

INFECTIOUS BRONCHITIS VIRUS S1 SPIKE GENE POLYMORPHISMS IN THE ARKANSAS DPI VACCINE

INFLUENCE MINIMUM INFECTIOUS DOSE AND S1 SPIKE PROTEIN BINDING TO HOST CELLS

by

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ABSTRACT

The Arkansas-type (Ark) IBV has continued to be the most commonly isolated serotype in commercial poultry for the past two decades in the United States. Experimental and field trials that used the live-attenuated Ark vaccine, Ark Delmarva Poultry Industry (ArkDPI), have shown that it exhibits poor replication in chickens and confers poor protection against challenge. Re-isolation of the ArkDPI virus from vaccinated birds has shown that certain polymorphisms arise and are selected for upon infection and replication in birds. Most of the polymorphisms observed are located in the S1 spike protein gene, suggesting that S1 spike is an important factor that determines virus infectivity and replication. Previous studies have shown that ArkDPI administered by the eye drop route achieves an adequate level of infection and replication in birds. When ArkDPI is delivered by spray however, infection and replication in birds is poor. We increased the doses of ArkDPI delivered by spray and observed that a 100x dose achieved the same level of infection and replication as ArkDPI administered by eye drop. Sequencing of the S1 spike gene from re-isolated from vaccinated birds showed the certain polymorphisms emerge as expected. Two polymorphisms most commonly observed in re-isolated ArkDPI vaccine viruses are a tyrosine to histidine change in position 43 (Y43H) and a deletion of an arginine residue at position 344 (Δ 344). The S1 spike protein is responsible for attachment to host cells and is a primary determinant of tropism and thus, changes in the S1 spike protein sequence could affect the virus' ability to bind to host tissues. To examine

this, we cloned, expressed, and purified ArkDPI S1 spike proteins carrying Y43H and/or Δ 344 changes and used recombinant S1 spike proteins in a protein histochemistry assay on trachea from mature birds and chorioallantoic membrane (CAM) tissues from embryonated eggs. We observed that the Y43H change enhanced binding to trachea, whereas the original ArkDPI vaccine spike (H43) had the highest binding to CAM. Western blot of recombinant S1 spike proteins showed that ArkDPI serotype-specific antisera was unable to recognize linear epitopes on S1 spike proteins with Δ 344, suggesting that Δ 344 change alters the antigenicity of the ArkDPI S1 spike. Taken together, these results demonstrate the emergence of polymorphisms in re-isolated ArkDPI vaccine viruses from chickens can be explained by the selective advantage offered by enhanced binding to host tissues, as in the case for Y43H; and by alterations in antigenicity, as in the case of Δ 344. Furthermore, the viruses with such polymorphisms appear to be in low numbers in the ArkDPI vaccine preparation and for this reason, rates of infection and replication are low upon spray vaccination with ArkDPI.

INDEX WORDS: Infectious bronchitis virus, S1 spike protein, Arkansas DPI

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DEDICATION

To my family and friends, with all my love

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CHAPTER 1

INTRODUCTION

Infectious bronchitis virus (IBV) is a highly transmissible respiratory disease in poultry. Millions of dollars are lost annually due to IBV outbreaks. Typical clinical signs of IBV are rales, wheezing, and open-mouth breathing (6, 10, 29). IBV typically causes loss of cilia activity, mucus accumulation, and necrosis in the trachea. Secondary infection with bacteria often follow an IBV infection, thereby leading to airsacculitis, at which point, a carcass is more likely to be rejected during processing. In layers, IBV causes poor egg production as well as abnormal growth in chicken embryos and some strains of the virus can cause a nephritis (6, 29).

IBV is a positive-sense single stranded RNA virus of the family *Coronaviridae*. Like many RNA viruses, IBV exhibits high genetic diversity. Many serotypes of IBV exist and cross-protection between serotypes is poor (6, 10, 29). Thus, commercial poultry operations typically use vaccines against multiple IBV serotypes. Live-attenuated vaccines have been in use in the market for many decades. Because IBV exhibits high diversity, many serotypes exist around the globe (12). The vaccine for one particular serotype, namely the Arkansas (Ark) serotype, has been shown to provide poor protection in vaccinated birds (14, 27). Indeed, sample submissions from the field to the diagnostic lab at the Poultry Diagnostic and Research Center (PDRC) are mostly of the Ark type (13), demonstrating that Ark strains frequently circulate in commercial flocks. Trials using commercially available live-attenuated IBV vaccines have shown that replication of the vaccine virus within birds is correlated with good protection against pathogenic IBV. As of date, ArkDPI (Arkansas Delmarva Poultry Industry) (11) is the only commercially available Ark-type vaccine. However, ArkDPI vaccine has been shown to replicate poorly (27) and to persist within a flock (14, 22). Moreover, it has been demonstrated that ArkDPI vaccine circulates in a flock and

undergoes multiple replication cycles (17), providing an opportunity for the vaccine to regain virulence. Failure of the ArkDPI vaccine to protect birds has been attributed to poor vaccine coverage and to the vaccine delivery (14, 27). When administered by the eye drop route, ArkDPI vaccine exhibits good replication in birds and protective immune responses are induced (27, 30). However, when using the hatchery spray cabinet method, the number of birds positive for vaccine virus is low, at about 30%, and little protection against challenge is observed (27).

One hypothesis to explain the poor performance of ArkDPI delivered by spray is that the hatchery spray cabinet causes mechanical damage to the virus particles due to high pressures and shearing forces at the supply lines and spray nozzle. It has been shown that this is not the case, however. While the virus titers of the vaccine drop as vaccine virus passes through the spray cabinet, there were no differences observed between Ark vaccine and Ma5, a Massachusetts-type vaccine that exhibits good replication in birds following spray application (15, 28). These data suggest that ArkDPI vaccine strains have some intrinsic defect in infection and replication in the host. It is thus conceivable that increasing the dose of vaccine virus could aid in enhancing vaccine coverage and vaccine virus replication in birds. Indeed, increasing the dosage of an Ark vaccine two-fold using a hatchery spray cabinet enhanced virus replication and vaccine efficacy, but not to the same degree as a single dose via the eye drop route (27).

Several groups have examined the genotypes present in ArkDPI as a vaccine preparation and as re-isolated vaccine virus from birds (1, 19, 23, 25, 27, 31). Vaccination with ArkDPI showed selection for certain polymorphisms in the IBV genome when the vaccine virus is re-isolated from the flock (18, 22, 31). Not surprisingly, most of the polymorphisms are found in the S1 spike protein gene (1, 22, 25) since, like other serotypes, the highest sequence diversity across the genome is observed in the spike glycoprotein gene (9, 24, 32). It is thus proposed that ArkDPI vaccine preparations are not genetically homogeneous and are composed of several subpopulations occurring at different frequencies (22, 31).

The spike glycoprotein of IBV is composed of two subunits: S1 at the amino terminus and S2 at the carboxy terminus (4, 5). The S1 spike protein is involved in receptor binding to host tissues while the S2 region anchors the spike protein and functions in membrane fusion (3, 33). Additionally, the spike protein is a major target of immune responses against IBV. Serotype-specific neutralizing antibodies often bind to S1 regions of the spike glycoprotein, though immunodominant regions have also been found in the S2 region (2, 8, 9, 20, 21). Small amino acid changes in the S1 region of the spike glycoprotein correlate with serotype divergence (2, 7). Since the S1 spike protein is responsible for binding to host tissues, these polymorphisms in ArkDPI S1 spike gene can potentially influence the ability of certain virus subpopulations to bind to host tissues and thus affect the replication of the virus in the host.

The overall goal of this research is to contribute to our understanding of how polymorphisms in the ArkDPI S1 spike protein influence vaccination success. Specifically, this research aims (1) to determine the minimum infectious dose of the ArkDPI vaccines given by spray, necessary to achieve adequate vaccine coverage, and (2) to determine the genotypes of ArkDPI vaccine viruses re-isolated from chickens given different vaccine doses. This research also aims (3) to examine the impact of polymorphisms on the ArkDPI S1 spike protein gene on binding to host tissues and antigenicity.

SPECIFIC AIMS

Infectious bronchitis virus (IBV) is one of the most economically important pathogens in poultry production. Live-attenuated IBV vaccines are typically given to the commercial flocks to prevent outbreaks. The use of spray is one of most common methods to administer live-attenuated vaccine since it is convenient and cost-effective. However, it has been demonstrated that ArkDPI delivery by spray does not provide adequate vaccine coverage and protection against homologous challenge with virulent IBV (27). Re-isolation of ArkDPI vaccine virus has also shown that certain polymorphisms arise, specifically in the S1 spike protein gene (18, 25, 31).

The overall goal of this research is to determine the influence of the polymorphisms in S1 spike gene to vaccination outcomes. Our specific goals are: (1) to determine the minimum infectious dose of the ArkDPI vaccines given by spray, necessary to achieve adequate vaccine coverage, (2) to determine the genotypes of ArkDPI vaccine viruses re-isolated from chickens given different vaccine doses, and (3) to examine the impact of polymorphisms on the ArkDPI S1 spike protein gene on binding to various host tissues and antigenicity.

Specific Aim 1. Determine the minimum infectious dose of ArkDPI vaccines given by spray, necessary to achieve adequate vaccine coverage.

Since Ark IBV vaccine given by the eye drop route adequately replicates to protect birds against challenge, our hypothesis is that the level of replication of an Ark IBV vaccine given by spray is not adequate because not enough vaccine viruses are getting to the chick. We tested this hypothesis by increasing the dose of the ArkDPI given by spray to determine if we can achieve the same level of replication observed when the vaccine is given eye drop. To this end, our approach is to first measure the level of replication of ArkDPI vaccine administered via the eye drop route, a vaccination method that has been shown to be protective against challenge. Choanal cleft swabs were taken from birds 7 days and 10 days post-vaccination. RNA

was extracted from the choanal swab samples to isolate IBV nucleic acids. Quantitative reverse-transcription PCR (qRT-PCR) was then used to measure virus load in each bird. Once a benchmark level of virus load is calculated, day-old chicks were vaccinated via the spray cabinet using higher dosages, starting at a 10-fold increase from the standard ArkDPI dose. Similar to the first experiment, qRT-PCR was used to measure virus load in birds at 7 days and 10 days post-vaccination. Dosages were adjusted accordingly to achieve a similar rate of coverage and virus replication when birds are vaccinated via the eye drop route.

Specific Aim 2. Determine the genotypes of ArkDPI vaccine viruses re-isolated from chickens given different vaccine doses.

Certain polymorphisms, specifically in the S1 spike protein gene, are observed upon re-isolation of ArkDPI from vaccinated birds (18, 25, 31). It is not clear whether the route of administration or the dose of vaccine has some influence on what type of polymorphisms arise or the frequency of certain polymorphisms. For this purpose, we amplified and sequenced full-length S1 spike protein genes from a subset of samples isolated from birds vaccinated by the eye drop route or by spray at various doses. The frequency of certain polymorphisms was then tallied and compared between vaccination route and dose. Our hypothesis is that similar polymorphisms at comparable frequencies will be found across various routes of administration and doses.

Specific Aim 3. Examine the impact of polymorphisms on the ArkDPI S1 spike protein gene on binding to various host tissues and antigenicity.

Our hypothesis is that sequence polymorphisms at amino acid positions 43 and 344 of the spike glycoprotein, which are observed in subpopulations of most ArkDPI IBV isolates, affects binding to mature tracheal tissues and to embryonic chorioallantoic membranes. Particularly, the presence of tyrosine instead of a histidine at amino acid position 43 (Y43H) has been shown to be critical for binding to tracheal

tissues (26). On the other hand, such polymorphisms would result in reduced binding to the embryonic chorioallantoic membrane since the major subpopulations of the vaccine virus grown in embryonated eggs do not contain those sequences.

Another polymorphism in the ArkDPI S1 spike protein gene is a deletion of an arginine residue at position 344 (Δ 344). The region where position 344 is found has previously been described as a major antigenic target of virus neutralizing monoclonal antibodies (16). Our hypothesis is that Δ 344 polymorphisms alters antigenicity of the S1 spike such that it is able to prevent antibody binding to the spike protein and thereby, offers a selective advantage for virus populations with Δ 344.

To examine the binding properties of the spike glycoprotein, recombinant ArkDPI S1 spike proteins, bearing Y43H and/or Δ 344 changes, were fused to a GCN4 trimerization domain at the carboxy-terminal end and tagged with a Strep-Tactin peptide. A signal peptide was also fused immediately upstream of the S1 fusion protein gene to allow its secretion into the media. Recombinant S1 spike proteins were then expressed by transfection of a mammalian cell culture and subsequently, purified by affinity column chromatography. Protein histochemistry using the recombinant S1 spike proteins was then performed on paraffin-embedded sections of mature chicken tracheal tissue as well as embryonic chorioallantoic membrane. To test antigenicity, recombinant S1 spike proteins were subjected to Western blot using ArkDPI serotype-specific serum. Both native and denaturing conditions were used to examine differences in conformation and linear epitopes.

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CHAPTER 2. LITERATURE REVIEW

2.1. INFECTIOUS BRONCHITIS VIRUS: INTRODUCTION

History and General information

Infectious bronchitis virus (IBV) was first described in 1931 as an acute respiratory disease in young birds (184). The researchers described hallmark clinical signs of IBV infection including mucus accumulation and rales, which was referred in the paper as “peculiar coarse chirp”. Experimental infection of birds was also performed using filterable material from respiratory exudates, indicating that the etiological agent was a virus (although the existence of viruses was unknown then). During those early days, IBV was mistaken for another respiratory virus, infectious laryngotracheitis virus (ILT) (22, 73). It was later demonstrated that IBV and ILT were distinct pathogens through cross-protection and cross-neutralization studies (9).

IBV is a positive-sense single stranded RNA virus of the family Coronaviridae, and genus *Gammacoronavirus* (108). Its genome is fairly large for RNA viruses at 28 kilobases. IBV Virus particles are typical of coronaviruses and are enveloped and spherical with club-like projections extending out of its envelope. As is typical of RNA viruses, IBV replication is error-prone and thus, high genetic diversity is observed among viral strains. IBV can also undergo recombination, leading to radical changes in its genome and subsequently, to novel strains.

The existence of serotypes was first discovered in 1957 through vaccine and challenge studies in birds, and virus neutralization tests in embryonated eggs (120). Connecticut and Massachusetts were the first serotypes identified. Today, many IBV serotypes have been described around the globe (111). Indeed, there are about a dozen serotypes reported in the field in the United States alone (114). Diversity in avian coronaviruses, including IBV, is complex and it has been proposed that a nomenclature

should be put into place (33). The nomenclature proposed is similar to that for influenza strains and is as follows: host species/location/isolate number/year.

Clinical signs and Pathogenesis

The infectious bronchitis virus (IBV) is an economically important pathogen of poultry. It causes a respiratory illness in birds with typical clinical signs such as rales, wheezing, and open mouth breathing (35, 53, 180). The disease is relatively mild in itself, but it leaves infected birds more susceptible to secondary bacterial infections (101, 117) and to harsh abiotic factors such as high ammonia. Airsacculitis is a common consequence of IBV infection and is a cause of condemnations in the processing plant. IBV infection in layers causes a drop in egg production and poor egg quality (35, 180). Effects on roosters have also been described. IBV has been associated with calcium stones in the epididymis of vaccinated roosters and decreased fertility (15, 110).

IBV has tropism for the ciliated cells and goblet cells of the trachea (1). Infection with IBV causes *ciliostasis* or a loss in ciliary beating. Ciliostasis leads to the cessation of the mucociliary escalator, mucus accumulation, and necrosis in the trachea (35, 180). Other than tracheal tissues, IBV can also replicate in other respiratory tissues such as the nares, trachea, lungs, and airsac. Some strains of IBV can also replicate in kidneys, causing nephritis; in reproductive tissues such as the oviduct; and in gastrointestinal tissues such as the esophagus, proventriculus, duodenum, jejunum, cecal tonsils, rectum, and cloaca (34, 35, 175). Virus infection and replication in the gastrointestinal tract is not associated with clinical disease or apparent pathology. However, the serotype QX, which was first described in China, is known to cause proventriculitis and kidney lesions including ureate deposition and inflammation (80, 226). Infection of chicken embryonic kidney cells (CEK) and tracheal organ cultures (TOC) with IBV showed elevated apoptosis and cell death (48).

The most common means of propagating IBV is in embryonated eggs by inoculation in to the allantoic fluid, although some strains have been adapted to *in vitro* or *ex vivo* culture systems such as CEK and TOCs (74, 180). A non-pathogenic strain Beaudette has also been developed from a Massachusetts-type strain after a least 150 passages in embryonated eggs (10, 143). This particular strain can grow in mammalian cells such as BHK and Vero cells unlike other IBV strains (165).

Epidemiology and control

Transmission of IBV occurs through aerosols and direct contact with respiratory secretions. For this reason, IBV is highly contagious and can easily spread in a flock (180). The distribution of IBV is throughout the globe, although particular serotypes circulate in a given geographic location (111).

Diagnosis. Since IBV is a respiratory pathogen, the virus can be detected in tracheal and lung tissues as well as tracheal or choanal swabs (93, 180). IBV can also be detected in kidneys if the a nephropathogenic strain is suspected, as well as in cecal tonsils and feces. There are many diagnostic methods to detect IBV. Virus isolation and reverse transcriptase polymerase chain reaction (RT-PCR) are preferred methods according to the World Organisation for Animal Health (93). Serology by hemagglutination inhibition test (HI), virus neutralization test (VN), and enzyme linked immunosorbent assay (ELISA) can be additionally used to characterize the IBV isolate at hand. Other tests that can be performed are histopathology of infected tissues such as trachea and immunohistochemistry to detect viral antigen production in infected tissues (93, 180).

Control measures. Vaccination and biosecurity are the most common control measures against IBV. Before the development of live-attenuated vaccines, poultry growers induced immunity in their flocks by inoculating a group of birds with IBV after which, they released the inoculated birds to in to the house to infect their flockmates (73, 95, 171).

Vaccination. The development of modern vaccines, both live-attenuated and inactivated forms, led to widespread use of vaccines to control the disease. Typically, birds are vaccinated with live-attenuated IBV at the day of hatch. Inactivated vaccines are also used to boost immunity especially for long-lived birds such as layers and breeders. It has been estimated that vaccination with live-attenuated H120 reduces transmission of homologous IBV types by 29-fold (62). However, many IBV serotypes exist and cross-protection between them is poor (57, 82, 98, 100, 169). It is thus important to carefully design vaccination programs that are appropriate for the flock type and geographic location of operations. Unfortunately, only Massachusetts-type IBV vaccines are permitted for use in many countries (93).

Live-attenuated vaccines are typically developed by continuous passage of IBV in embryonated eggs (93, 180). Attenuation can also be expedited by heat-shock treatment. Here the virus is incubated at 56°C and inoculated into embryonated eggs. This strategy has been successfully applied to the development of a GA08 IBV vaccine (116).

Vaccination with an inactivated vaccine alone does not provide good immunity and requires priming with a live-attenuated vaccine (93). Vaccine failure with live-attenuated vaccination can also occur if the vaccine is not properly applied or the dose given is inappropriate (93, 181).

Serotype, Genotype, and Protectotype. Careful design of a vaccination program is necessary to prevent outbreaks from occurring. In the best scenario, one would test all available vaccines to all known strains circulating in the area where the flock is reared. This is however not practical or economically feasible. There is thus a need to characterize a property of the virus, for example the sequence of the spike gene (38), that correlates well with protection .

As previously mentioned, the existence of serotypes in IBV was first discovered in 1957 (120) and since then many serotypes have been identified (35, 111). IBV exhibits high antigenic variation as a reflection of its ability to undergo rapid genetic changes. IBV strains of the same serotype often exhibit good protection. Likewise, cross-protection between different serotypes is poor (57, 82, 98, 100, 169).

Serotyping provides good information about the IBV strain in question but is labor and resource intensive.

The development of nucleic acid-based diagnostic techniques led to more rapid determination of the genotype of the IBV strain at hand. Examples of nucleic acid-based methods (genotyping) are restriction fragment length polymorphism analysis (131) and real time RT-PCR (28, 179). Sequencing of the spike IBV gene revealed that hypervariable regions exist, specifically in the S1 spike gene (126, 156). The hypervariable regions in S1 spike gene were later demonstrated to correlate with epitopes of neutralizing antibodies, suggesting that genetic sequencing can be used to identify IBV (38). Experimental challenge studies further showed that the S1 spike sequence comparison is a better predictor of immunity in chickens than serotyping by virus neutralization (132). Indeed, protection against IBV requires both antibody responses as well as cell-mediated responses (50, 185, 186).

It has been shown that small changes in the S1 spike gene can have profound effects on cross-neutralization and cross-protection (34, 97, 191). For example, GA98 and DE072 are two strains that share >90% sequence identity in the S1 spike gene at positions 39-178, but virus neutralization tests reveal that the strains do not cross-react (133). Alternatively, several vaccine trials have also shown that a degree of cross-protection can be observed from strains that are serologically distinct (139, 175). For instance, it has been shown that the strains D274 and 793/B exhibited 93% protection against ciliostasis in tracheal organ cultures (TOCs), where cross-neutralization was only 16% (65). Similarly, New Zealand T and Australian A strains, which are serologically distinct, showed comparable protection against ciliostasis in the trachea as homologous challenge (139). In this same study, Lohr *et al* proposed that it is more practical for the poultry industry to categorize IBV strains into “protectotypes”, which is composed of IBV strains that exhibit adequate cross-protection in chickens.

