

**CHARACTERIZATION OF ANTIGENIC VARIANTS OF  
INFECTIOUS BURSAL DISEASE VIRUS**

by

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## **ABSTRACT**

Infectious bursal disease virus (IBDV) is a double stranded RNA virus, which targets developing B cells in the bursa of Fabricius. IBDV is the etiological agent of infectious bursal disease (IBD), a disease which results in varying degrees of immunosuppression in poultry. The severity of immunosuppression is associated with the age at which birds are infected. When birds are infected at a young age (<21 days of age) immunosuppression is long-lasting and severe. IBDV vaccines are administered to broiler breeder flocks, and maternally derived antibodies are passed vertically to broilers. Broilers should be protected by maternally derived antibody from day of hatch until approximately twenty-one days of age. This passive immunity is vital to protect young birds at the age at which infection would result in the most severe immunosuppression.

Bursal surveys are performed regularly to determine the state of IBDV in flocks across the United States through the collection and histological rating of affected bursas. Results of surveys over the past seven years have shown young flocks affected with IBDV when protection should have been afforded by maternally derived antibodies. Lack of early antibody protection suggests the presence of new isolates that are antigenically distinct from current vaccine viruses.

Field IBDVs were chosen from flocks affected with IBD at younger than twenty-four days-of-age for serological testing in search of an IBDV to act as a new potential vaccine candidate. Virus stocks and antiserum were created in specific-pathogen-free leghorn chickens. Preliminary virus neutralization assays were performed to determine if prevalent vaccine virus Delaware Variant E would effectively neutralize field isolates. Results showed poor neutralization indices, demonstrating that field isolates were antigenically distinct from Delaware Variant E. Isolates which were poorly neutralized were characterized utilizing virus neutralization assays to determine the efficacy of antiserum for cross neutralization of virus stocks in search of an isolate that elicits highly cross neutralizing antibodies. Field IBDVs neutralized each other to varying extents. Two highly cross-protective isolates, AVS-MB and AVS-TV were found. These IBDVs will be evaluated further as potential vaccine candidates. AVS-MB and AVS-TV antiserum neutralized 10/10 and 9/10 evaluated IBDVs respectively. Two other field IBDVs, AVS-SCH and AVS-RRV did not effectively neutralize any of the evaluated IBDVs. All other field isolates and contemporary strains cross-neutralization potential fell between these ranges.

After serological evaluation, the whole genomes of sixteen IBDVs with varying levels of cross-neutralization potential were sequenced using Illumina high-throughput technology with the objective of finding correlations between genotype and serotype or pathotype. IBDV encodes for five viral proteins. VP1 is an RNA dependent RNA polymerase, VP2 is a capsid protein which has previously been associated with antigenicity, VP3 is a multi-functional capsid protein, VP4 functions

as a viral protease, and VP5 is a non-structural protein. Overall, the IBDV genome was highly conserved for all five viral proteins (>90%). The most conserved proteins were VP1 (98.3-100.0%) and VP4 (98.8-100.0%) were most conserved, followed by capsid proteins VP2 (96.0-100%) and VP3 (97.9-100.0%), while VP5 had the lowest percent identity (90.4-100.0%).

Amino acid sequence alignments were created for all viral proteins. Specific amino acid mutations in capsid protein VP2, which has previously been associated with antigenicity, were identified. A predictive protein structure 3D models was created for a Delaware Variant E sequence with insertion of a pair of amino acid changes, D318N and A321E. This mutation resulted in a conformational change in an antigenically significant region of VP2.

The results obtained here show that contemporary field IBDVs neutralize each other to varying extents, and two of the evaluated isolates, AVS-MB and AVS-TV elicited highly cross-neutralizing antibodies. Genome sequencing showed a number of amino acid changes in field isolates, and at least one of the amino acid changes resulted in a conformational change when evaluated in a 3D protein model.

