balloon-like cysts characteristic of False Layer Syndrome, supporting the theory that IBV causes a range of reproductive lesions that result in defective oviducts and production loss. Histological lesions seen in challenged groups included lymphoplasmacytic inflammatory infiltrates, exudate in the oviduct lumen, and lymphoid nodule formation. The highest percentage of severe histology lesion scores were seen in the 3-day challenge groups for both viruses, further indicating that age at infection impacts disease severity. IBV DMV/1639 and M41 were detected with the DMV-specific or Mass-specific riboprobe RNAscope technique in the oviduct epithelium, confirming IBV infection as the cause of oviduct damage observed. The data collected in the first experiment confirms that IBV DMV/1639 causes cystic oviduct and indicates that age at infection plays a role in the pathogenesis of False Layer Syndrome. Understanding the impact that virus strain and timing of infection have on pathogenesis is crucial to developing effective prevention strategies.

Vaccination is the primary method used to control disease caused by IBV, but False Layer Syndrome is still seen in vaccinated hens in the field. We hypothesized that timing of vaccination and infection with challenge virus influences the development of cystic oviducts and False Layer Syndrome regardless of vaccine serotype used. Since there is not a commercial vaccine available for DMV/1639, we used a either a Mass or Ga08 vaccine

for the at-hatch vaccinations. These vaccines are commonly used in layer pullets in the field to protect against IBV. There was no appreciable difference in gross lesion development between the vaccinated and unvaccinated groups challenged with DMV/1639. This seems to contradict the observation that adding a vaccination at hatch can decrease the incidence of false layer syndrome in a flock in the field [da Silva et al., 2021], but maternal antibody status and challenge load must also be considered. Similar gross lesions were identified in this study when compared to the previous [Mueller Slay, 2022].

Oviducts that were grossly abnormal and contained fluid but were not clearly cystic were termed “fluid-filled.” Fluid-filled lesions were detected in all groups. Vaccinated groups had a higher percentage of fluid-filled lesions compared to unvaccinated groups, which might suggest that the fluid-filled oviduct is a lesser lesion or that it is cyst development delayed by vaccination. Due to the terminal nature of oviduct analysis, it is not known whether these oviducts will continue development into functional oviducts or into cyst development. The histopathological scoring system for this experiment reflected the previous experiment, though a “dilated oviduct” category was added to yield a maximum potential score of 4 but there did not appear to be a relationship between histological lesion scores and vaccination status. In this study, vaccination status had little to no effect on cystic oviduct formation and histological lesions associated with early DMV/1639 challenge in maternal antibody negative SPF pullets.

Commercial layer pullets have maternally derived IgY antibodies against IBV for their first two weeks of life while their immune system is still developing [Broadfoot et al., 1956]. SPF pullets do not contain these maternal antibodies. It’s possible that the absence of maternal antibodies makes the birds in our experiment more susceptible to IBV-related

oviduct damage despite being vaccinated. Additionally, the challenge load given to birds in this experiment is known to be strong. Such a strong challenge load might not accurately reflect the amount of challenge virus birds are exposed to in the field and as a result the challenge virus may overcome any potential effects of vaccination.

In this experiment, groups that received a Mass vaccination at hatch and a subsequent Mass/Conn vaccination at 14-days of age developed gross and histological lesions in the oviduct despite never being exposed to challenge virus. The consensus in literature on the ability of vaccines to cause reproductive disease in hens is debated. Broadfoot et al state that a commercial attenuated IBV vaccine did not cause abnormal oviducts [1956], but some low passage live vaccines have produced cysts in chicks exposed in the first week of age [Crinion, 1972]. An early live vaccination in chicks without maternal antibodies may explain the increased incidence of affected oviducts seen in this experiment. The data collected in this experiment indicates that vaccination alone does not offer sufficient protection against False Layer Syndrome. Additional factors such as viral challenge load and maternal antibody status should be considered when trying to protect a flock.

In the third component of this study, we hypothesized that the presence of maternal antibodies, when combined with vaccination, would offer the most protection against cystic oviduct formation as a result of DMV/1639 challenge at three days of age when compared to maternal antibody negative SPF birds. The SPF birds did not have maternal antibodies on day of hatch against IBV. The commercial birds did have maternal antibodies against IBV at hatch, though the average ELISA titer at hatch was lower than expected. By the end of the study at 30 weeks, birds that had maternal antibodies against IBV at hatch had lower

percentages of affected oviducts than SPF birds without maternal antibodies. Vaccinated, maternal-antibody positive birds had the lowest percentages of cystic oviducts. All hens with cystic oviduct had bright yellow fat pads that may be related to the hens age and ovary development by the time of final necropsy. Historically, birds with false layer syndrome have increased visceral fat and pigmented the same color of the free yolk in the abdominal cavity [Broadfoot et al., 1956].

Overall, the commercial birds had lower percentages of cystic oviducts compared to SPF groups in similar treatments. These commercial birds were maternal antibody positive at hatch and these results demonstrate a potential pattern between maternal antibody status and prevalence of cystic oviduct in adulthood. This is supported by the work of Broadfoot et al. [1956] who describe the potential protective effects of maternal antibodies against reproductive lesions caused by early IBV challenge. Cystic oviducts were detected in all challenged groups. Vaccinated groups had a lower percentage of oviduct lesions compared to unvaccinated groups. The titer of the DMV/1639 challenge virus was lower in this study than in previous studies, further supporting our conclusions that challenge load influences percentage of cystic oviducts formed. In the field, a lower incidence of cystic oviducts is seen when birds are vaccinated at hatch [da Silva et al., 2021]. The challenge titer used in this study may be closer to what birds experience in the field.

In this experiment, the SPF group that received a Mass vaccination at hatch developed gross lesions in the oviduct despite never being exposed to challenge virus. In both this study and the previous, the vaccine-related oviduct lesions occurred in maternal antibody negative birds. It is interesting to note that the commercial maternal antibody

positive birds that received the same vaccination did not develop any oviduct lesions, indicating that maternal antibodies may play a role in the protection of the immature oviduct when birds are vaccinated at hatch.

At 21 weeks of age, the titers against IBV increased in all vaccinated and/or challenged groups. In times of stress, such as onset of lay, birds may experience repeat infection of IBV from virus in the environment or in previously infected birds. This is supported by the negative serum samples in the unvaccinated, unchallenged commercial group at 21 weeks of age. There does not appear to be a pattern between sampling timepoint post-challenge and antibody titer levels. Serological assays for IBV such as ELISAs may not be specific or sensitive enough to accurately detect the strain being investigated, especially considering ELISA assays cannot differentiate between serotypes [Jackwood et al., 2011]).

The age of birds at infection, challenge load, vaccination status, and maternal antibody presence influence development of cystic oviduct related to False Layer Syndrome caused by early IBV challenge. The data collected in the third portion of this study indicates that maternal antibodies do play an immunological role in the prevention of cystic oviduct formation as a result of early IBV infection. Vaccination of layer breeders may play a critical role in the prevention of False Layer Syndrome in progeny layer pullet flocks and, in the face of an outbreak of False Layer Syndrome, attention should be made to the vaccination schedule of the breeders and adjustments made if necessary to increase maternal antibody levels in the progeny.

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