Thus far, it appears that serotype, genotype, and protectotype are related classifications for IBV strains. However, serotype, genotype, and protectotype do not correlate with each other perfectly and

conflicts may be observed for certain strains. It would thus be important to characterize newly discovered IBV strains with more than one criterion, if possible.

2.2. INFECTIOUS BRONCHITIS VIRUS: GENOME AND LIFE CYCLE

Classification

The infectious bronchitis virus (IBV) belongs to the order Nidovirales, family Coronaviridae, subfamily Coronavirinae, and genus Gammacoronavirus (108). The Coronaviridae family has the largest RNA genomes known, with genome sizes ranging from 26-32 kilobases (kb). Other genera in the Coronaviridae are Alphacoronavirus and Betacoronavirus, both of which infect primarily mammals. Members of the genus Gammacoronavirus and Deltacoronavirus, on the other hand, infect primarily avian species. Interestingly, a coronavirus called SW1, belonging to the genus Gammacoronavirus was isolated from the diseased liver of a 13-year-old captive beluga whale (151). Other species in the genus Gammacoronavirus infect chickens, turkey (90), quail (49), pheasants (42), and guineafowl (135).

Genome and gene products

IBV virions are enveloped and pleomorphic with a diameter of about 150 nm (11, 35, 66). Its genome is a single positive-sense, single-stranded RNA genome that is about 28 kb in size (141). Genomic IBV RNA is capped and polyadenylated, and can thus function as both viral genomic RNA and messenger RNA. Capping and polyadenylation allows for the translation of genes necessary for virus replication and allows genomic IBV RNA to be infectious upon entering host cells (140). The organization of the IBV genome is typical of other coronaviruses, with about the first two-thirds (20 kb) coding for the genes involved in replication of the genome and last one-third (8 kb) coding for structural proteins. The genome organization of IBV is shown on Figure 1.

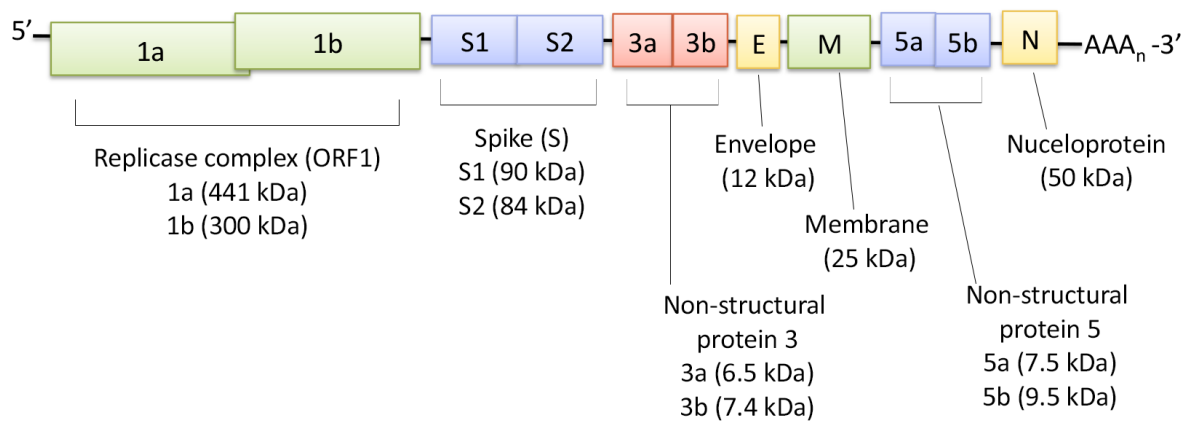


Figure 1. Genome organization of infectious bronchitis virus. Proteins involved in replication of the viral genome and transcription are encoded by ORF1a (1a) and ORF1b (1b) genes. The structural proteins are encoded by the spike protein (S), envelope protein (E), membrane protein (M), and nucleoprotein (N). Note that the length of boxes representing each gene is not to scale. The ORF1a and ORF1b are approximately 20 kb in length and the downstream genes are about 8 kb altogether.

Replicase proteins. The replicase proteins in coronaviruses are encoded by the ORF1 gene that is about 20 kb in size and is found at the 5' end of the genome (71). The ORF1 gene codes for two polypeptides called ORF1a and ORF1b. A frameshift within ORF1 results in the translation of ORF1a and ORF1b (24). The sequence UUUAAAC is found between the ORF1a and ORF1b gene and has been shown to facilitate frameshifting by ribosomal slippage (25). For IBV, ORF1a codes for a polyprotein called pp1a that has a size of 440 kDa; and ORF1a and ORF1b together code for a larger polyprotein called pp1ab that is 740 kDa in size (71). Proteolytic processing of the two polyproteins results in a total 15 non-structural proteins (nsp) numbered 2 (IBV does not have nsp1) through 16 (35).

Among the proteins encoded by ORF1 are proteases, helicase, and RNA-dependent RNA polymerases that function in the replication of the genome and transcription of viral genes. Helicase

activity was identified in the non-structural protein 13 (nsp13) in the severe acute respiratory syndrome coronavirus (SARS-CoV) (109). A mutation in the helicase nsp13 has been shown to prevent the transcription of subgenomic RNAs (75). The RNA-dependent RNA polymerase (RdRp) is encoded on nsp7 to nsp10 genes (70, 146, 196, 227). RdRp facilitates the synthesis of the viral genome copies and transcription. Other proteins in ORF1 are an endonuclease encoded by nsp15 gene (12, 13); and membrane-anchoring proteins encoded by nsp4 (164) and nsp6 (163) genes.

A particular protein that is unique among coronaviruses is a 3' → 5' exoribonuclease (ExoN), which has been identified as one of the pp1ab cleavage products called nsp14. The ExoN activity in nsp14 has been shown to have a proofreading function, which is thought to be essential for maintaining the coronaviruses' large genome. In the murine hepatitis virus (MHV), mutations in the active site from conserved amino acid residues to alanine residues in nsp14 was shown to have a significant impact in the fidelity of virus genome replication (68). Additionally, the nsp10 protein has been demonstrated to interact with nsp14 and promote its proofreading function (193).

There are two proteases involved in processing of pp1a and pp1ab namely, picornavirus 3C-like protease (3CL^{pro}, also called M^{pro} or viral main protease) and papain-like protease (PL^{pro}) (230). The 3CL^{pro} protease is encoded by nsp5 gene in ORF1 and is located upstream of the junction between ORF1a and ORF1b. The 3CL^{pro} protease cleaves itself from the polyprotein and subsequently, cleaves proteins downstream of 3CL^{pro} (76, 138). On the other hand, the PL^{pro} is encoded by the nsp3 gene in ORF1 and cleaves upstream proteins (71, 230). Interaction of PL^{pro} to the nucleoprotein (N) has been shown to be important in localizing N protein to sites of genomic RNA replication (104).

ORF1 is a major determinant of pathogenicity in IBV. Replacement of the ORF1 genes derived from mammalian cell line-adapted, Massachusetts (Mass)-type Beaudette strain into the genome of a pathogenic Mass-type strain M41 resulted in a non-pathogenic virus (5). Likewise, a chimeric virus with an M41 spike in a Beaudette genome backbone was found to be non-pathogenic (97). This

demonstrates that structural proteins of IBV do not contribute to virulence and that ORF1 genes are responsible in large part to the virulence of IBV. Indeed, passage of virulent viruses in embryonated eggs for vaccine production often results in changes in the ORF1 (4).

Spike protein. The spike protein is a structural protein of coronaviruses and is found as large bulbous structures projecting away from the virion. The arrangement of spike proteins on a coronavirus particle is reminiscent of a crown and is the namesake of the Coronaviridae (“corona” meaning crown in Latin). The spike protein of coronaviruses exists as a homotrimer (32, 63). In the case of IBV, spike is translated as a single polypeptide and is cleaved by cellular proteases into two subunits called S1 and S2. The two polypeptides S1 and S2 associate in the nascent spike monomer by non-covalent interactions. Identification of a multi-basic site with consensus sequence RR-X-RR led to the discovery of the involvement of cellular proteases such as furin in the processing of the spike protein (40). The amino-terminal subunit S1 is responsible for binding of the virus onto host cells (37); while the carboxy-terminal subunit S2 is responsible for fusion of virus and host cell membranes (19).

Coronaviruses have a highly diverse genome, specifically in the spike protein gene. Between the S1 and S2 spike proteins, the S2 spike is relatively well conserved among various IBV strains, whereas S1 exhibits high sequence diversity among known IBV strains (191). Interestingly, sequence analysis of common IBV serotypes showed that conservation of the S2 spike gene sequence was found to be highly similar between IBV isolates of the same serotype, but not among different serotypes, suggesting that S2 may influence the repertoire of antibodies produced against the spike protein (29).

The coronavirus spike protein is a class I virus fusion protein (19, 213). The hallmark of class I fusion proteins is the presence of hydrophobic heptad repeats in the fusion peptide. For coronaviruses, the fusion peptide resides in the S2 spike protein (60). The S2 spike protein of IBV has two heptad repeats (HR) called HR1 and HR2, as well as a transmembrane domain that tethers the spike onto the viral envelope. Binding of the S1 spike protein onto host cell receptors is thought to induce a

conformation change in the spike trimer and followed by a rearrangement of the S2 spike into a trimer of hairpins, allows for fusion of virus and host cell membranes and for the entry of the viral genome into the host cell (213).

The S1 spike is a major determinant of tropism in IBV and other avian coronaviruses (214). Indeed, it has been demonstrated that the turkey coronavirus (TCoV) is a recombinant virus that has IBV-like genomic backbone and a spike protein gene from a yet unidentified virus (112). TCoV is an enteric virus and causes diarrhea and enteritis in turkeys. The acquisition of the spike protein is thus implicated in a change in host species (chicken to turkey) as well in a tissue tropism (respiratory to enteric). A similar phenomenon has also been reported for an avian coronavirus identified in guinea fowl (135). There is no evidence that the spike protein contributes to virulence since it has no impact on disease severity upon infection with chimeric viruses carrying different spike protein genes (5, 6, 26, 30, 97). Interestingly, the S1 spike protein in other coronaviruses has been implicated with virulence, demonstrating distinct differences in the lifestyles among coronavirus species. For example, in porcine epidemic diarrhea virus (PEDV), mutations in the S1 spike gene were associated with decreased disease in pigs even though the infected pigs had comparable viremia (47).

Neuraminidase treatment of host cells prevents infection, demonstrating that IBV requires α -2,3 sialic acids to bind to host cells (217). It has been postulated that coronaviruses use carbohydrates such as sialic acid as a primary receptor; and another secondary protein receptor, e.g. ACE-2 (angiotensin-converting enzyme 2) for the SARS-CoV, that allows for a high affinity interaction of the spike protein and host receptors (134). A secondary protein receptor, if it exists, has yet to be identified for IBV.

IBV spike protein is a major target of immune responses against IBV. Specifically, the S1 spike protein is the main target of virus neutralizing antibodies (126). Serotype as well as cross-protection are thus correlated with the sequence relatedness in the S1 spike gene (191), although some exceptions do

exist (133). Further discussion of the immune responses against spike is found in the section on immune responses against IBV (page 31).

Nucleoprotein. The nucleoprotein (N) gene is a well-conserved gene in the IBV genome and other coronavirus genomes (216). Diagnostic ELISA tests have utilized the N protein as a target antigen (44). N protein, as its name suggests, binds to the viral genomic RNA, forming a ribonucleoprotein complex (142). This ribonucleoprotein complex is subsequently packaged into the virion (51, 153). The RNA-binding domain of N protein facilitates packaging of the ribonucleoprotein complex into virus particles (127). Phosphorylation of the nucleoprotein has been proposed to allow the recognition of viral RNA (45).

In coronaviruses, assembly of viral particles occurs in a specialized region in the cytoplasm (86, 189). Interestingly, the N protein was shown to localize to the cytoplasm and nucleolus (94). In the cytoplasm, the N protein functions in packaging viral genomes into virion. However, localization of the N protein in the nucleolus suggests that the N protein has functions other than packaging genomic RNA. Indeed, it has been demonstrated that the N protein causes cell cycle perturbations that may maximize translation of viral mRNAs (219, 224). Furthermore, the N protein of IBV appears to interact with nucleolin, providing a mechanism for the nucleolar localization of the N protein (46). Another protein that was shown to interact with the N protein is the nsp3 viral protease. This interaction appears to be important in replication of the viral genome (103).

Envelope and membrane proteins. As in most coronaviruses, the envelope protein (E) and membrane (M) protein are small proteins that are found inserted in the viral membrane. The M and E proteins function together in assembly and budding of virions. Indeed, co-expression of the M and E proteins is sufficient to form virus-like particles (VLPs) (18, 208). The spike protein and nucleoprotein were dispensable for the formation of VLPs. It has been shown that the expression of E protein alone can drive the formation and budding of VLPs (144). Nonetheless, the M protein is required for

localization of the E protein to the Golgi apparatus, where budding of virions occur (54). Different conformations of M protein were identified and shown to be important in influencing virion morphology (159). Interaction of the M and E proteins is facilitated by their cytoplasmic domains and was demonstrated to be important for their roles in virion assembly and budding (55, 137).

Other gene products. Less is known about the biological functions of the ORF3a, ORF3b, ORF5a, and ORF5b. It has been shown that these proteins are non-essential for replication of IBV in culture. For example, deletion of the ORF3a and 3b did not impact virus replication of the Beaudette strain in chicken kidney cells, in embryonated eggs, or in tracheal organ cultures (96, 188). Likewise, ORF5a was found to be non-essential for replication of the Beaudette strain in Vero cells (225). However, these proteins are conserved so it is thus proposed that they are likely to be important *in vivo*.

Virus replication

The first step in the life cycle of IBV is the attachment of the spike protein onto host cells, which triggers a conformational change in spike then fusion of the virus and host cell membranes occurs through the action of the S2 spike protein. The genomic RNA then enters into the cytoplasm, where the viral genome serves as template for RdRp production by the host cell. The viral RdRp transcribes the viral mRNAs from the genome, viral proteins are produced by the cellular machinery and virions are assembled. The genomic RNA is capped and polyadenylated, and can thus serve as an mRNA as well as a template for the production of anti-sense genomic RNA which intern is used to replicate the viral genome (35, 71, 72, 230).

The polyproteins of the replicase complex are translated from the viral genomic RNA then proteolytically processed into non-structural proteins (nsp) of the replicase complex. The replicase complex is responsible for the replication of the viral genome and transcription of subgenomic RNAs (35, 71, 72, 183, 230). A leader sequence of about 70 bp is found at the 5' untranslated region (UTR) of the

genome and at the 5' end preceding each open reading frame (ORF) (23, 195). The 5' leader sequence is used as a primer to produce a nested set of 3' co-terminal subgenomic mRNAs. The gene at the 5' end of these subgenomic mRNAs is the only ORF translated into protein (119, 183, 218, 231). Like most RNA viruses, translation of viral proteins is facilitated by host cell machinery.

Coronavirus genome replication and transcription occurs at cytoplasmic membranes (71). Specifically, viral RNA synthesis has been associated with double membrane vesicles and convoluted membranes in the cytoplasm (86, 194). Budding and assembly of the virus particles occurs at the Golgi apparatus, endoplasmic reticulum, and other intermediate compartments. Exocytosis then mediates the exit of virions from the host cell (71).

Diversity and evolution

Coronaviruses are one of the most dynamic virus families. Its members exhibit high diversity and frequent cross-species transmission. RNA viruses, like coronaviruses, are known for having much higher rates of mutation than DNA viruses and cellular organisms (99). It is estimated that the rates of synonymous mutations in RNA viruses to be between 10^{-7} to 10^{-2} substitutions/site/year. This rate of mutation was shown to be dependent upon the length of infection and the mode of transmission of the viral species in question (92). Recombination of viral genomes is also another means of expanding virus diversity (190). The ability of RNA viruses to rapidly change their genetic material allows RNA viruses to exist as a heterogeneous collection of virus populations called quasispecies (67, 192). Many groups have shown that subpopulations within IBV serotypes exist (113, 149, 160, 204).

The mutation rate for several IBV serotypes has been estimated. Variations in the rate of mutation are observed among different serotypes. For example, the rate of mutation in common serotypes found in the United States were measured by full genome sequencing; and were found to be 1.6×10^{-2} to 7.4×10^{-3} substitutions for California (Cal), and 9.8×10^{-4} to 1.6×10^{-6} substitutions/site/year

for Connecticut (Conn) and Massachusetts (Mass) serotypes (150). The disparity in the mutation rates between Cal versus Conn and Mass was proposed to be an effect of the widespread use of live-attenuated vaccines for Conn and Mass serotypes.

Across the coronavirus genome, the S1 spike gene exhibits the greatest sequence diversity (191). Indeed, three hypervariable regions have been identified in the S1 spike gene (39, 129). The mutation rate in the hypervariable region is much higher than the rest of the genome and has been estimated to be 1.4×10^{-2} substitutions/site/year for the DE072 strain (133).

Recombination is another important source of virus diversity, especially for coronaviruses (88, 210). For non-segmented single-stranded RNA viruses like IBV, the mechanism for recombination is through template switching, wherein the RdRp picks up viral genome replication from one RNA molecule to another (190). This mechanism requires that a single host cell to be infected with more than one virus strain. Early evidence that coronaviruses recombine came from experimental co-infections with two murine hepatitis virus (MHV) strains (124, 145), and by transfection of synthetic RNA into MHV-infected cells (136).

Evidence for recombination in IBV can be traced back in the early 1990's from sequencing of field strains D207 from Netherlands, 6/82 from Great Britain, and KB8523 from Japan (130). Indeed, many common IBV strains such as Conn and Cal were identified as recombinants of other strains (201). The same study also showed that the common recombination breakpoints were found in the nsp-2, -3, and -16 genes in ORF1, as well as in the spike protein gene. The extensive use of multiple live-attenuated vaccines against IBV has also been a source of recombination. A study in China demonstrated multiple recombination events at the spike gene of the ck/CH/LHLJ/140906 isolate. The origin of the recombinant viruses were traced to 4/91 and H120 live-attenuated vaccine strains, which are commonly administered together in the field (228).

There are many factors that contribute to the emergence of novel IBV strains. Although the coronaviruses have the potential for high diversity, the emergence of a novel strain should require positive selection to drive its survival and expansion. Examples of factors that can contribute to the emergence of novel strains are the immune status of existing hosts, and the availability of new host species and/or target tissue. One could argue that vaccine development through passaging of virulent strains in embryonated eggs takes advantage of the propensity of IBV to adapt to different hosts. Continuous passage of IBV in culture systems, e.g. cell culture and embryonated eggs, appears to drive virus populations towards similar genotypes. For instance, passage of ArkDPI in primary chicken kidney cells lead to a more homogenous population (84). Nonetheless, alternate passaging of the strain 793/B between chickens and embryonated eggs demonstrated that certain polymorphisms in the spike are associated with a specific host, and that a turnabout of a mutation occurs when the virus is allowed to grow in a different host (43).

In 1998, a novel strain of IBV was isolated in Georgia that had high sequence similarity of about 90% in the S1 spike gene to that of the DE072 vaccine virus, but was serologically distinct from DE072. It was thus postulated that the origin of GA98 was DE072 vaccine virus that had been circulating in the field and was selected as an escape mutant from antibodies generated by vaccinated birds (133). This is an example of how the immune responses against IBV can place positive or negative selection on viral subpopulations.

The presence of novel coronaviruses in other avian species has also been examined as a potential source of virus diversity. A survey in England showed that wild birds could be infected by diverse coronaviruses. Interestingly, researchers also detected IBV-like viruses that are closely related to the vaccine strain H120, demonstrating that there are avian coronaviruses that can infect domestic poultry and wild bird species (102). It remains to be determined if these wild bird coronaviruses have the potential to infect domestic poultry. As previously mentioned, sequence analysis of the turkey

coronavirus (TCoV) was shown to be a recombinant virus with an IBV genomic backbone and a spike gene that is of unknown origin (112). It is possible that other avian species could be the source of the novel spike and further investigation needs be done. Nonetheless, whether a novel, emergent coronavirus would be virulent or non-virulent would be dependent on whether the genetic changes incurred increases the virus' chance to propagate in its host species (27, 59).

2.3. IMMUNE RESPONSES AGAINST IBV

Overview of the immune responses against IBV

The immune responses against IBV are typical of viruses that cause acute, respiratory disease. Adaptive immune responses against IBV are Th1 biased and are primarily based upon the generation of neutralizing antibodies and the development of cell-mediated responses via cytotoxic T cells (35, 89, 161, 211). Responses in mucosal surfaces of the chicken respiratory tract are thought to play a large role in protection against IBV (161). Less is known of innate responses against IBV. Nonetheless, transcriptome analysis of tissues from IBV infected birds have shown that IBV causes the production of pro-inflammatory cytokines such as type I interferons and interleukin-1 β (IL-1 β) (89, 162, 211).

Figure 2 below illustrates the overall kinetics of immune responses, with respect to the course of IBV infection in trachea. The sequence of events is as follows: As early as 1 day post infection (dpi), pro-inflammatory cytokines such as IL-6 and IL-1 β are produced. At 3 to 5 dpi, virus production is at its peak, followed by damage to the ciliated cells of the trachea at 5 to 7 dpi. At this point, IBV infection typically causes cell death in tracheal epithelium and, in the case of nephropathogenic strains, in kidney tubuli (118). Lymphocytes also infiltrate the trachea at this point in the infection. The influx of cytotoxic T cells as well NK cells into the trachea precedes a decline in virus titers and in ciliary damage at 7 dpi (50, 161). Finally, antibodies are produced at 14 dpi and are kept at a basal level for several weeks (162). For vaccine efficacy experiments, the amount of virus replication and disease is typically measured at 5 dpi in the United States or at 4 - 7 dpi in Europe since both viral titers and disease as measured by ciliostasis and histopathology are high (61).

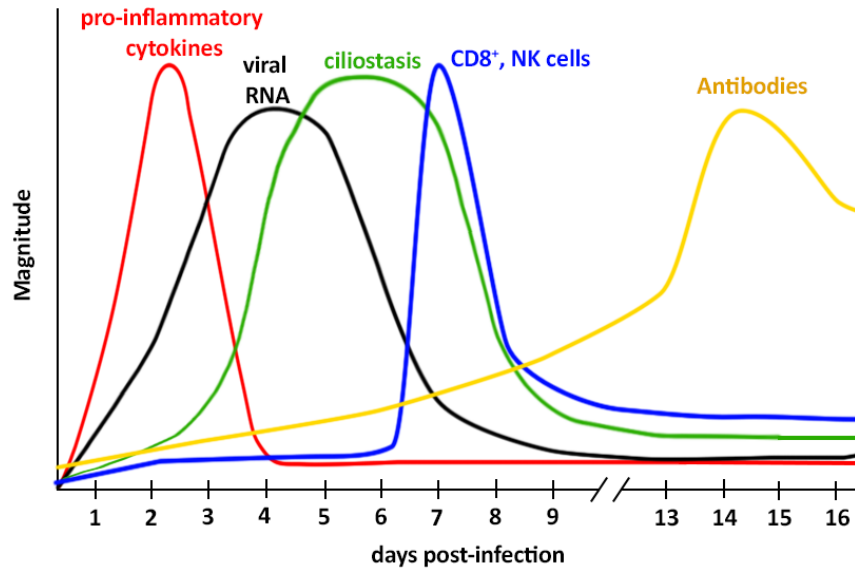


Figure 2. Kinetics of immune responses in the trachea with relation to virus replication (RNA) and to development of disease. Pro-inflammatory cytokines such as IL-6, IL-1 β and TGF β are detected as early as 1 day post-infection (dpi) and can continue to rise until 3 dpi. This is followed by a peak of virus load as measured by RRT-PCR at 3-5 dpi and subsequently, a decline in virus load at 7 dpi. Ciliostasis and histopathological lesions trail the peak of virus titers and begin develop at 4 dpi and maximize at 5-7 dpi. The decline of ciliary damage is associated with an increase of cell-mediated immune responses in the trachea as measured by CD8⁺ T cell levels and the level of the cytotoxic protein granzyme homolog A transcript. Antibodies are detected much later in infection. Peak antibody titers are observed at 14 dpi; after which, antibody levels decline but generally stay at a detectable level for several weeks. *Adapted from data generated by Okino et al (161, 162).*

Innate responses

Microarray analysis early after infection revealed that certain innate responses such as interferon-stimulated genes, chemokines, and pattern-recognition receptors (PRRs) like TLRs and RIG-I, are up regulated (56, 89). Likewise, an increase in the expression of PRRs LGP2, MDA5, and TLR3 was observed in chicken embryo kidney cells (CEK) at 9 hours post-infection (48). Other innate immune genes which were shown to be up regulated include type I interferons, interferon stimulated genes (ISGs), components of the complement system, and components of the inflammasome like IL-1 β .

Several players of the innate immune system have been implicated in restricting IBV. This includes the mannan-binding lectin (MBL), which has been shown to activate complement and inhibit replication in the trachea (121) as well as enhance proliferation of CD8⁺ T cells (122). Like most viral infections, chicken type I interferon has a protective role against IBV. For instance, a 50% reduction in viral load is observed when type I interferon is administered 1 day before challenge in *in vivo* and *in vitro* systems (167). Likewise, avian beta defensins (AvBDs) have been shown to have antiviral activity against IBV (220).

Adaptive immune responses: Humoral and Cell-mediated immunity

Infection with pathogenic or attenuated IBV leads to the development of both antibody as well as cell-mediated immunity. As mentioned earlier, adaptive responses against IBV are Th1 biased, as is typical of acute viral infections (89, 211). In experimentally infected chickens, large numbers of CD8⁺ but few CD4⁺ T cells are often found in affected tissues such as trachea, kidney, and lung (174). These CD8⁺ T cells recognize virus-infected cells and subsequently, target them for lysis. Later after infection or vaccination with IBV, antibodies against IBV are generated in the systemic as well as local mucosal antibody pools. The isotypes generated are of IgG (IgY) isotype for systemic responses and IgA isotype

for mucosal responses. A more detailed discussion on protective immune responses can be found in a succeeding section.

Antigenic targets of immune responses against IBV

Characterization of immune responses against IBV has shown that it primarily targets structural proteins of IBV namely, S, M and N. Specifically, it has been shown that S, M, and N contain epitopes that are involved in cell-mediated responses, whereas antibody responses primarily target S, particularly the S1 subunit, and N proteins but not M (105, 106). Indeed, a study has been done wherein they tested DNA vaccines using plasmids that contain the S1, M, or N protein gene. They administered each plasmid singly or in combination. Vaccination with the S1 plasmid elicited high antibody titers, while vaccination with S1 or M plasmid correlated with CD4⁺ and CD8⁺ T cell responses. Vaccination with all three plasmids resulted in an amplified immune response and in 85% protection as measured by a reduction in detected virus. When plasmids are administered singly, the S1 plasmid provided 74% protection while the N and M plasmids provided 65% and 40% protection respectively (222). The role of the two major antigenic proteins namely, the spike and nucleoprotein, will be discussed below.

Spike glycoprotein. The S glycoprotein is a structural protein that is found as club-like projections extending out from the virus particle. It is responsible for virus' tropism for tracheal and/or kidney cells (36, 77, 215). The spike protein is a homotrimer wherein each polypeptide has two subunits: (i) S1, which is responsible for attachment to host cells; and (ii) S2 which is involved in fusing viral and host cell membranes (19, 31, 32). It has long been accepted that spike is important in inducing protective immunity against IBV, albeit limited in cross protection (57, 82, 98, 100, 169). It has been demonstrated, for example, that replacement of the spike protein gene from the pathogenic strain M41 for that of the apathogenic strain Beaudette is sufficient to provide protection against M41 (97).

Small polymorphisms in the S1 spike correlate with serotype relatedness (14, 38). Indeed, sequence similarity in S1 between two serotypes provides a good estimate of protection. The issue is however that the spike protein gene undergoes rapid genetic changes, due perhaps to selective pressure from the immune system. As little as 2-4% or 10-15 amino acid changes are sufficient to switch serotypes (34, 97, 191). It is of note however that a small proportion of birds may be poorly protected even if S1 genes between the two viruses have high sequence identity (41).

Antibodies generated against IBV primarily target the S protein. In particular, serotype specific, neutralizing antibodies often bind to the S1 subunit (39, 41, 126, 152, 155). Epitopes targeted by neutralizing antibodies are mostly found in the hypervariable regions of the S1 subunit (126). It is thought that the hypervariable regions exhibit high sequence diversity because it receives selective pressure from the immune system to change rapidly. Of interest, certain epitopes on spike are associated with protection against lesions in the kidney (107). This again highlights the importance of spike in tissue tropism and in protection.

Immunization with purified S1 protein and not N or M proteins elicited protection against a challenge with virulent IBV (105). However, it must be noted that this study immunized with protein alone. Thus, one would expect that only antibodies are made against the antigens and that cell-mediated immunity was not induced.

Cell mediated immunity has also been shown to target the spike protein. In particular, it has been demonstrated that cytotoxic T cell (CTL) activity (50) and nitric oxide release (166) are induced if target cells express either the spike glycoprotein or nucleocapsid protein.

Plasmid DNAs containing the S1 (123) or the full-length spike glycoprotein gene (7) have been used as experimental vaccines. Unfortunately, DNA vaccines are not efficacious when delivered as a single immunization *in ovo*. However, prime-boost strategy by the intramuscular route using a plasmid containing the S1 gene was sufficient to provide some protection (123). In the same study by Kapczynski

et al., they noted that the level of antibodies produced against S1 is modest, demonstrating that cell-mediated immunity is likely the mode of protection afforded by DNA vaccines. Vaccination with the S1 protein via an adenovirus vectored vaccine has also been tested and has been demonstrated to achieve good efficacy (206). Likewise, vaccination with the more conserved S2 protein, either as a subunit protein vaccine (107) or in a virus-vectored vaccine (207), has also shown some efficacy. Cryo-electron tomography of the MHV spike trimer showed that the S2 spike protein has a highly conserved region that is accessible and is thus a potential target for broadly neutralizing antibodies (209).

Nucleocapsid. The N protein of IBV is a structural protein that is involved in binding to the genome allowing it to be packaged into the virus particle. Less is known about the immune responses against N but it has been demonstrated that it plays a part in cell-mediated immunity and protection against virulent IBV. Indeed, epitopes on N protein have been identified and have been shown to stimulate proliferation of blood mononuclear cells (17, 187) and to induce cytotoxic T cell responses (50, 185, 187). Nitric oxide release has also been associated with the cytotoxic T cell killing of target cells that express the N protein (166).

The location of epitopes on the N protein appears to be serotype dependent. There are reports that epitopes on the nucleocapsid are found at amino acids 71-78 (17, 107). On the other hand, other studies have shown that T cell epitopes are found on the carboxy-terminal end of N (50, 187). The N gene that the investigators used in this case was from another serotype.

Several studies have attempted to create IBV vaccines using the N protein as antigen. For instance, the N protein has been used as a subunit protein vaccine. In this study, priming with bacterially expressed N protein followed by a boost with an inactivated vaccine, increased hemagglutination (HI) and virus neutralization (VN) titers. Increases in proliferative responses of blood mononuclear cells upon re-stimulation with IBV antigens were also observed. These responses have been shown to be protective against infection with a virulent strain of IBV (16). Aside from subunit vaccines, DNA vaccines are an

attractive platform for using N protein as an antigen because DNA vaccines are capable of inducing cell-mediated immunity. Indeed, when birds were immunized with a plasmid contain the N protein gene, they provided an increase in T cell numbers in the blood. An increase in antibody titers was also seen, albeit lower than S protein specific antibodies. Furthermore, 85% protection was achieved when birds were vaccinated with the plasmid containing the N protein gene together with plasmids containing the S and M protein genes (222).

Protective responses

Vaccination is the primary preventive measures that are taken to prevent IBV outbreaks in commercial poultry operations. There are generally two types of vaccine given to birds (35). Live-attenuated vaccines are given at day of hatch to meat-type birds as well as to long-lived birds like breeders and layers. Vaccination with live-attenuated IBV provides good protection but is typically short-lived and begins to wane at 9 weeks after vaccination (58, 87). For this reason, inactivated vaccines are often given to long-lived birds to boost immunity. Protection offered by most vaccines designed against IBV exhibit some to very little cross-protection across serotypes (57, 82, 98, 100, 169). Thus, control of IBV in the field can be challenging because the vaccine should match circulating field strains.

The old paradigm for many acute viral infections is that neutralizing antibodies provide sufficient protection against infection and disease. For some viruses like small pox, virus-neutralizing antibodies provide adequate protection (69, 170). This is not the general case however because cell-mediated immunity can play a significant role in providing protection against infection and disease (170). It is evident that for IBV, humoral and cell-mediated responses are necessary for protection. For example, inactivated vaccines provide little protection against ciliostasis or drop in egg production without a priming vaccination with live-attenuated vaccines (21, 147, 157). Inactivated vaccines are capable of eliciting good antibody responses but are unable to induce cell-mediated immunity. Thus, if birds are

vaccinated only with an inactivated vaccine, immune responses elicited are primarily humoral. Further evidence shows that mucosal antibodies in tears or systemic antibodies in sera do not correlate well with protection (58, 83, 105, 221). Moreover, removal of the bursa of Fabricius, the organ in birds where B cells mature, does not impact cytotoxic T cell responses and does not impact mortality upon primary infection, although higher morbidity is observed (52, 185). Along these lines, it can be said that antibodies have a significant role in immunity but are not sufficient to provide complete protection against disease.

Cell-mediated immunity has been demonstrated to be important in protection against IBV. In particular, cytotoxic CD8⁺ T cells are implicated in eliminating virus-infected cells by releasing molecules that induce cell lysis, thereby facilitating clearance of the virus. For IBV infections, the peak of CTL responses correlates with a rapid decline in viral load (50, 161) (also see *Figure 1*). Moreover, CD8⁺ T cells harvested from birds infected with IBV were found to be effective at lysing IBV-infected cells *in vitro* (50) as well as protective against clinical disease (166). In corollary, depletion of CD8⁺ T cells but not CD4⁺ T cells correlate with significant reduction in viral load (50, 161, 162). Furthermore, inhibition of T cell activation by cyclosporine treatment induced mortality up to 43% in infected birds, whereas birds experimentally infected with IBV typically do not succumb from IBV infection alone (182). Further studies have also demonstrated that the subset of T cells that is involved in protective responses is the $\alpha\beta$ T cell subset. A study by Seo *et. al.* has shown that adoptive transfer of $\alpha\beta$ T cells but not of $\gamma\delta$ T cells are protective against challenge with a virulent strain of IBV (186). Functional CTL epitopes have been identified in the S1 spike. These peptide epitopes were cloned into a plasmid and used as a DNA vaccine that was administered by the intramuscular route. Vaccine trials showed 90% protection in vaccinated birds (198).

In an effort to improve immune responses, vaccines have been designed that co-administer cytokines incorporated into a viral vector (200, 212) or as an additional gene insert into a DNA plasmid

containing the S1 gene (197, 199). Here, the cytokines were previously identified as a positive contributor to protection and were thus used as an adjuvant. The cytokines used were Th1 cytokine interferon gamma (IFN γ), chemokine granulocyte macrophage colony stimulating factor (GM-CSF), and T cell maturation cytokine IL-2. The addition of these cytokines into vaccines enhanced immune responses and increased protection.

Role of maternal antibodies in protection and vaccination. Maternal antibodies are always an issue when it comes to vaccination of chicks with replication-competent vaccine viruses. It is possible that maternal antibodies could severely impair vaccination. Previous studies have shown that about 1% of systemic IgM and IgA are transferred from the dam to chick while approximately 30% of IgG (IgY) is transferred from dam to chick (91). Moreover, it is estimated that even lower levels of IgM and IgA leak into the chick's mucosal system (97, 154). A rapid decline of maternal antibodies begins to occur at 14 days of age (97, 154).

With respect to IBV, maternal antibodies do not appear to inhibit vaccination with live-attenuated vaccines and thus, protection can still be expected from the progeny of vaccinated hens (83, 154, 173). However, it is generally accepted that exceptionally high antibody titers could lead to a significant leak of neutralizing antibodies against IBV into the chick's mucosal system (154, 173). Furthermore, maternal antibodies can also limit the spread of pathogenic IBV in internal organs such as the spleen, gonad, kidney, lung, cecal tonsils, and cloacal bursa. This suggests that antibodies against IBV, which are present systemically in the blood, are effective in neutralizing virus particles and in preventing infection of internal organs (158).

Mucosal immunity. IBV is respiratory disease and thus, mucosal immunity plays a major role in protective responses. Humoral responses as well as cell-mediated responses are both involved with

mucosal immunity. Indeed, antibodies in the trachea and tears can be detected upon intraocular or intratracheal vaccination with IBV (85, 202). Furthermore, it is thought that leakage of maternal antibodies into the mucosa restricts replication of pathogenic and vaccine viruses (154). IgA, IgM, and IgG levels in the tears correlate well with protection as measured by histopathological scores of trachea, ciliostasis, and reduction in virus load (161). In addition, cytotoxic CD8⁺ T cells in conjunction with the production of pro-inflammatory cytokine IFN γ were shown to correlate with protection (161). Since mucosal immunity is necessary for protection against IBV, the ideal vaccination routes are oculonasal and intratracheal. These vaccination routes can be labor-intensive and expensive however. Thus, live-attenuated vaccines are administered by spray in the hatchery. It has also been suggested administration of DNA vaccines via a mucosal route would enhance its efficacy (123).

Innate responses determine disease versus immunization. Attenuation of pathogenic IBV strains is the primary means of developing serotype-specific vaccines. Passage in embryos is one of the most common methods for attenuation. Attenuation is associated with the loss of any clinical signs upon infection of birds and a lower rate of replication. Many nucleotide sequence changes are observed when comparing the genomes of attenuated viruses versus its parental strain. Genes associated with attenuation are the replicase proteins, spike protein, and non-structural protein (nsp3) (4, 168). *In vitro* passage of pathogenic strains showed marked reduction in innate immune responses as measured by the up-regulation of (i) avian beta defensins, which are cationic microbicidal peptides; and (ii) Toll-like receptors, which function in immune surveillance and are pattern recognition receptors (PRRs) (220).

It is hypothesized that non-structural proteins such as nsp3 are involved in evasion of the innate immune system and that attenuation causes alteration in nsp3 that lead to the activation of the innate immune system (168). As a result, attenuated strains are immediately sensed by the immune system and rapidly cleared by the host. Nsp3 protein in particular has been shown to inhibit interferon by

preventing the phosphorylation of STAT1 (125). The molecular mechanism of innate immune evasion may involve one or more of the protein domains identified in the nsp3 protein: (i) acidic domain, which is predicted to be an RNA binding domain. It has been shown that some RNA viruses such as influenza, reovirus, and rotavirus express proteins that can sequester dsRNA, thereby preventing PRRs to bind to it and activate downstream signaling (2, 20); (ii) papain-like protease (PLP2) domain, which is a protease that is conserved among coronaviruses. For SARS (64) and MHV (229), PLP2 has been demonstrated to be an interferon antagonist and its mode of action is preventing interferon regulatory factor 3 (IRF3) from localizing to the nucleus. In this manner, transcription of interferon response genes is prevented; and (iii) ADP-ribose-1 phosphatase domain, which is likewise found in non-structural proteins of other coronaviruses. This domain has been shown in SARS to be important to evade antiviral effects type I interferon (128).

Host factors that contribute to disease outcomes. The major histocompatibility complex (MHC) contains molecules that are involved in distinguishing self versus non-self and are engaged in antigen presentation (223). One specific subset of MHC molecules called the class I MHC molecules (MHCI) have been shown to influence disease outcomes in viral infections. In fact, viruses have also been shown to down regulate MHCI expression in an effort to evade detection by the immune system (176).

The MHCI genes in the chicken genome are encoded in the region called the B locus. For IBV, certain B haplotypes are associated with resistance or susceptibility to clinical illness due to IBV infection. The B2, B5, and B8 haplotypes are associated with resistance against IB disease while susceptibility is correlated with the B12 and B19 haplotypes (8, 79). Note that whether a bird is susceptible or resistant can also depend upon which serotype infects the bird.

Immune deficiency with a viral etiology can also influence disease outcomes in IBV-infected birds. Chickens that were infected with chicken anemia virus (CAV) or infectious bursal disease virus

(IBDV) were found be more susceptible to IBV infection with increased viral loads in trachea and tears, delayed IgA production, increased clinical signs, and more severe histopathological lesions (205).

Concluding remarks

Vaccine trials have consistently showed that protective responses against IBV are integrated. Efficacious immunity requires a combination of both antibody and cell-mediated responses. Live-attenuated vaccines have thus far provided good protection inducing both humoral and cell-mediated immunity. However, it does not circumvent the issue of limited cross-protection between serotypes. The development of a universal IBV vaccine would be of great interest not only to the poultry industry but to the scientific community at large. More in depth study of the protective responses that are common between serotypes would be highly informative for the design of a universal vaccine. Furthermore, since antibodies are not predictive of protection, there is a need to develop well-defined immune correlates of protection that can accurately predict vaccine success.

2.4. ARKANSAS-TYPE IBV

History

Arkansas (Ark) was first identified as a distinct serotype in 1973 (78). The new IBV serotype was named Ark99 for the state (Arkansas) where the sample was taken and for the sample number (#99). In the United States, live-attenuated Ark vaccine is extensively used in the poultry industry. Despite this, the Ark is highly prevalent in the United States. A retrospective study of diagnostic sample submissions to the Delmarva peninsula and Southeastern United States revealed that Ark is the most common serotype isolated in the field (114, 160, 205). Currently, there is only one strain of vaccine available with an Ark serotype. This vaccine strain is called ArkDPI or Arkansas Delmarva Poultry Industry. ArkDPI was developed by >100 passages in embryonated eggs. Initial studies demonstrated that 50 passages of virulent ArkDPI in embryonated eggs was sufficient to eliminate clinical signs, but still render infected birds seropositive (81).

Emergence of subpopulations and consequences to vaccination

As previously mentioned in the section Diversity and Evolution (page 24), IBV like many RNA viruses exhibits rapid change brought about by high mutation rates and frequent recombination events (88, 99). The ability of RNA viruses to rapidly change is exploited by vaccine development wherein attenuation in birds is achieved through passage of virulent parental virus in embryonated eggs. Passage of ArkDPI in embryonated eggs led to a decrease in virulence in infected birds, which is associated with particular sequence changes in the replicase gene (ORF1) as well as the spike protein gene (4).

RNA viruses such as IBV exist as a diverse collection of subpopulations that can be genotypically distinct. It has indeed been demonstrated that the ArkDPI vaccine has certain subpopulations with associated polymorphisms found on the S1 spike gene (149, 160, 203). Adaptation of ArkDPI vaccine to chicken kidney cells (CEK) led to a more homogenous population, wherein less polymorphisms are

observed (84). This demonstrates that a particular subpopulation can predominate if there is a strong selective pressure to adapt to a particular host.

The existence of viral subpopulations is not unique to ArkDPI and has also been detected in other serotypes such as VicS in Australia, as well as Massachusetts, Delaware, and Connecticut in the United States (149, 172). It has also been shown that alternating passages of 793/B in embryos and chickens led to the selection of polymorphisms in the S1 spike that are associated with either embryos or chickens respectively (43). This finding highlights the ability of IBV to propagate as a diverse group of viral populations and to carry over subpopulations that are suboptimal in replicating in the existing host but may be optimal for a future, potential host.

Sequencing of field samples identified as Ark IBV have shown that many of these samples resemble ArkDPI vaccine, indicating that ArkDPI vaccine is somehow persisting in the flocks (114, 160, 205). Indeed, ArkDPI has been shown to exhibit rolling replication wherein the ArkDPI vaccine virus is transmitted from a successfully vaccinated bird to a naïve bird within the flock (115, 148). Rolling replication is a consequence of poor vaccination coverage since birds are not uniformly vaccinated leaving naïve, non-vaccinated birds present to serve as a pool of susceptible hosts. The danger of rolling replication of the vaccine is that essentially, the vaccine virus is back-passaged in birds and allows for reversion of the vaccine from non-pathogenic to virulent (93).

Examination of ArkDPI-like strains isolated from the field revealed that there is better genetic and antigenic relatedness between field isolates compared to that of the reference ArkDPI vaccine, suggesting that these field isolates are a selected vaccine subpopulation and/or may have been circulating in birds for an extended period of time allowing antigenic drift to occur (160). Cutting (reducing) the vaccine dose is a common practice with poultry growers. However, it has been shown that vaccine dose reduction to $\frac{1}{2}$ or $\frac{1}{4}$ of the recommended dose is insufficient to protect birds from

airsacculitis (181). This can further lead to rolling replication and reversion of the ArkDPI to virulence since vaccination coverage when partial doses are used is reduced.

Experimental and field trials using ArkDPI vaccine have shown that ArkDPI exhibits a poor level of infection and replication (115, 177). The route of administration in particular influences protection; mass application of ArkDPI by hatchery spray or by drinking water was not sufficient to provide adequate protection against challenge (177). In the same study by Roh *et al*, hatchery spray vaccination at day of age with Massachusetts (Mass) and GA98 exhibited significantly higher levels of vaccine virus replication than ArkDPI. Other groups have shown a similar phenomenon wherein Mass was shown to replicate in spray-vaccinated birds at earlier time points and was detected up to 14 days post-vaccination but ArkDPI was detected at a later time point and persisted up to 28 days post-vaccination (3). It has been proposed that ArkDPI is more susceptible to damage brought about by shearing forces during spray vaccination. This is not the case however since the reduction of viral titers in samples taken before and after spray for ArkDPI are not significantly different than a Mass vaccine (178).

Re-isolation of ArkDPI vaccine from vaccinated birds demonstrated that certain polymorphisms are selected for when the vaccine virus is allowed to replicate in chickens (149, 160, 177, 203). It has further been shown that the polymorphisms selected for upon vaccination of chickens are rare in the ArkDPI vaccine virus preparation. The role of these polymorphisms and the subpopulations in ArkDPI vaccination will be further examined by studies described herein.

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CHAPTER 3

MINIMUM INFECTIOUS DOSE DETERMINATION OF THE ARKDPI

INFECTIOUS BRONCHITIS VIRUS VACCINE DELIVERED

BY HATCHERY SPRAY CABINET¹

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SUMMARY

The Arkansas-Delmarva poultry industry (ArkDPI) infectious bronchitis virus (IBV) vaccine is effective when administered by eye drop, where the vaccine virus is able to infect and replicate well in birds and is able to induce protection against homologous challenge. However, ArkDPI vaccine is ineffective when applied by hatchery spray cabinet using the same manufacturers' recommended dose per bird. For this study, we aimed to determine the minimum infectious dose for spray-administered ArkDPI vaccine, which we designate as the dose where the spray-administered ArkDPI vaccine achieves the same level of infection and replication as eye drop-administered ArkDPI vaccine. To this end, we used increasing doses of commercial ArkDPI vaccine to vaccinate 100 broiler chicks at day of hatch, using a commercial hatchery spray cabinet. At 7 and 10-days post-vaccination, the choanal cleft was swabbed and real time RT-PCR was performed to measure the number of infected chicks and virus levels. We observed that the level of infection and replication with spray vaccination matches with that of eye drop vaccination when chicks received 100 times the standard dose for the commercial ArkDPI vaccine. We further examined the S1 spike gene sequence from a subset of re-isolated ArkDPI vaccine virus samples and observed that certain nucleotide changes arise in vaccine viruses re-isolated from chicks, as previously reported (10, 12, 18). Interestingly, for chicks that became infected, these nucleotide changes occur in all of the groups tested, regardless of the vaccine dose the chicks received. This suggests that the ArkDPI vaccine has a certain virus subpopulation that, while successful at infecting and replicating in chicks, represents only a minor virus subpopulation in the original vaccine. Thus, the minimum infectious dose for the ArkDPI vaccine using a hatchery spray cabinet appears to be dependent on the amount of this minor subpopulation reaching the chicks.

Key words

Infectious bronchitis virus, ArkDPI, IBV vaccines, minimum infectious dose

Abbreviations

IBV: Infectious bronchitis virus

ArkDPI: Arkansas Delmarva Poultry Industry

Mass: Massachusetts

RRT-PCR: real time, reverse-transcriptase polymerase chain reaction

INTRODUCTION

In the United States, the Arkansas-type (Ark) infectious bronchitis virus (IBV) is one of the most common IBV serotypes isolated from chickens in the field (5, 13). The Arkansas-Delmarva Poultry Industry (ArkDPI) strain is the only commercially available attenuated live vaccine against the Ark IBV serotype. It has been shown that ArkDPI vaccine can persist within a flock (6) and that poor vaccine coverage results in rolling replication, which is characterized by lateral transmission of the vaccine virus among birds (9). The ArkDPI vaccine has also been associated with delayed replication within a flock, relative to other serotypes of IBV vaccine (1, 6). Indeed, many field isolations of Ark-type IBV appear to be closely related genetically to ArkDPI vaccine virus (13).

Eye drop administration of ArkDPI has been shown to provide good vaccine coverage as well as good protection against homologous challenge (14). However, when ArkDPI vaccine is delivered by mass applications such as a hatchery spray cabinet, poor vaccine coverage within the flock and little protection against homologous challenge has been seen (6, 14, 16). One hypothesis to explain poor vaccine coverage is that its virions may be sensitive to mechanical damage as the virus particles pass through the lines and nozzle in a hatchery spray cabinet. Though some reduction in titer was observed in the ArkDPI vaccine, mechanical damage is likely not an issue because the reduction in titer was not different from a fully efficacious Massachusetts-type (Mass) IBV vaccine (15). An alternative hypothesis is that the chicks are simply not getting enough ArkDPI vaccine by spray vaccination. To this end, we sought to determine the “minimum infectious dose” or the dose at which spray-administered ArkDPI vaccine achieves the same level of infection and replication as eye drop-administered ArkDPI vaccine. We vaccinated chicks at day of hatch using a commercial hatchery spray cabinet with increasing doses of ArkDPI vaccine and measured virus levels in the chicks at 7 and 10-days post-vaccination using real time RT-PCR.

Re-isolation of the ArkDPI vaccine virus from vaccinated chicks has demonstrated that certain nucleotide changes occur in the S1 spike gene, suggesting the existence of a virus subpopulation that is able to more efficiently infect and replicate in chicks (11, 12, 19). To identify what virus subpopulation(s) were replicating in the chicks, we performed S1 spike gene sequencing from swabs taken from vaccinated chicks to examine the genotypes of re-isolated ArkDPI vaccine viruses.

MATERIALS AND METHODS

Experimental design. One-day old broiler chicks were vaccinated with a commercially available ArkDPI (Arkansas Delmarva Poultry Industry) vaccine using a single-nozzle hatchery spray cabinet. Three separate experiments were conducted. For each experiment, one hundred chicks were placed in a hatchery chick basket, spray vaccinated with either a 1X dose, 10x dose or 100x dose in 7mL then immediately transferred into Horsfall isolation units. For each experiment, a group of chicks were also vaccinated with ArkDPI vaccine by eye drop, using the manufacturer's recommended dose. In addition, we vaccinated chicks by the eye drop route and using the hatchery spray cabinet method with the manufacturer's recommended dose of a Massachusetts-type (Mass) vaccine. Eye drop vaccination was performed on 20 chicks by placing 100 μ L of vaccine equivalent to one dose recommended by the manufacturer directly into the eye using a pipet. Choanal swabs were taken from each bird at days 7 and 10 post-vaccination. Swabs were placed in 1-mL of sterile PBS (pH 7.4) and stored at -80°C until analysis. Protocols used for the bird experiments were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Real-time reverse-transcription polymerase chain reaction (RRT-PCR). Measurement of infectivity and virus replication levels in the chicks was performed using RRT-PCR. Viral RNA was extracted from swab samples using the MagMax-96 Total RNA isolation kit (Life Technologies, Thermo Scientific; Massachusetts, USA) and the Kingfisher magnetic particle processor (Thermo Scientific, Finland) per manufacturers' protocols. Five microliters of each RNA sample was added to 20 μ L of master mix from the AgPath-ID One step RT-PCR kit (Applied Biosystems, Thermo Scientific). We used the IBV-specific primers, S1 5' GU391 forward primer (5' - GCT TTT GAG CCT AGC GTT - 3') and S1 3' GL533 reverse primer (5' - GCC ATG TTG TCA CTG TCT ATT G - 3'), and TaqManH dual-labeled probe IBV5'G (5'-FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ-3'), as described previously (2).

S1 spike gene sequencing and analysis. The S1 spike genes were amplified using the 5' oligo and 3' degenerate primers (8) together with the Titan One Tube RT-PCR kit (Roche). Samples were sent to the Georgia Genomics Facility (Athens, GA) for standard Sanger sequencing using the S1 oligo 5' (5' – TGA AAC TGA ACA AAA GAC - 3') and S1 3' deg (5' – CCA TAA GTA ACA TAA GGR CRA - 3') as previously described (8). Additionally, Ark99/DPI-Int-F primer (5'- TTT CTG TGA CTA AAT ATC CTA AG -3') was designed and used to sequence the middle portion of the S1 spike gene. The spike gene for each sample was sequenced with three reads and assembled using Geneious software (Version 8.0.5, Biomatters Ltd). Each contig was aligned to the full length S1 spike protein gene of ArkDPI with GenBank accession number ADP06471.2. The frequency of nucleotide sequence changes and corresponding amino acid changes were then determined using Geneious and CodonCode Aligner (Version 5.1.5, CodonCode Corp).

RESULTS AND DISCUSSION

ArkDPI is the only commercially available vaccine against Ark-type IBV. The ArkDPI vaccine is efficacious when it is administered by eye drop, but it fails to protect against homologous challenge when it is delivered by a hatchery spray cabinet or by drinking water methods (6, 14). Spray cabinet vaccination is associated with a low level of replication that is delayed relative to other IBV vaccine strains (1, 6). It thus appears that the chicks are not getting an infectious dose when they are vaccinated with a hatchery spray cabinet. The objective of our study was to determine the “minimum infectious dose” of ArkDPI administered by a hatchery spray cabinet. Herein, we define the minimum infectious dose as the dose of ArkDPI vaccine delivered by spray that achieves the same level of infection and replication in chicks that are vaccinated with the manufacturers recommended dose by eye drop.

Determination of the minimum infectious dose. We performed eye drop vaccination of chicks with the manufacturer’s recommended dose of ArkDPI vaccine to serve as the benchmark level of infection and replication. For eye drop vaccination, the number of birds positive for vaccine virus was 88% with those positives a mean Ct value of 32.24 ± 0.37 at 7 days post-vaccination (Figure 1A). The number of birds positive for vaccine virus drops to 48% with a mean Ct value of 34.19 ± 0.44 for positive samples at 10 days post-vaccination. In contrast, the percent of birds positive for vaccine virus that were vaccinated with Mass using the eye drop route was 100% at days 7 and 10 post-vaccination with mean Ct values of 28.73 ± 0.30 and 29.74 ± 0.52 for positive samples, respectively. Likewise, spray vaccination with Mass resulted in 100% and 99% of the birds positive for vaccine virus with mean Ct values of 27.04 ± 0.16 and 29.34 ± 0.23 for positive samples at 7 and 10 days post-vaccination, respectively.

The doses of ArkDPI tested in a hatchery spray cabinet was 1x, 10x, and 100x the manufacturer’s recommended dose. As expected, the 1x dose of ArkDPI exhibited a poor level of infection and replication with 15% testing positive for vaccine virus and with a mean Ct value of 32.36 ± 0.77 for

positive samples at 7 days post-vaccination. At 10 days post-vaccination at 1x dose, only 22% were positive for vaccine virus with a mean Ct value of 30.99 ± 0.59 for positive samples. Likewise, chicks vaccinated with a 10x dose by spray had poor levels of infection and replication with only 27% ($Ct = 34.64 \pm 0.28$) and 19% ($Ct = 34.72 \pm 0.35$) of birds positive for vaccine virus at 7 and 10 days post-vaccination respectively. We observed much higher levels of infection and replication with chicks vaccinated with a 100x dose, which had approximately 88% ($Ct = 31.69 \pm 0.23$) and 90% ($Ct = 31.56 \pm 0.25$) of the chicks positive for vaccine virus at 7 and 10 days post-vaccination, respectively. The level of infection at 7 days post-vaccination was 88% when ArkDPI was delivered by eye drop at the standard dose (1x) or by spray at 100x dose. Thus, the minimum infectious dose of ArkDPI vaccine delivered by hatchery spray cabinet for this study is 100x the manufacturers' recommended dose.

Considering only positive samples, the mean Ct values for ArkDPI was not significantly different between eye drop spray cabinet administration (Figure 1B). This suggests that, if a bird receives vaccine, ArkDPI can replicate to an appreciable level. Likewise, the data also suggest that the level of infection is dependent on the dose of vaccine delivered to birds and not on the ability of ArkDPI to replicate in the chick. It is also interesting to note that the mean Ct values for birds vaccinated with Mass, regardless of route of administration, are generally higher than with ArkDPI, indicating that Mass appears to have a higher replicative ability in birds than ArkDPI.

S1 gene sequence analysis. The S1 spike is a structural protein found on the surface of coronavirus particles. It is primarily responsible for binding to host tissues (3) and is the main target of virus neutralizing antibodies (4, 7). Moreover, the S1 spike gene contains hyper-variable regions where the highest sequence diversity is observed across the entire genome (4, 20).

Re-isolations of ArkDPI from vaccinated chicks have shown that many nucleotide sequence changes occur at the S1 spike gene, specifically at hyper-variable regions (11, 12, 19). The rapid

emergence of these viruses indicates that the ArkDPI vaccine contains virus subpopulations, which appear to have differences in the ability to infect and/or replicate in chickens. For our study, we examined the sequence of the S1 spike genes from chicks vaccinated with ArkDPI via the eye drop route or via hatchery spray cabinet at various doses. Each sequence was assembled and compared to the reference ArkDPI S1 spike gene in GenBank. As expected, we observed specific nucleotide changes in re-isolated ArkDPI vaccine virus (Figure 2). Interestingly, the frequency of nucleotide changes was similar between groups. For instance, position 127 in the reference ArkDPI strain is a T; whereas in all of the groups tested, most of the samples ($\geq 50\%$), position 127 contains a C. The nucleotide changes observed in re-isolated ArkDPI vaccine virus is evidence of a virus subpopulation that is competent at infecting and replicating in birds, as previously proposed (11, 12, 19). Our data suggests that poor vaccine coverage with spray-administered ArkDPI is a result of having an insufficient number of virus particles belonging to the competent virus subpopulation that is successful in infecting and replicating in chicks (Figure 3A). When chicks are vaccinated with a critical number of vaccine virus particles (in this study a 100x dose), the minimum infectious dose, of the competent virus subpopulation is achieved (Figure 3B). It is interesting to note that we did not observe an increase in clinical signs as after vaccination with 10x or 100x dose.

In summary, we have demonstrated the minimum infectious dose for the ArkDPI vaccine administered by hatchery spray cabinet is 100x of the manufacturers' recommended dose. Re-isolated ArkDPI vaccine viruses showed similar nucleotide changes in the S1 spike gene, regardless of route of administration or dose. This indicates that a certain virus subpopulation is competent to infect and replicate in chicks, providing further evidence of the existence of these virus subpopulations in the ArkDPI vaccine.

This study highlights the importance of developing well-defined vaccination protocols and implementing them properly. Indeed, it has been demonstrated that vaccination of birds with less than

a full dose does not protect birds from homologous challenge with virulent IBV (17). Obviously it is not economically feasible to vaccinate birds with 100x dose of ArkDPI vaccine. There is thus a need to further enhance existing vaccination strategies or to develop an alternative Ark-type IBV vaccine.

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Table 3-1. Nucleotide changes and their corresponding amino acid changes in the S1 gene of vaccine virus re-isolated from vaccinated chicks

Letters and numbers enclosed in parenthesis correspond to amino acid residues. Rows in gray indicate those positions where the nucleotide or amino acid in the re-isolated ArkDPI is the predominant ($\geq 50\%$) nucleotide or amino acid.

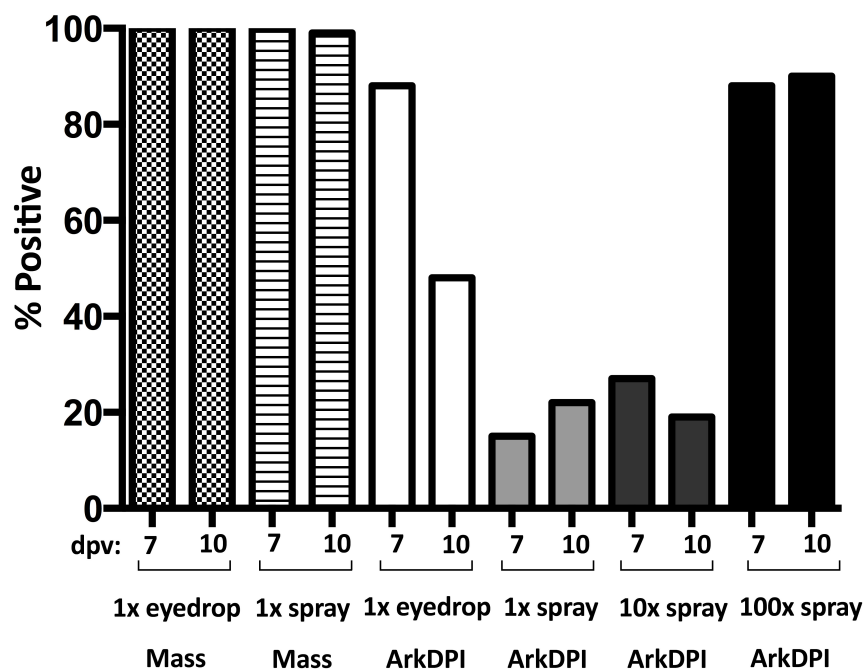
Position	Primary ArkDPI vaccine	Re-isolated ArkDPI vaccine
121	C	T
(41)	(H)	(Y)
127	T	C
(43)	(Y)	(H)
167	A	G
(56)	(N)	(S)
226	C	T
(76)	(L)	(F)
233	C	T
(78)	(A)	(V)
355	T	C
(119)	(S)	(P)
388	A	G
(130)	(S)	(G)
414	G	A
(138)	(M)	(I)

511	T	C
(171)	(Y)	(H)
593	A	C
(198)	(K)	(T)
602	G	A
(201)	(G)	(Q)
610	A	G
(204)	(T)	(A)
637	T	G
(213)	(S)	(A)
770	T	C
(257)	(I)	(T)
968	G	C
(323)	(R)	(T)
976	T	A
(326)	(Y)	(N)
1033	TAA	deletion
(344)	(R)	(deletion)
1100	A	T
(367)	(Q)	(L)
1157	G	A
(386)	(R)	(H)
1173	C	T

(391)	(silent)	(-)
1184	A	G
(395)	(E)	(G)
1192	C	G
(398)	(Q)	(E)
1195	C	T
(399)	(H)	(Y)
1446	C	T
(482)	silent	(-)

Figure 3-1.

A.



B.

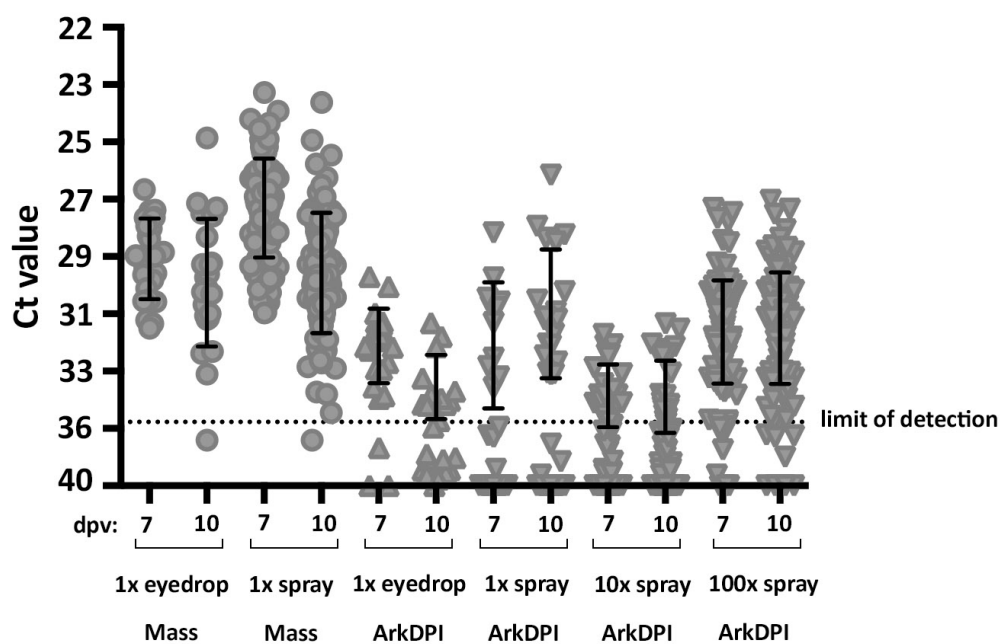


Figure 3-1. Determination of the minimum infectious for the ArkDPI vaccine administered by hatchery spray cabinet

Choanal swabs were taken at 7 and 10 days post-vaccination from 100 chicks vaccinated at day of hatch using the vaccine, dose, and route as indicated. Real time RT-PCR was performed for all samples to measure virus levels. (A) To measure the level of infection in vaccinated birds, the number of birds positive for vaccine virus was counted using the cutoff Ct value of 36 (limit of detection). (B) To measure levels of replication, the mean Ct value was calculated from samples that tested positive for vaccine virus. The dashed lines indicate the limit of detection with real time RT-PCR. Note that all samples are represented on the scatter plot. The number of birds positive for vaccine virus was 88% at day 7 post-vaccination with ArkDPI administered by eye drop (1x dose) or spray at 100x dose, demonstrating that, in this study, the minimum infectious dose for ArkDPI administered by hatchery spray is 100x of the standard dose.

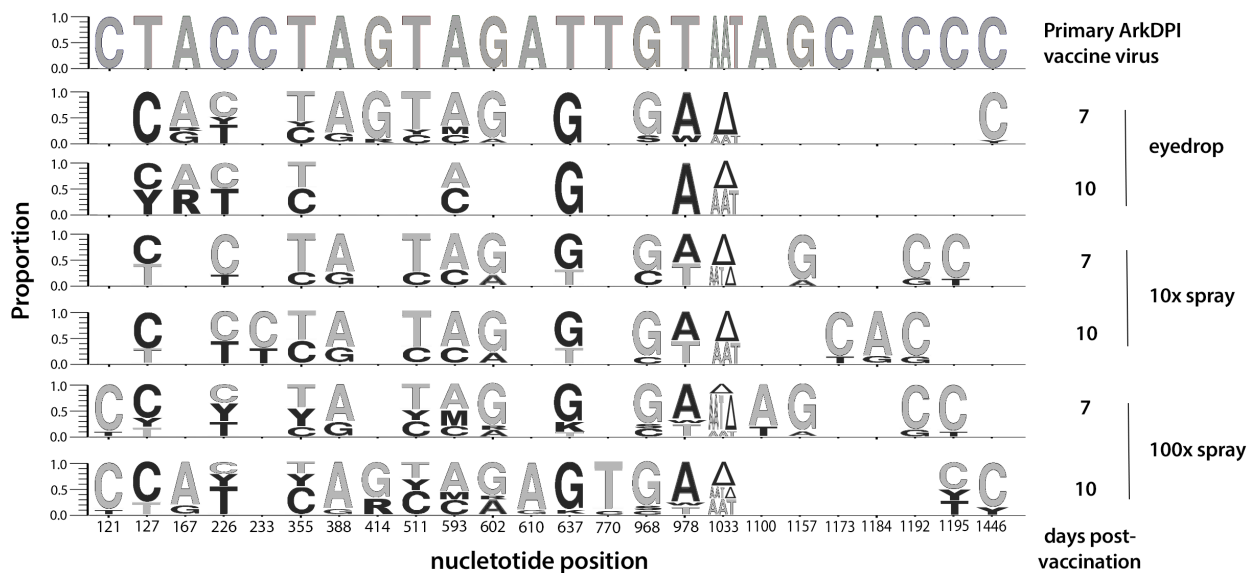
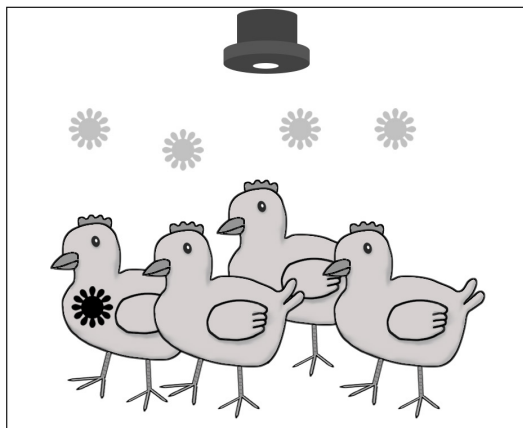
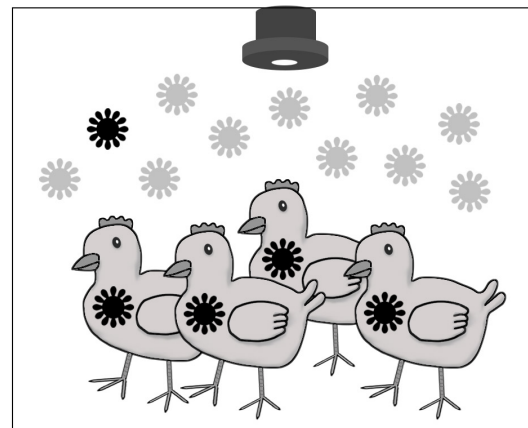


Figure 3-2. A summary of the polymorphisms found in the S1 spike gene of ArkDPI vaccine virus

The height of the letters indicates the proportion of samples with the nucleotide base indicated by the letter. Grey letters indicate nucleotides that are found in the reference ArkDPI sequence (top row) while black letters indicate a nucleotide change from the reference sequence. Single letter IUPAC codes are used for the nucleotide bases. Additionally, Greek letter delta (Δ) indicates a deletion. The minimum number samples considered per group are: 12 for eye drop at 7 days-post vaccination (dpv); 2 for eye drop at 10 dpv; 4 for 10x spray at 7 dpv; 7 for 10x spray at 10 dpv; 10 for 100x spray at 7 dpv; and 13 for 100x spray at 10 dpv.



A. Below the minimum infectious dose



B. At or above the minimum infectious dose

Figure 3-3. Good vaccine coverage is achieved when birds are vaccinated with a dose at or above the minimum infectious dose

The ArkDPI vaccine has been shown to contain different virus populations, one of which is predominant in the vaccine preparation (shown as blue virus particles). Re-isolation of the ArkDPI vaccine virus has shown that certain virus subpopulations (shown as red virus particles) are selected for in the chick. When chicks are vaccinated below the minimum infectious dose, not enough viruses of such subpopulation are available to vaccinate the chicks. This leads to poor vaccine coverage (A). On the other hand, when chicks receive vaccine at or above the minimum infectious dose, enough of the virus subpopulation is available and good vaccine coverage is achieved (B).

CHAPTER 4

POLYMORPHISMS IN THE S1 SPIKE PROTEIN

OF THE ARKANSAS-TYPE INFECTIOUS BRONCHITIS VIRUS

SHOW DIFFERENTIAL BINDING TO HOST TISSUES AND ALTERED ANTIGENICITY¹

¹ Leyson C.L., M. França, M.W. Jackwood, and B.J. Jordan. To be submitted to *Virology*.

ABSTRACT

Sequencing avian infectious bronchitis virus spike genes re-isolated from vaccinated chicks revealed that many sequence changes are found on the S1 spike gene. In the ArkDPI strain, Y43H and $\Delta 344$ are the two most common changes observed. This study aims to examine the roles of Y43H and $\Delta 344$ in selection *in vivo*. Using recombinant ArkDPI S1 proteins, we conducted binding assays on chicken tracheas and embryonic chorioallantoic membrane (CAM). Protein histochemistry showed that the Y43H change allows for enhanced binding to trachea, whereas the ArkDPI S1 spike with H43 alone was able to bind CAM. Using Western blot under denaturing conditions, ArkDPI serotype-specific sera did not bind to S1 proteins with $\Delta 344$, suggesting that $\Delta 344$ alters antigenicity of S1. These findings are important because they propose that specific changes in S1 enhances virus fitness by more effective binding to host tissues (Y43H) and by evading a vaccine-induced antibody response ($\Delta 344$).

Keywords

Infectious bronchitis virus, Arkansas-type vaccine, spike glycoprotein, S1 spike

INTRODUCTION

Infectious bronchitis virus (IBV) is a member of the Coronavirus family, genus Gammacoronavirus. IBV is a highly contagious respiratory pathogen in chickens though some IBV strains may be nephropathogenic and can cause lesions in the kidney. IBV has a narrow host range; chickens are the only species known to be naturally infected. Outbreaks of IBV can cause significant economic loss, particularly for commercial egg producers (5, 11).

Like many members of Coronaviridae, IBV exhibits high genetic diversity. Many serotypes of IBV are known and cross-protection between serotypes is poor (9, 15, 30). For this reason, poultry growers must match their vaccination program with circulating IBV strains in their area. In the United States, the most common serotype isolated in the field is the Arkansas (Ark)-type IBV (16). Currently, only ArkDPI (Arkansas Delmarva Poultry Industry) vaccine is commercially available against Ark-type IBV. The ArkDPI vaccine was originally attenuated by passage of the virus in embryonated eggs 100+ times (12). Experimental and field trials using ArkDPI vaccine have shown that ArkDPI does provide adequate protection (17, 27). One reason may be because ArkDPI vaccine virus does not replicate well in chicks when applied by hatchery spray cabinet (28). Moreover, it has been shown that the ArkDPI vaccine persists in the flock (17) causing a rolling reaction, wherein transmission of the vaccine virus continuously occurs between birds (20).

Re-isolation of ArkDPI virus from vaccinated birds has shown certain sequence changes are frequently observed (21, 25, 31). Certain nucleotide changes are attributed to particular manufacturers but some are common to most ArkDPI re-isolated from vaccinated birds. The existence of these nucleotide changes support the concept that ArkDPI contains viral subpopulations that differ in capacities to infect and replicate in birds (32).

Most of the nucleotide changes observed in re-isolated ArkDPI vaccine virus is found on the S1 spike protein (1, 21, 23, 25, 31, 33). The spike protein of coronaviruses is responsible for attachment and entry into host cells. It has two subunits; S1 and S2. The S1 subunit is primarily responsible for attachment to receptors on host cells (4, 6) while the S2 subunit is responsible for the fusion of virus and host membranes (3).

The S1 spike protein contains the receptor-binding domain (26) and is primarily responsible for host and tissue tropism (35, 36). Epitopes of neutralizing antibodies are also primarily found in S1 (7). Thus, the S1 spike also has significant influence in protective immune responses against IBV. Examination of the S1 spike protein from various serotypes demonstrated that the amino terminal region of S1 exhibits the highest sequence diversity across the genome (19, 24, 34). Rapid mutation in the S1 spike, together with selective pressure, allows the virus exist as viral subpopulations (14).

Two amino acid changes in the S1 spike protein are commonly found in most of the studies that looked at the spike gene sequences of ArkDPI vaccine virus re-isolated from chickens. These are a change from tyrosine to histidine at position 43 (Y43H) and a deletion of an asparagine residue at position 344 (Δ 344). We hypothesize that these amino acid changes provide the virus a selective advantage in the chicken host. To study this, recombinant S1 spike proteins that contained Y43H and/or Δ 344 changes were used in protein histochemistry to examine binding to host tissues. Recombinant S1 spike protein with the Y43H change demonstrated differential binding to mature chicken tracheas and to embryonic chorioallantoic membrane tissue. Additionally, S1 spike protein with Δ 344 was unable to bind ArkDPI serotype-specific sera, demonstrating that Δ 344 alters antigenicity. Taken together, these results help to explain the selection of these polymorphisms in re-isolated ArkDPI vaccine viruses. It appears that the Y43H and Δ 344 changes offer selective advantages over the ArkDPI vaccine virus by

enhancing binding to host tissue, thereby increasing infectivity (Y43H) and by evading the immune response by altering antigenicity of the spike ($\Delta 344$).

RESULTS AND DISCUSSION

Many studies have demonstrated that certain polymorphisms in the spike gene can often be found in viruses isolated from chickens that are vaccinated with the ArkDPI vaccine (1, 21, 23, 25, 31, 33). Two of the most common polymorphisms are in the S1 region of the spike gene, specifically at amino acid position 43 where a tyrosine to histidine is changed (Y43H) and at amino acid position 344 where an asparagine residue is deleted (Δ 344).

The goal of this study was to investigate the role of those two polymorphisms found in the spike gene of re-isolated ArkDPI vaccine in binding to host tissues and in reactions with serotype-specific antibodies. To this end, four recombinant ArkDPI S1 spike proteins were cloned, expressed, and purified. These are (1) primary ArkDPI vaccine spike (Y43/N344), (2) ArkDPI spike with the Y43H change, (3) ArkDPI spike with the Δ 344 change, and (4) ArkDPI spike with both the Y43H and Δ 344 changes. As a control, a Strep-tagged GFP protein was also produced to function as a control for transfection efficiency as well as a control for protein histochemistry.

Expression and purification of S1 proteins

Codon-optimized S1 genes were cloned into a mammalian expression vector and transfected into HEK 293 cells. The S1 proteins were purified using StrepTactin affinity chromatography and visualized by Western blot analysis using StrepTactin (Figure 1). Also shown in Figure 1 are recombinant proteins that were digested with PNGase F. As expected, deglycosylated S1 proteins digested with PNGase F were observed to be approximately 60 kDa in size. However, fully N-glycosylated S1 proteins were observed to be about 260 kDa, which is larger than previously reported for the S1 proteins from other IBV serotypes. This could indicate

that the recombinant ArkDPI S1 protein has glycosylation sites that are different from other IBV serotypes.

Protein histochemistry with recombinant S1 proteins on mature trachea

Binding of the spike onto formalin-fixed tracheal and chorioallantoic membrane (CAM) tissues was examined using protein histochemistry. To detect binding, recombinant spike proteins were pre-complexed with a horseradish peroxidase-conjugated StrepTactin. After which, the complexes were applied onto slides containing sections of mature chicken trachea (Figure 2A, 2B) or embryonic CAM (Figure 3A, 3B).

Protein histochemistry on mature tracheas showed that the S1 spike binds primarily on the ciliated cells as expected, since IBV has a tropism for ciliated epithelial cells. The highest binding to trachea was observed for the spike containing the Y43H mutation. The primary ArkDPI vaccine spike (Y43/N344), the spike with Δ 344, and the spike with both sequence changes (Y43H and Δ 344) have considerably less binding to the mature tracheal tissue. It is interesting that the S1 spike protein containing both Y43H and Δ 344 had poor binding to trachea. This shows that while Y43H enhances binding to trachea, it cannot enhance binding in the presence of Δ 344 perhaps due to a conformational change associated with the deletion.

The results of protein histochemistry assays on tracheas are consistent with the hypothesis that viral subpopulations containing the Y43H change have a spike that has an enhanced ability to bind to chicken tissues i.e. trachea. In this manner, viral subpopulations with Y43H are able to infect the host better than the primary ArkDPI vaccine virus (Y43/N344). Amino acid position 43 has previously been described as one of the four amino acid positions that are critical for binding of the Massachusetts-type IBV S1 spike onto chicken tracheal tissues (26). Additionally, Figure 4 shows a multiple sequence alignment in the region where the four critical

amino acids reside. The histidine at position 43 is conserved among many of the common IBV serotypes, highlighting its importance in spike. Indeed, H43 is also conserved in the Ark99 strain, which belongs to the same serotype as ArkDPI.

Protein histochemistry with recombinant S1 proteins on CAM

Passage of virus in embryonated eggs is a common strategy to attenuate IBV for vaccine production. Moreover, commercial IBV vaccine is produced by propagating the vaccine virus in embryonated chicken eggs. The ArkDPI vaccine was developed and is produced in this same fashion. A virulent ArkDPI strain was passaged in embryonated eggs about 100 times before it was attenuated and sold as a commercial vaccine. Like many other poultry viruses, IBV has been shown to replicate in the chorioallantoic membrane (CAM) of embryonated eggs (2, 10). For this reason, binding of S1 spike on CAM was also examined.

Protein histochemistry on CAM using the recombinant S1 spike proteins showed that only the primary ArkDPI vaccine S1 spike (Y43/N344) was able to significantly bind onto CAM (Figure 3A, 3B). This result is consistent with the hypothesis that the spike protein of the ArkDPI vaccine virus is well-adapted to bind and infect embryonic tissues.

Antigenicity of recombinant S1 proteins

The S1 spike protein is the main target of neutralizing antibodies against IBV (7, 19, 22). Epitope mapping of the S1 spike has shown that there are five antigenic sites targeted by neutralizing monoclonal antibodies (18). About three quarters of the monoclonal antibodies tested had epitopes found in the region spanning amino acids 290 to 400 of the S1 spike protein.

As mentioned above, the $\Delta 344$ polymorphism is often observed in ArkDPI sequences re-isolated from vaccinated chickens. We postulate that, since amino acid position 344 sits in a prominent antigenic region, the $\Delta 344$ change could alter antigenicity of the ArkDPI S1 spike protein. To test this hypothesis, immunoblots on the recombinant S1 spike proteins were performed using serotype-specific antisera against virulent ArkDPI (S1 spike contains N344) under native and denaturing conditions. Under native conditions, there appears to be no difference in the extent of binding between the recombinant S1 spike proteins (Figure 5A). In contrast, western blot of S1 spike proteins electrophoresed under denaturing conditions revealed that S1 spike proteins with $\Delta 344$ alone or together with Y43H are unable to bind ArkDPI serotype-specific antisera (Figure 5B). The primary ArkDPI S1 vaccine spike (Y43/N344) and the spike with Y43H were able to bind ArkDPI serotype-specific antisera under denaturing conditions.

Neutralizing antibodies found in serotype specific antisera against spike generally recognize conformationally-dependent epitopes. However, denaturation of the spike protein associated with the vaccine virus grown in embryonated eggs still bound to serotype specific antisera indicating that at least one linear epitope was recognized. The $\Delta 344$ change in S1 abolished this binding. It has yet to be determined if antibodies that bind to a linear epitope/s associated with $\Delta 344$ are neutralizing.

The ArkDPI serotype-specific antisera used for the immunoblots was taken from birds infected with virulent ArkDPI virus grown in embryonated eggs, whose S1 spike gene sequence contains an asparagine at position 344. The lack of binding to denatured S1 proteins with $\Delta 344$ suggests the deletion of asparagine could offer a selective advantage to viral subpopulations with $\Delta 344$ by helping to evade antibodies that bind to spike.

Inactivated vaccines are frequently given to breeder birds after priming with a live-attenuated vaccine. Since the predominant virus in the vaccine preparation has N344, breeder birds would likely have antibodies that can recognize ArkDPI S1 spike with N344. Thus, it appears that maternal antibodies present in chicks provides a selection pressure against viruses with N344 in spike, allowing for the emergence of viruses with Δ 344. Additionally, we would predict that birds vaccinated with ArkDPI with the Δ 344 change would not generate antibodies against virulent ArkDPI virus, which does have an asparagine at position 344.

Summary and conclusions

In this study, the roles of Y43H and Δ 344 polymorphisms in the S1 spike on binding to the host tissues and antigenicity were examined. Binding experiments on host tissues showed that Y43H enhances the ability of the spike protein to bind to mature tracheal tissue but not to embryonic CAM. On the other hand, the primary ArkDPI vaccine S1 spike protein (Y43/N344) has an inverse binding behavior, wherein it binds robustly to CAM but not to mature trachea.

The second set of experiments looking at the antigenicity of S1 spike proteins demonstrated that Δ 344 alters linear epitopes on spike, thereby abolishing spike's interaction to antibodies present in ArkDPI serotype-specific antisera. Taking the results altogether, we postulate that turnabout of viral populations upon vaccination of chicks can be explained, at least in part, by the selective advantage offered by amino acid changes found in the spike protein. These polymorphisms enable a virus population either to bind robustly onto different host tissues, in the case of Y43H, or to evade neutralization by antibodies from the host, in the case of Δ 344. In this manner, these amino acid changes enhance the virus population's overall fitness in vaccinated chicks. Furthermore, the primary ArkDPI vaccine population is maintained in the vaccine preparation because its spike is well suited to infection of embryonic tissues.

MATERIALS AND METHODS

Construction of plasmids with recombinant S1 fusion protein gene.

The S1 spike glycoprotein gene from an Arkansas Delmarva Poultry Industry (ArkDPI) vaccine isolate (accession number ADP06471.2) was obtained from the database of the National Center for Bioinformatics (NCBI). Plasmids (pUC57-Amp) containing codon-optimized ArkDPI S1 genes with one or two amino acid changes (Y43H and/or Δ 344) were obtained from Genewiz (New Jersey, USA). The amino acids 19-540 of the ArkDPI S1 spike gene were fused to the GCN4 trimerization domain (13) in frame to allow for oligomerization (designated as S1-GCN4). The sequence of the GCN4 trimerization domain is RMKQIEDKIEEIESKQKKIENEIARIKKLVPRGSLE. NheI and BsaI restriction sites were appended at the 5' and 3' ends of S1-GCN4, respectively. The plasmid pEXPR-IBA42 (IBA Life Sciences; Goettingen, Germany) was used as the plasmid vector since it allows for the production and secretion of Strep-tagged proteins. In order to digest with the methylation-sensitive restriction enzyme BsaI, pEXPR-IBA42 and pUC57-Amp plasmids containing the S1 genes were maintained in SCS110 cells (Agilent; California, USA).

The vector pEXPR-IBA42 and pUC57-Amp plasmids containing the S1-GCN4 fusion protein genes were digested with NheI at 37°C for one hour. Digested DNAs were then purified using the GeneJet PCR Purification kit (Thermo Scientific; Massachusetts, USA). After which, purified DNA fragments were digested with BsaI at 37°C for 1 hour. Digested DNAs were then electrophoresed in an agarose gel. Appropriate bands were excised and digested DNAs were purified using the GeneJet Gel Purification kit (Thermo Scientific). To ligate S1-GCN4 fusion genes into pEXPR-IBA42 vector, digested fragments were combined at a 1:3 (vector: insert) ratio and added to a ligation reaction mix containing T4 ligase (Thermo Scientific). Ligation products were incubated at 4°C overnight and then transformed into chemically competent JM109 *E. coli* cells (Promega; Wisconsin, USA) using the heat shock method described by the manufacturer.

The transformation reaction mix was plated on Luria-Bertani agar plates supplemented with 100 µg/mL carbenicillin. Overnight cultures were prepared from several bacterial colonies obtained and plasmid extraction was performed using the GeneJet plasmid miniprep kit (Thermo Scientific). To identify positive transformants, plasmid minipreps from each bacterial colony were subjected to digestion with NheI and subsequently, sequencing by Sanger method at the Georgia Genomics Facility (University of Georgia). A large batch of plasmids to be used for transformation of HEK 293 cells was prepared using the GeneJet Maxiprep kit (Thermo Scientific) following the manufacturer's recommended protocol.

Expression and purification of recombinant S1 fusion proteins.

HEK 293 cells were transfected with plasmids coding for ArkDPI S1 fusion protein using Turbofect (Thermo Scientific) according to the manufacturer's recommended protocol. The DNA:OptiMEM:Turbofect ratio used was 6 µg:0.8 mL:10 µL for every 10^6 cells. Culture media was harvested 6 days after transfection and centrifuged at 3,000 x g for 15 minutes at 4°C to pellet any carried over cells. Supernatants were subjected to affinity chromatography with a StrepTactin-Sepharose column (IBA Life Sciences) using the manufacturer's recommended protocol. Presence of S1 fusion proteins was confirmed by performing Western blot using StrepTactin-HRP (IBA Life Sciences) following the manufacturer's recommended protocol. Proteins were digested with PNGase F (New England Biolabs; Massachusetts, USA) to remove N-glycosylations and to accurately measure molecular weight in Western blot. Briefly, we denatured S1 fusion proteins in 1x Glycoprotein denaturation buffer (New England Biolabs) for 10 minutes at 100°C. PNGase F digestion was then performed using 1 U PNGase F enzyme for a 20-µL reaction for 1 hour at 37°C. Purified S1 fusion proteins were quantified using a Nanodrop spectrophotometer (Thermo Scientific).

Protein histochemistry.

Protein histochemistry was performed as previously described (26, 36). A trachea was obtained from a 6-week old commercial broiler chicken and chorioallantoic membrane (CAM) was obtained from 18-day old commercial chicken embryos. All tissues were fixed in 10% formalin for 48-72 hours, embedded in paraffin, and cut into 4- μ m thick sections. Slides were de-paraffinized and antigen retrieval was performed in citrate buffer (10 mM citrate buffer, pH 6.0) for 45 minutes, with the use of a steamer. After antigen retrieval, the slides were blocked with 10% normal goat serum in PBS for 30 minutes at room temperature. Endogenous peroxidase activity was also blocked using BLOXALL blocking solution (Vector Labs; California, USA) for 10 minutes at room temperature. The recombinant S1 fusion proteins were pre-complexed with the StrepTactin-HRP at a 1:200 ratio (StrepTactin: S1 spike) for 30 minutes on ice. A concentration of 70 μ g/mL of recombinant S1 spike proteins was used for each slide. Slides were incubated with the recombinant S1 spike proteins overnight at 4°C. The slides were then washed thrice with PBS and signal was developed using the Vectastain ABC kit (Vector Labs). After 10-15 minutes, the substrate was washed off with distilled water three times. Slides were counterstained with hematoxylin for 2 minutes and examined by light microscopy..

Images of stained slides were taken with an Olympus BX41 microscope (New Jersey). Five tissue sections were placed on each slide and a representative image was taken for each tissue section at a 40x magnification. Quantification of the signal was performed on these images using the Image J software (29).

Production of serotype-specific sera and western blot.

Specific pathogen free chickens at 3-8 weeks old were used to generate serotype specific sera. Each bird was intratracheally inoculated with 1×10^5 50% embryo infectious dose

(EID₅₀) of the ArkDPI challenge strain. After two weeks, birds were boosted by intravenous inoculation with 1×10^5 EID₅₀ of the ArkDPI challenge strain. Terminal bleeds were then performed at 3 weeks after boosting. Subsequently, the sera were pooled and inactivated at 56°C for 30 minutes. Virus neutralization tests were performed to confirm that the sera only reacted to ArkDPI. For Western blotting of our S1 fusion proteins, we used the ArkDPI serotype specific serum at a 1:1000 dilution as a primary antibody and a horseradish peroxidase-conjugated goat anti-chicken IgG (Sigma) at a 1:4000 dilution.

Experimental strategies described in this work were adapted from previously published papers (8, 26, 36)

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Table 4-1. Summary of protein histochemistry assays

	DPI	Y43H	$\Delta 344$	$\Delta Y43H$ & $\Delta 344$
Trachea, mature	+	+++	+	+
CAM	+	-/+	-/+	-/+

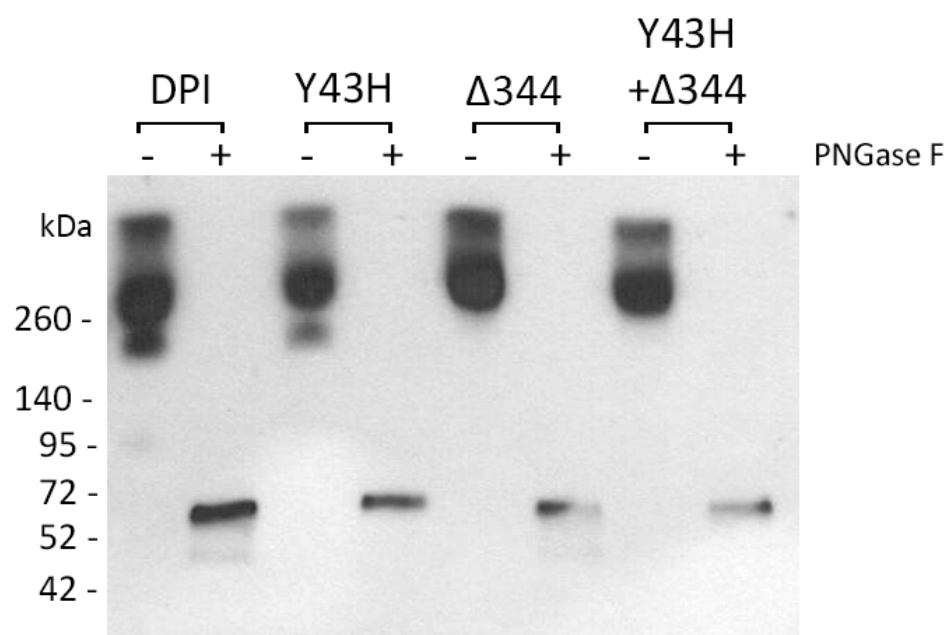


Figure 4-1. S1 fusion proteins were successfully expressed and purified from HEK 293 cells

PNGase digestion was performed to remove N-glycosylation from the recombinant proteins. As expected, unglycosylated S1 fusion proteins migrated to a band 60 kDa in size, while the fully glycosylated S1 fusion proteins ran at a molecular mass of about 260 kDa.

Figure 4-2.

A.

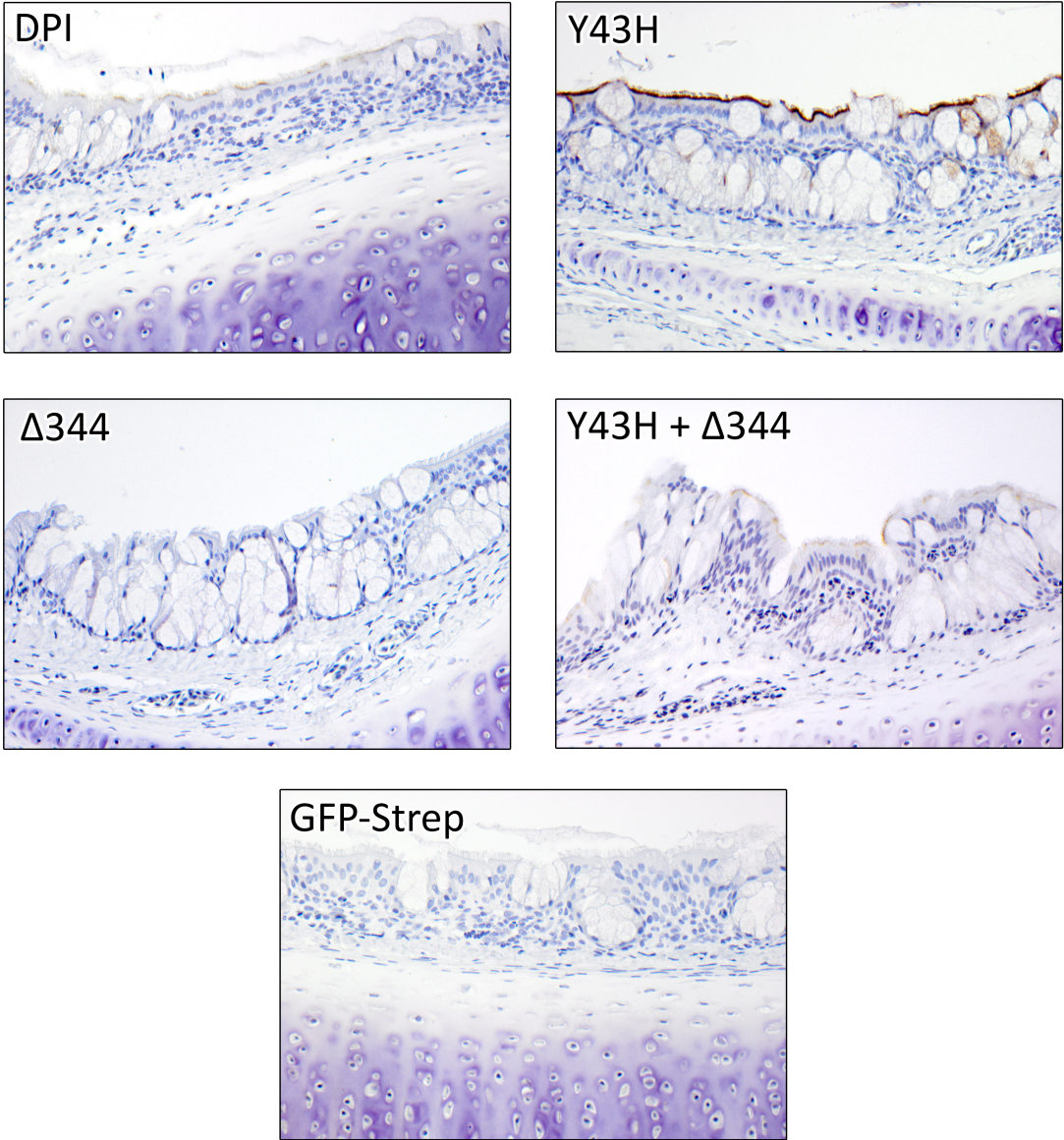


Figure 4-2 continued

B.

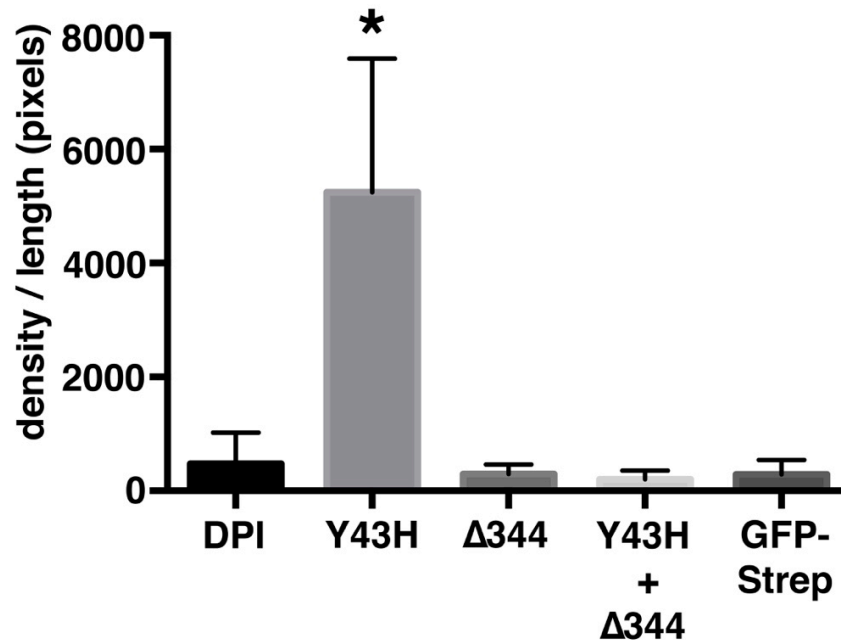


Figure 4-2. The amino acid position 43 is critical for spike binding onto tracheal tissues

Binding of recombinant S1 spike proteins on tissue sections of mature tracheal tissue was examined using protein histochemistry. (A) Four S1 spike proteins were used for the binding experiments namely, the primary ArkDPI S1 spike (DPI), the ArkDPI spike with the Y43H change (Y43H), the ArkDPI spike with a deletion at position 344 ($\Delta 344$), and finally, the ArkDPI spike with both Y43H and $\Delta 344$ changes. StrepTactin-tagged GFP protein (GFP-Strep) was used as a negative control. To detect binding, we pre-complexed the S1 spike proteins with horseradish peroxidase-conjugated StrepTactin and developed a brown signal using DAB as a substrate. Quantification of the signals was performed by densitometry using the ImageJ software (B). Highest binding on mature trachea was observed for Y43H spike. *Statistical analysis was done in GraphPad Prism software. *Indicates $p < 0.05$ when compared to other groups.*

Figure 4-3

A.

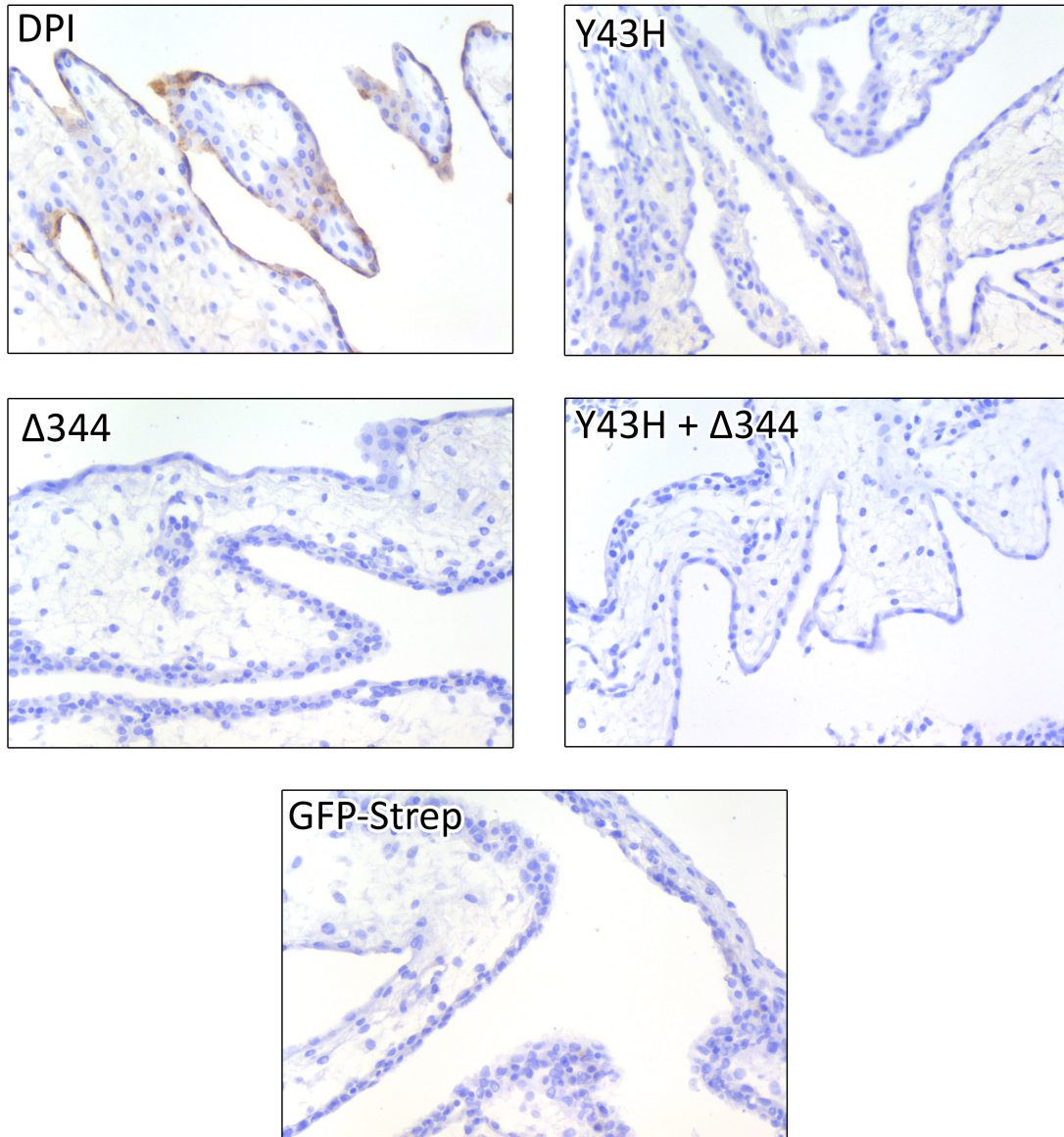


Figure 4-3 continued

B.

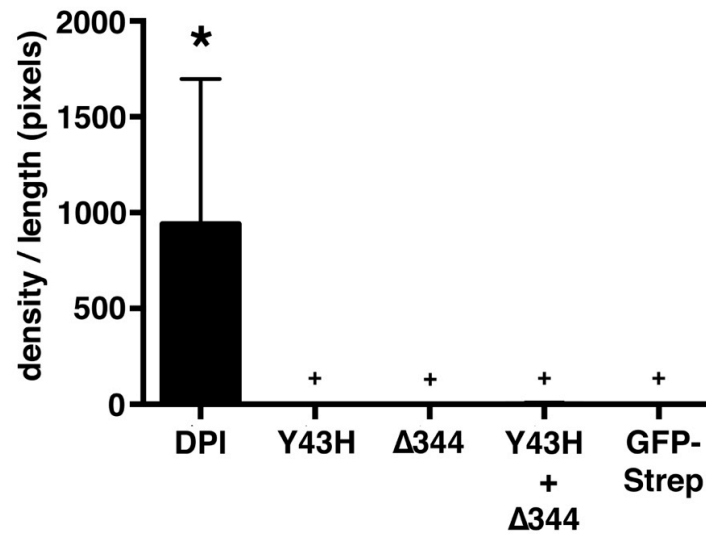


Figure 4-3. The primary ArkDPI spike exhibits highest binding to embryonic tissue

Protein histochemistry on embryonic tissue chorioallantoic membrane (CAM) was performed using purified recombinant S1 spike proteins (A). As with the protein histochemistry with tracheal tissues, the four S1 proteins were used: the primary ArkDPI S1 spike (DPI), the ArkDPI spike with the Y43H change (Y43H), the ArkDPI spike with a deletion at position 344 (Δ 344), and the ArkDPI spike with both Y43H and Δ 344 changes. As a negative control, StrepTactin-tagged GFP protein (GFP-Strep) was also used in protein histochemistry. S1 spike proteins were pre-complexed with horseradish peroxidase-conjugated StrepTactin prior to addition onto CAM tissue sections. Binding signal was developed using DAB as a substrate, thereby producing a brown color. Quantification of the brown signal was performed by densitometry using the ImageJ software (B). Substantial binding to CAM was only observed for the primary ArkDPI vaccine spike protein. *Densitometry was performed using the ImageJ software. Statistical analysis was done in GraphPad Prism software. *Indicates $p < 0.05$ when compared to other groups. +Indicates very low level of signal detected.*

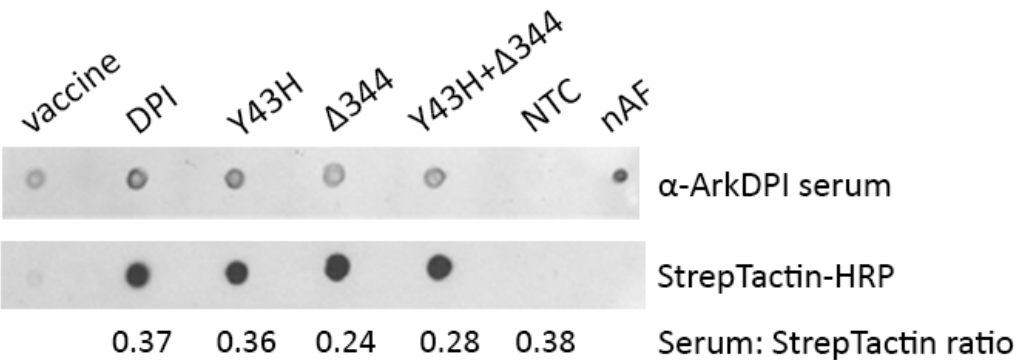
	36				43												60												70						
ArkDPIS1	P	G	H	G	W	H	L	Y	G	G	A	Y	A	V	V	N	V	S	S	E	N	N	N	A	G	T	A	P	S	C	T	A	G	A	I
Ark99S1	P	G	H	G	W	H	L	H	G	G	A	Y	A	V	V	N	V	S	S	E	N	N	N	A	G	T	A	P	S	C	T	A	G	A	I
USA/GA08/GA08/08	P	G	L	G	W	H	L	H	G	G	A	Y	A	V	V	N	V	S	S	E	T	N	N	A	G	S	S	S	S	C	T	A	G	A	I
GA13/14255/14	P	F	D	G	W	H	L	H	G	G	A	Y	E	V	I	N	T	T	Q	E	F	N	N	A	G	L	N	S	E	C	T	A	G	A	I
Mass/H120/55	P	P	D	G	W	H	L	H	G	G	A	Y	A	V	V	N	I	S	S	E	S	N	N	A	G	S	S	S	G	C	T	V	G	I	I
Mass/Ma5	P	P	D	G	W	H	L	H	G	G	A	Y	A	V	V	N	I	S	S	E	S	N	N	A	G	S	S	S	G	C	T	V	G	I	I
Mass/Mass41/41	P	P	N	G	W	H	L	H	G	G	A	Y	A	V	V	N	I	S	S	E	S	N	N	A	G	S	S	P	G	C	I	V	G	T	I
USA/Conn/Conn46/51	P	P	N	G	W	H	L	H	G	G	A	Y	A	V	V	N	T	S	I	E	S	N	N	L	R	E	-	-	-	C	I	V	G	I	I
USA/GA98/0470/98	P	P	N	G	W	H	M	Q	G	G	A	Y	K	V	V	N	K	T	T	I	S	Y	T	N	Q	E	-	-	-	C	T	I	G	V	I
consensus	P	P		G	W	H	L	H	G	G	A	Y	A	V	V	N		S	S	E	S	N	N	A	G				C	T		G		I	

Figure 4-4. Multiple sequence alignment of the partial S1 spike protein

The sequence above shows amino acid positions 35-70. Previous studies have shown there are four amino acid positions (38, 43, 63, and 69) that are important for binding of the spike on tracheal tissue (2, 26). *Multiple sequence alignment was performed using ClustalW and eBioX software.*

Figure 4-5.

A.



B.

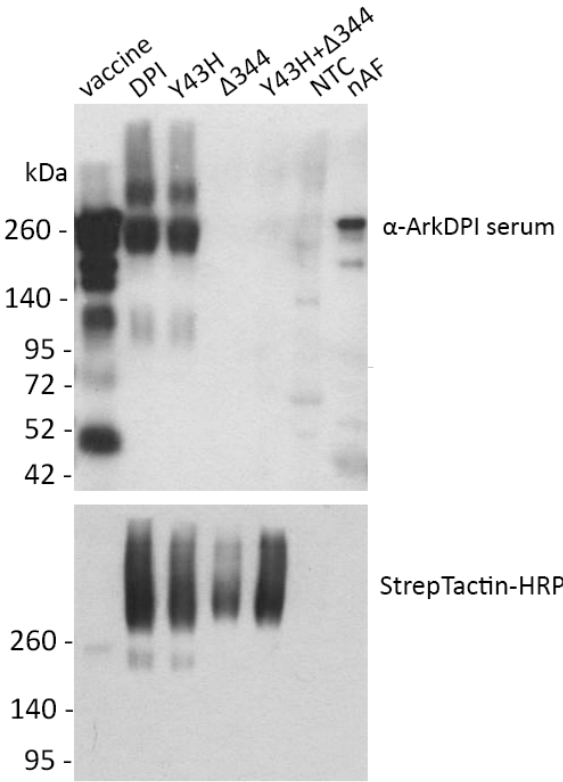


Figure 4-5. Immunoblot analysis of recombinant S1 proteins

ArkDPI serotype-specific antiserum was used to examine antigenicity of recombinant S1 spike proteins. (A) Dot blot was performed under native conditions. An equal amount of protein (350 ng) was spotted onto a nitrocellulose membrane strip and subsequently probed with ArkDPI serotype-specific sera and StrepTactin, as a control. No differences were observed in the amount of antibody binding between recombinant spike proteins. (B) Conventional Western blot under denaturing conditions. We observed that denatured S1 spike proteins with $\Delta 344$ singly or in combination with Y43H were unable to bind to ArkDPI-serotype specific antisera. *Densitometry analysis was done using ImageJ software. NTC = non-transfected control, nAF = normal, non-infected allantoic fluid*

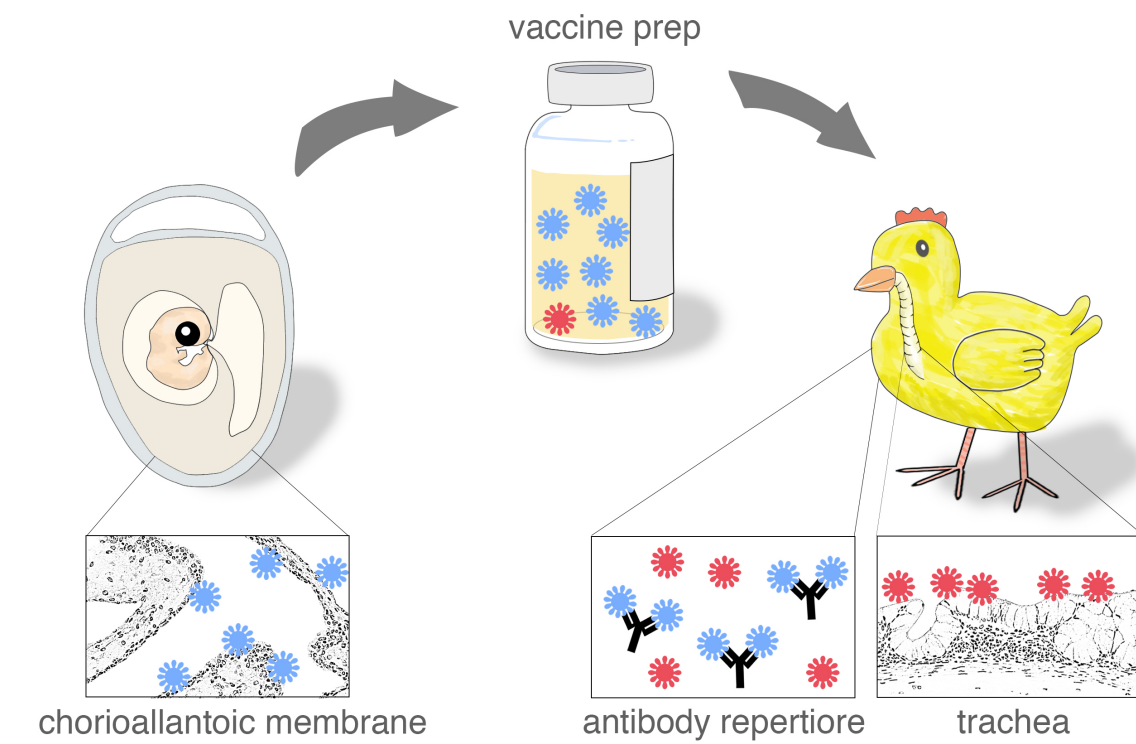


Figure 4-6. Graphical abstract

CHAPTER 5

INSIGHTS FROM MOLECULAR STRUCTURE PREDICTIONS

OF THE INFECTIOUS BRONCHITIS VIRUS S1 SPIKE GLYCOPROTEIN¹

¹ Leyson, C.L., B.J. Jordan, and M.W. Jackwood. To be submitted to *Infection, Genetics and Evolution*.

ABSTRACT

Infectious bronchitis virus is an important respiratory pathogen in chickens. The IBV S1 spike protein is a viral structural protein that is responsible for attachment to host receptors and is a major target for neutralizing antibodies. To date, there is no experimentally determined structure for the IBV S1 spike. In this study, we sought to find a predictive tertiary structure for IBV S1 using I-TASSER, which is an automated homology modeling platform. We found that the predicted structures obtained were robust and consistent with experimental data. For instance, we observed that all four residues (38,43, 63, and 68) shown to be critical for binding to host tissues, were found on the surface of the predicted structure of Massachusetts (Mass) S1 spike. Together with antigenicity index analysis, we were also able to show that Ma5 vaccine has a higher antigenicity index close to the receptor-binding region than M41 vaccine, thereby providing an possible mechanism on how Ma5 achieves better protection against challenge. Examination of the predicted structure of the Arkansas IBV S1 spike also gave insights on the effect of polymorphisms at position 43 on the surface availability of receptor binding residues. This study showcases advancements in protein structure prediction and contributes useful, inexpensive tools to provide insights into the biology of IBV.

Keywords (max 6)

Infectious bronchitis virus (IBV), Spike glycoprotein, S1 spike, protein structure prediction, Massachusetts IBV, Arkansas IBV

Abbreviations

IBV: Infectious bronchitis virus

I-TASSER: Iterative threading assembly refinement

S1: spike glycoprotein subunit 1

NTD: N-terminal domain

CTD: C-terminal domain

RBD: receptor binding domain

Highlights

- Tertiary structure of the S1 spike glycoprotein of IBV was predicted using I-TASSER
- Antigenic regions and receptor-binding residues were mapped on the predicted structure of S1
- Mapping of important regions in S1 offer insights on outcomes of IBV vaccination

RESULTS AND DISCUSSION

Infectious bronchitis virus (IBV) is a highly transmissible respiratory virus in poultry. It belongs to the family Coronaviridae and genus Gammacoronavirus (12). Biosecurity and vaccination are the main control strategies against IBV, and vaccination has proven to be an effective strategy to prevent IBV outbreaks. Typically, broiler chickens are vaccinated by spray with live-attenuated IBV vaccine at the day of hatch. However, some problems still occur. Poor vaccine coverage and lateral transmission among birds are examples of vaccination issues encountered in the field (14).

The spike glycoprotein is one of the structural proteins of all coronaviruses. In electron micrographs, it appears as large bulbous projections radiating from the virus particles and makes up the hallmark “crown-like” appearance that is characteristic of the Coronaviridae. The spike glycoprotein is a major determinant of host and tissue tropism (34). Binding of the spike protein to host cells is dependent on α -2,3 sialic acid, though a secondary protein receptor has been proposed (35). The spike protein is composed of two subunits (4); the S1 subunit, which is responsible for binding the virus to host cells (6); and the S2 subunit, which is responsible for mediating viral-cellular membrane fusion (3).

The S1 spike subunit of IBV also serves as the major target of virus neutralizing antibodies (8, 9, 17, 21, 22) as well as an important antigen for cell-mediated immunity (10). The S1 subunit gene contains the greatest region of genetic diversity within the IBV genome. Within the S1 spike gene, three hypervariable regions have been identified as hotspots for nucleotide sequence changes (17, 23, 25). It is interesting to note however, that as little as 2-4% or 10-15 amino acid changes are sufficient to alter serotypes (5, 11, 31). Thus, it appears that small changes in the structure of S1 may result in global changes in the protein's structure, which may have implications on binding to host cells and on immune responses against the virus.

Several three-dimensional structures are currently available for the spike protein of other coronaviruses, particularly those for members of the genera Alphacoronavirus and Betacoronavirus

(Protein Data Bank; rcsb.org). However, none exists for the IBV or any other viral species in the genus Gammacoronavirus. It has recently been proposed that the spike protein of all coronaviruses share a common evolutionary origin and that it is reasonable to predict structure from existing structural data, despite having a sequence identity below 30% (18). Furthermore, the same study proposed that Alpha-, Beta-, and Gamma-coronaviruses likely share protein domains, namely the N-terminal domain (NTD) and C-terminal domain (CTD) in the S1 spike protein. Thus, it is possible to map these proteins and identify regions that function as epitopes or in receptor binding. For our study, we sought to produce a three-dimensional structural model of the S1 spike glycoprotein of various IBV vaccine serotypes and to map regions in the S1 spike that are of significant interest, such as the receptor binding region.

To produce a structural model for S1 spike, we attempted to perform conventional homology using SWISS-MODEL (2). The first step was to perform an alignment between the S1 spike protein of IBV and a receptor-binding domain (RBD) of a coronavirus with known crystal structure. Among coronavirus species examined, the human coronavirus NL63 (Alphacoronavirus, PDB ID: 3KBH) had the highest sequence similarity to IBV S1, albeit at 17%. An amino acid alignment between IBV and NL63 S1 proteins was performed in preparation for homology modeling. However, we found that the NL63 RBD aligned to IBV S1 positions 239-377, which did not correspond with the region previously identified that contains the critical residues responsible for binding to the host (27). This was not surprising since there is only 17% sequence identity between NL63 and IBV S1 spike proteins. Moreover, it appears that the RBDs of NL63 and IBV are found on different regions of the S1 spike. For NL63, the RBD is found in the CTD (36), whereas the RBD of IBV S1 has been mapped to the NTD (27). It has been proposed that NTD and CTD of coronaviruses have distinct capacity in terms of receptor function since NTDs from various coronavirus species primarily interact with carbohydrates such as sialic acid, while CTDs primarily interact with protein host receptors (19). It is thus likely that IBV has a receptor-binding strategy that is distinct from NL63. Since using the conventional homology modeling was not feasible, we sought to find alternatives.

I-TASSER (Iterative Threading Assembly Refinement) is an automated program that performs a series of multiple homology modeling across a database of known structures and uses additional algorithms to refine structure and to infer function (29, 37, 38). An online service for I-TASSER is currently available at <http://zhanglab.ccmb.med.umich.edu/I-TASSER/>. We submitted IBV S1 sequences of two vaccine virus serotypes namely, the Massachusetts (Mass) and Arkansas (Ark) serotypes. The S1 sequences we submitted spans from amino acid 19 through the last amino acid before the furin cleavage site that delineates S1 and S2 spike protein subunits (aa 533 – 538 in Mass). The signal peptide of S1 spike is found at amino acid positions 1-18 and was removed from the sequence submitted to I-TASSER since it is not present in the mature S1 spike protein. Among the top 5 models predicted by I-TASSER, we chose the model with the highest C-score, which is a score that estimates the quality of the models produced. Figure 1 shows the overall structure of the S1 spike protein for Ma5 (Mass) and ArkDPI (Arkansas Delmarva Poultry Industry) vaccine strains. We observed that the predicted structures have a dumbbell shape with two distinct domains that are composed primarily of β sheets. Interestingly, the minimal binding domain, which spans amino acids 19-272 (27), mapped as one of the distinct protein domains. This observation is consistent with the proposal that all coronavirus S1 spike proteins have two functional domains, NTD and CTD, as previously mentioned. Cryo-electron microscopy structures of the homotrimeric spike protein have been determined for two other coronavirus species, mouse hepatitis virus (33) and human coronavirus HKU1 (16). The S1 spike in these structures is V-shaped with the NTD and CTD as distinct domains composed of β sheets, similar to the predicted structures obtained for IBV.

Aside from the minimal binding domain, we also highlighted amino residues (38, 43, 63, 69) that were identified as critical for binding to host tissues (27). We observed that these amino acids mapped to the surface of the S1 spike, consistent with being part of the receptor binding site (Figure 2). It is of note however that these residues were identified in M41, which is a Mass-type IBV. Other amino acid

residues may play a critical role in binding to the host in serotypes other than Mass. For instance, amino acids 261-272 in ArkDPI (Figure 1B) are two beta-strands that are located in the CTD, suggesting that there are probably differences in the receptor-binding domains across serotypes.

Examination of predicted structure of Mass IBV vaccines. The Mass-type IBV vaccine is the most commonly used vaccine across the globe. There are several genotypes of Mass vaccines commercially available. Among them are Ma5, M41, and H120 vaccines. Vaccine trials in our laboratory comparing two vaccine strains, Ma5 and M41, have shown that Ma5 achieves better protection against challenge than M41 (unpublished results). One hypothesis to explain this observation is that Ma5 has greater antigenicity than M41. In order to test this, we calculated the Jameson-Wolf antigenicity indices (15) for the S1 spike protein at each amino acid position and compared numerical values of the antigenicity scores between the two Mass-type vaccine strains at each amino acid position (Figure 3). The overall difference in antigenicity between Ma5 and other vaccine strains was also obtained by taking the absolute value of the difference in antigenicity score at each amino acid position and calculating the mean. We found that the overall antigenicity indices did not differ exceedingly with only 0.0592, 0.0185, and 0.0593 values between Ma5 and M41, H52, or H120, respectively. Most of the regions where we did find differences in antigenicity indices coincide with hypervariable regions in S1, as expected (7) (Figure 3). The differences in antigenicity indices between Ma5 and M41 were mapped onto their respective predicted tertiary structure (Figure 4). Interestingly, we found that that Ma5 had a higher antigenicity index at a region close to the residues critical for binding to host tissues (Figure 4). This suggests that better protection with Ma5 may be explained by its enhanced ability to induce neutralizing antibodies close to the receptor-binding region.

Examination of predicted structure of Ark IBV vaccines. Ark IBV is the most common serotype isolated in the United States (13, 26). Currently, ArkDPI is the only commercially available vaccine against Ark IBV. However, ArkDPI has been shown to replicate poorly in birds (28) and to persist within a flock (14). Re-isolation of ArkDPI vaccine virus from vaccinated birds reveals that certain polymorphisms emerge, particularly in the S1 spike gene (1, 20, 24, 26, 28, 32). One of the most common polymorphisms observed in re-isolated ArkDPI is a tyrosine to histidine change at position 43 (Y43H). As mentioned above, amino acid position 43 is one of the residues that are critical for binding to host tissues. A histidine is conserved at position 43 (H43) in many other serotypes of IBV (Figure 5A). However, position 43 has been substituted with a tyrosine in the reference sequence of the ArkDPI vaccine (GenBank accession ADP06471.2). Upon re-isolation of ArkDPI vaccine virus from chickens, S1 spike sequencing reveals that almost all re-isolated ArkDPI vaccine virus has H43. This suggests that H43 has some impact in the ability of IBV to infect chickens, likely by influencing the binding properties of S1 spike. To examine the impact of Y43H, we submitted the S1 spike of ArkDPI with Y43 or H43 to the I-TASSER server. The predicted tertiary structure revealed that position 43 is buried in the structure of ArkDPI S1 spike with Y43 or H43 (Figures 5B and 5C). However, ArkDPI with H43 allowed for a conformational change to expose amino acid residues 38 and 63, which has been implicated in binding to host tissues. Our binding experiments on host tissues have shown that a tyrosine (Y) at position 43 allows S1 spike to bind strongly to chorioallantoic membrane in embryos, whereas a histidine (H) at the same position allows S1 spike to bind well on mature chicken trachea (unpublished results). These findings proved an explanation to the emergence of the Y43H polymorphism in ArkDPI vaccine virus re-isolated from chickens.

We have shown in this study that the prediction of three-dimensional protein structure using I-TASSER can offer insights that aid in explaining experimental data as well as phenomena observed in the field. *In silico* analysis has advanced to a point where protein structure prediction has become robust.

Indeed, the availability of such structure predictions is especially important to proteins that are difficult to empirically determine their structure. Applying this type of analysis to other IBV serotypes will offer a fast, inexpensive way to design experiments and to predict outcomes of sequence changes observed in the field.

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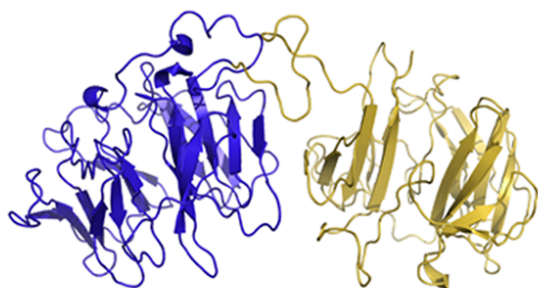
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A.



B.

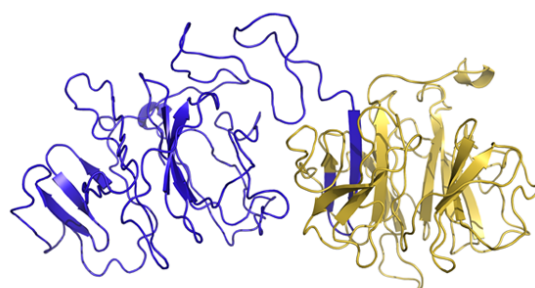


Figure 5-1. Predicted molecular structure of the S1 spike protein has two distinct domains

The S1 spike protein sequences from a Massachusetts (Ma5) and an Arkansas (ArkDPI) serotype vaccine viruses were submitted to I-TASSER (38). The signal peptide (amino acid positions 1-18) has been removed from submitted sequences to reflect the mature peptide sequence. Predicted three-dimensional structures of Ma5 (A) and ArkDPI (B) S1 spike proteins exhibited two distinct domains. The minimal receptor-binding domain (27) has been mapped to amino acids 19-272, which are shown in the structures as blue. Interestingly, the minimal receptor domain forms a distinct domain for all IBV S1 spike proteins examined. This observation is consistent with the proposition that the S1 spike of all coronaviruses have two domains namely, the N-terminal and C-terminal domains (19). *Visualization of protein structures was done using PyMol (30). GenBank accession number for Ma5 is AAS67647.1 and for ArkDPI is ADP06471.2.*

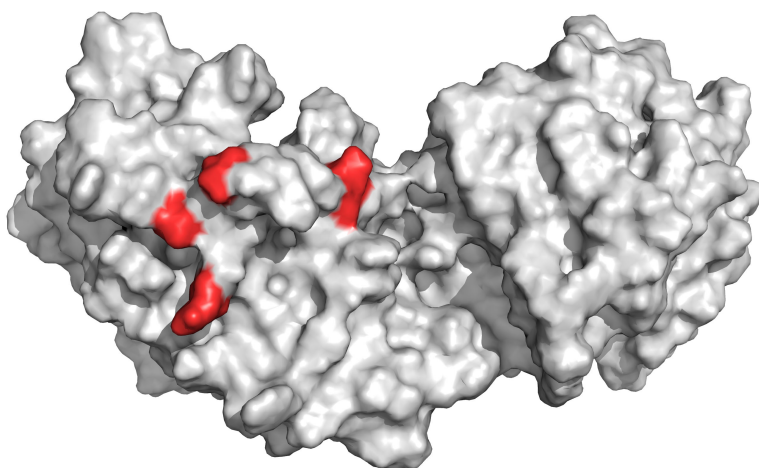


Figure 5-2. Residues critical for binding to chicken tissues map to a localized region on the surface of S1. The predicted molecular structure for Ma5 (Mass) vaccine S1 spike protein is shown as a surface representation to examine residues exposed to the solvent. Amino acids identified as critical for binding to host tissues (27) are colored red. All four amino acids (N38, H43, P63, T69) mapped to the surface, consistent with its function in binding to host receptors. *Visualization of protein structures was done using PyMol (30).*

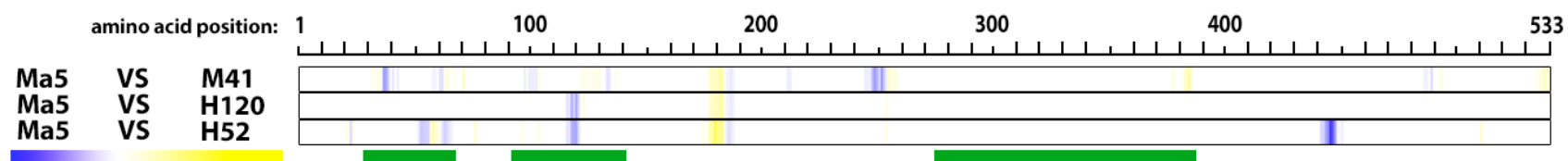
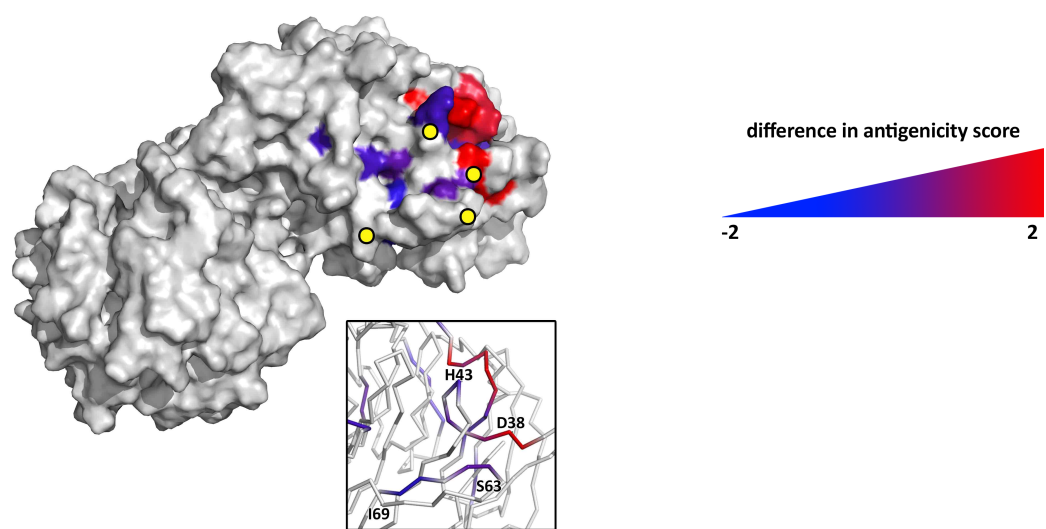


Figure 5-3. Antigenicity indices were compared between two Mass IBV vaccine strains, Ma5 and M41

The Jameson-Wolf antigenicity index (15) was applied to the S1 spike amino acid sequence of four common Mass-type IBV vaccines namely: Ma5, M41, H120, and H52. The antigenicity score for each amino acid position in Ma5 was numerically compared to that of M41, H120, or H52. Shown here is a heat map of regions where antigenicity scores were found to be different between Ma5 and other Mass-type IBV vaccine strains. Regions in blue represent amino acid positions where Ma5 has a higher antigenicity score than M41, H120, or H52; whereas regions in yellow represent regions where M41, H120, or H52 has a higher antigenicity score than Ma5. The green bars at the bottom represent known hypervariable regions in S1 spike (7). *Jameson-Wolf antigenicity indices were calculated using Protean from DNASTar (Wisconsin, USA). The heat map was generated in Microsoft Excel (Washington, USA).*

Figure 5-4

A.



B.

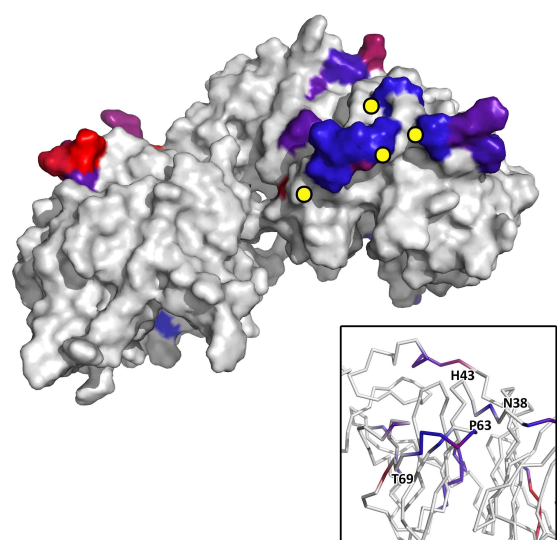


Figure 5-4. Greater antigenicity indices in the receptor-binding region help explain better efficacy among Massachusetts-type IBV vaccine

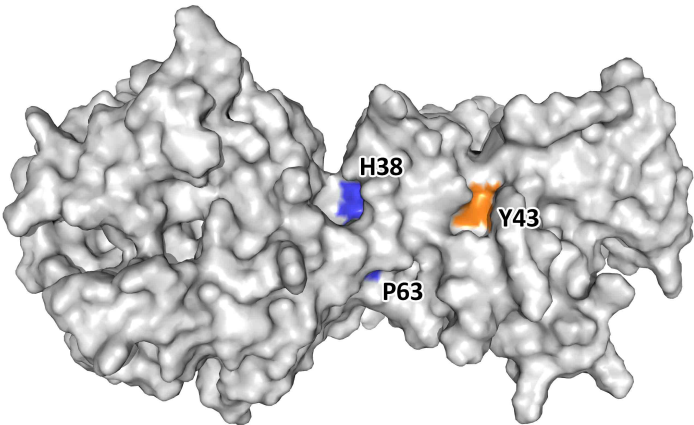
The antigenicity index scores for Ma5 and M41 were determined by the method previously described by Jameson and Wolf (1988). Antigenicity scores obtained were compared at each amino acid position between Ma5 and M41. The differences were then overlaid onto the predicted molecular structure of Ma5 (A) or M41 (B). A heat map was used to indicate the magnitude of the difference wherein blue represents the lowest value and red presents the highest value. The amino acid residues shown to be critical for binding are indicated by yellow dots (surface model) or by residue name and number (inset). The regions where Ma5 was found to have antigenicity scores are in (A), while regions where M41 was found to have greater antigenicity score are in (B). We observed that Ma5 had higher antigenicity scores close to the receptor-binding region. This may help explain better efficacy with Ma5 vaccine, since this model suggests that Ma5 elicits more neutralizing antibodies that target the receptor-binding region. *Visualization of protein structures was done using PyMol (30).*

Figure 5-5

A.

	38			43						63			68																						
Mass/Ma5	P	P	D	G	W	H	L	H	G	G	A	Y	A	V	V	N	I	S	S	E	S	N	N	A	G	S	S	S	G	C	T	V	G	I	I
Mass/H120	P	P	D	G	W	H	L	H	G	G	A	Y	A	V	V	N	I	S	S	E	S	N	N	A	G	S	S	S	G	C	T	V	G	I	I
Mass/H52	P	P	D	G	W	H	L	H	G	G	A	Y	A	V	V	N	I	S	S	E	S	N	N	A	G	S	S	S	G	C	T	V	G	I	I
Mass/M41	P	P	N	G	W	H	L	H	G	G	A	Y	A	V	V	N	I	S	S	E	S	N	N	A	G	S	S	P	G	C	I	V	G	T	I
ArkDPIS1	P	G	H	G	W	H	L	Y	G	G	A	Y	A	V	V	N	V	S	S	E	N	N	N	A	G	T	A	P	S	C	T	A	G	A	I
consensus	P	P	D	G	W	H	L	H	G	G	A	Y	A	V	V	N	I	S	S	E	S	N	N	A	G	S	S	S	G	C	T	V	G	I	I

B.



C.

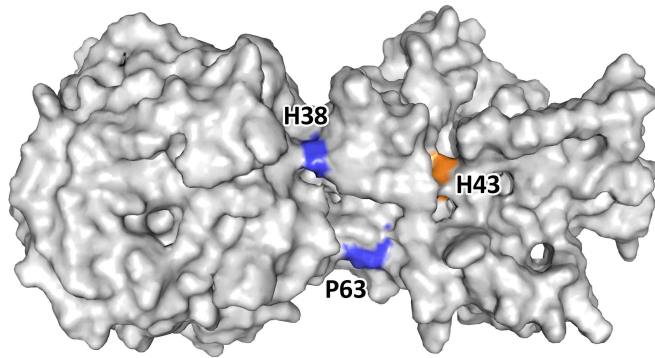


Figure 5-5. Y43H polymorphism in the ArkDPI S1 spike protein potentially alter the receptor-binding region

The amino acids 38, 43, 63, and 68 have been shown to be critical for S1 spike binding to host tissues (27). A multiple alignment showing amino acid positions 36 to 70 from Mass and Ark serotypes are found in (A). In most serotypes, a histidine (H43) is well conserved. In the ArkDPI vaccine however, position 43 has a tyrosine (Y43). Predicted tertiary structure of the ArkDPI S1 spike S1 revealed that positions 38, 43, and 63 are occluded from the surface of the S1 spike while position 68 is completely buried in the structure as a part of a β -strand. On the other hand, the predicted structure of ArkDPI with H43 has amino acids 38 and 63 on its surface, perhaps allowing it to interact with host receptors and to bind more effectively to host cells. *Multiple alignment was done using ClustalW algorithm and eBioX software. Visualization of protein structures was done using PyMol (30).*

CHAPTER 6

SUMMARY AND CONCLUSIONS

Over 80 years after its discovery in chickens (25), infectious bronchitis virus (IBV) remains one of the most important respiratory pathogens in poultry (1, 4, 23, 26). Many serotypes of IBV exist and for this reason, its control can be challenging. Like other coronavirus species, IBV has the propensity to undergo rapid genomic changes through high mutation rates as well as recombination (11, 12, 15, 29, 30). In the United States, IBV isolated from the field predominantly belong to the Arkansas serotype (Ark) (7, 19, 27). ArkDPI (Arkansas Delmarva Poultry) is the only Ark IBV vaccine that is currently commercially available. It has been shown however that it does not provide adequate protection; particularly when ArkDPI is applied by spray (8, 21). Lateral transmission of the vaccine virus also occurs in flocks vaccinated with ArkDPI and is associated with poor protection against challenge with virulent Ark IBV (13). Eye drop vaccination with ArkDPI has been shown to induce good levels of infection and replication, and to provide adequate protection against challenge. However, when ArkDPI is applied by spray, ArkDPI exhibits poor rates of infection and replication in vaccinated birds (21). One hypothesis that has been tested is that ArkDPI is more susceptible to mechanical damage due to the spray cabinet. This does not seem to be the case however, since the reduction in titer is no less than it is for Massachusetts, another type of IBV vaccine, which is efficacious following spray vaccination (22).

Determination of the minimum infectious dose. Vaccination with an inadequate dose is likely to lead to vaccine failure and in some cases, may cause adverse vaccine reactions. Indeed, it has been shown that partial doses of ArkDPI vaccine is inadequate to provide protection against virulent Ark viruses (24). Nonetheless, as a means to reduce costs, some poultry producers vaccinate with partial doses of ArkDPI that is $\frac{1}{2}$ or even $\frac{1}{4}$ of the recommended dose. For an eye drop vaccination, the bird

receives the full volume of vaccine into its system. However, for spray vaccination, a portion of the vaccine dose is lost; For example, some droplets may fall into the chick basket or simply dissipate into the air. In the case for ArkDPI, it appears that birds vaccinated by spray do not receive an adequate amount of vaccine. To this end, we sought to determine the minimum infectious dose of the ArkDPI vaccine when delivered by hatchery spray cabinet. Here, we defined the minimum infectious dose as the amount of ArkDPI applied by spray, which achieves the same level of infection and replication as ArkDPI applied by eye-drop. Real time RT-PCR was used to measure virus levels from choanal swabs sampled from vaccinated birds. We found that a dose that is 100x of the manufacturer's recommendations was able to achieve the same level of infection and replication as eye drop vaccination.

Genotypes of ArkDPI vaccine viruses re-isolated from chickens. Re-isolation of the ArkDPI vaccine virus has shown that certain polymorphisms emerge, particularly in the S1 spike protein gene (14, 18, 19, 28). This phenomenon is certainly not unique to ArkDPI. However, there are particular polymorphisms that are consistently reported in re-isolated vaccine viruses. The detection of such polymorphisms is evidence for the existence of virus subpopulations in the ArkDPI vaccine. To test which polymorphisms emerge when birds are given various doses of ArkDPI, we amplified the full length S1 spike protein gene from choanal swab samples and performed sequencing. As expected, we found that certain polymorphisms are well represented in re-isolated ArkDPI vaccine viruses. Interestingly, the predominance of such polymorphisms was not different from birds vaccinated by eye drop, or by spray vaccination with 10x and 100x doses. This finding supports the hypothesis that certain viral subpopulations in ArkDPI have enhanced ability to infect and replicate in birds and thereby become predominant in re-isolated ArkDPI vaccine viruses.

Polymorphisms in the S1 spike protein have differential binding to host tissues. The S1 spike protein of coronaviruses functions in attachment to host cells and is a major determinant of host and tissue tropism (6, 31, 32). Additionally, the spike protein is important in immune responses against IBV

and is the major antigen of virus neutralizing antibodies (2, 3, 10, 16, 17). Two polymorphisms in S1 spike protein gene of re-isolated ArkDPI vaccine viruses are common among different sources of ArkDPI vaccine: a tyrosine to histidine change at amino acid position 43 (Y43H) and a deletion of an arginine residue at amino acid position 344 (Δ 344). Our hypothesis is that the Y43H and Δ 344 changes in the S1 spike affect spike binding to host tissues, or alter antigenicity. In this regard, we produced a recombinant S1 spike protein using a strategy adapted from previous studies (5, 31). We produced four recombinant spike proteins: the primary ArkDPI S1 spike whose sequence is derived from the vaccine virus; the ArkDPI S1 spike with the Y43H change; the ArkDPI S1 spike with Δ 344; and finally, the ArkDPI S1 spike with both Y43H and Δ 344.

To examine the binding characteristics of these S1 spike proteins, we performed protein histochemistry on mature tracheal tissues as well as on embryonic chorioallantoic membrane (CAM). We found that the Y43H change enabled significantly more binding to tracheal tissue compared to the primary ArkDPI spike or to ArkDPI S1 spike proteins with the Δ 344 change. This finding suggests that enhanced binding to mature trachea by S1 with Y43H contributes to the selection of viral subpopulation with the Y43H changes when birds are vaccinated with ArkDPI. Indeed, the amino acid position 43 of the S1 spike protein was identified as one of the four amino acids critical for binding to tracheal tissue (20). Using I-TASSER (33), we sought predicted structural models of the S1 spike proteins and showed that the amino acids that are critical for binding are found at the surface of the S1 spike protein, consistent with its function in receptor binding. Furthermore, the structural model also predicts that the Y43H change alters the position of amino acids shown to be critical for binding to tracheal tissue, making them more accessible on the surface.

Binding experiments with CAM, showed significant binding only for the primary ArkDPI spike, supporting the concept that the primary ArkDPI vaccine virus is well adapted to grow in embryonated eggs. The ability of the primary ArkDPI vaccine virus to strongly bind to CAM bolsters its propagation in

the vaccine preparation, enabling this population of viruses to become the majority in the vaccine preparation.

To examine the antigenicity of ArkDPI S1 spike proteins, we performed Western immunoblots using an ArkDPI serotype-specific serum. Dot blot under native conditions showed that there are no significant differences in the ability of the ArkDPI serotype-specific serum to recognize the four recombinant S1 spike proteins. However, when we performed a Western blot on denatured S1 spike proteins, we found that ArkDPI spikes with $\Delta 344$, as a single change or in conjunction with Y43H change, were not recognized by the ArkDPI serotype-specific sera. It is of note that the ArkDPI serotype-specific sera was raised in chickens that were inoculated with virulent ArkDPI challenge strain that does not have $\Delta 344$ but instead, has an arginine at position 344. Mapping of epitopes of virus neutralizing monoclonal antibodies has demonstrated that 75% of the antibodies tested target a region where position 344 is located, at positions 291-398 (9). This finding suggests that antibodies elicited by the ArkDPI vaccine selects for escape mutants, one of which is viruses with the $\Delta 344$ change in the S1 spike gene.

Taking our binding and antigenicity experiments together, we can construct a model wherein propagation of the major ArkDPI virus population is supported by its ability to bind well to CAM and thereby efficiently infect embryos; while minor subpopulations, represented by viruses having Y43H and $\Delta 344$ changes, remain at low numbers. Upon vaccination of chickens, minor subpopulations are selected by enhanced binding to mature trachea, in the case of Y43H; or by escape from antibodies in the host, in the case for $\Delta 344$ or both.

The implications of our studies help to explain why ArkDPI vaccine, as it is currently applied in the field, exhibits poor vaccine coverage and poor infection and replication in birds. Evidently, the major population in ArkDPI replicates well in embryonated eggs but is not well suited to infect and replicate in birds. Increasing the dose of commercially available ArkDPI to 100x is a possible strategy to increase vaccination coverage but, unfortunately, it is not economically feasible. Clearly, there is a need to

develop alternative Ark IBV vaccines that do not have the same issues as the ArkDPI vaccine. Vaccines of other serotypes do not exhibit the same issues as the ArkDPI vaccine and thus we are optimistic that this goal can be achieved.

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