## Chapter 1

### REVIEW OF THE LITERATURE

#### 1.1 History

The first large scale outbreak of infectious bursal disease (IBD) was recognized in the Delmarva peninsula in 1957 with initial outbreaks occurring near Gumboro, Delaware, U.S.A. (40, 113). In 1962, A.S Cosgrove published an article in the journal *Avian Diseases* discussing this new, highly transmissible virus referred to as Gumboro disease, or avian nephrosis due to the acute tubular degenerative lesions seen in the kidneys of affected birds (40, 113). Clinical signs observed in the 1957 outbreak included depression and signs of enteric distress. Upon necropsy, renal lesions, excess mucus in intestines, and enlarged bursas of Fabricius were observed (40). The presence of renal lesions led to the assumption that Gumboro disease was caused by a variant, nephrogenic strain (Gray strain) (33) of infectious bronchitis virus (IBV). However, this association resulted from a concurrent infection with both IBV and IBDV (59, 87) when subsequent testing found that IBV immunized birds still presented with changes in the bursa when infected with Gumboro disease (131). Winterfield *et. al* isolated two agents in embryonating chicken eggs (113, 240) and determined that one isolate- an IBV Gray strain- caused nephritic lesions, while the other caused bursal lesions (113, 237, 238, 239). After it was discovered that the

bursal aspect of Gumboro disease was caused by a specific infectious agent, Allen Edgar termed the name ‘infectious bursal disease’ in 1961 (59).

Further research was performed throughout the 1960s and 1970s to characterize the causative agent of infectious bursal disease. The causative agent of infectious bursal disease was determined to be viral in origin in 1969 (38, 181, 182) and the etiological agent was better characterized by Nick, Cursiefen and Becht in 1976 (164). Allen *et al.* discovered the immunosuppressive effect of IBD on young birds in 1972 by observing the effects of IBDV infection on birds vaccinated against Newcastle disease virus (NDV), finding that NDV vaccinated birds infected with IBDV at a young age were poorly protected against NDV (40). In 1980, a second serotype of IBDV (serotype 2) was reported, although it was determined to be non-pathogenic in chickens, and causes a sub-clinical infection in turkeys (137).

High mortality rates caused either directly by IBD or by secondary infection due to immunosuppressive effect led to a pressing need for development of a vaccine effective against IBDV. Edgar produced an unattenuated vaccine consisting of bursal homogenates in 1963 (59), which allowed targeted, planned infection of birds. Since the original Edgar vaccine still caused infection in birds, Mouthrop and Snedeker-Wills selected a mild field isolate for propagation in embryonic chicken eggs (186) in search of an isolate for a milder vaccine. With the assistance of Hiram Lasher, Mouthrop and Snedeker-Wills’ isolate was utilized for the creation of the first licensed IBDV vaccine, “Bursa Vac” in 1967 (113). Shortly after the licensure of “Bursa Vac,”

the strains Winterfield 2512 (239), Edgar (59), and the Vero cell adapted Lukert strain (11) were utilized for vaccine development (113).

Prior to the mid-1980s, IBD was well controlled by vaccination procedures. However, in 1984, a new IBD outbreak emerged in vaccinated broiler flocks across the Delmarva Peninsula (186, 200). The newly arisen field virus isolates were able to break through maternal antibody protection for previously identified IBD virus strains (185, 186). Through *in vivo* testing, the field isolates were found to cause low mortality and minimal bursal inflammation and resulted in sub-clinical infection accompanied with rapid bursal atrophy followed by severe immunosuppression (184). The IBD field isolates were termed variants, while the older viruses were referred to as classical or standard type (186). IBD vaccines were developed using the field isolate Delaware Variant E, which was originally reported by Rosenberger in 1985 (185). Infection with Delaware Variant E was found to produce high levels of cross protection against other variant field isolates and classic virus, making it a good vaccine candidate (155).

In 1987, field isolates of IBDV associated with high rates of mortality (30-70%) and severe clinical signs were isolated in Holland, Belgium, and the UK. These isolates were classified as very virulent IBDV (vvIBDV) (202). vvIBDV affects the thymus and bone marrow as well as the bursa (2). Since the initial outbreak, vvIBDV has disseminated throughout most poultry-producing countries including the United States. vvIBDV was first isolated in the U.S.A in California in 2008, it has not been found in other states at this time (175).

Since the 1980s, IBD has been well controlled through vaccination in the United States. However, since the mid-2000s there have been increased incidences of IBD in young birds at an age when they should be protected by maternal antibody. This situation mirrors the emergence of variant type IBDV in 1984. Therefore, new vaccine candidates must be considered for effective protection against newly arising field isolates that appear to be antigenic variants.

## **1.2 Etiology**

The etiological agent of IBD is infectious bursal disease virus (IBDV), which is classified under the genus *Avibirnaviridus* in the family *Birnaviridae* (25, 46, 49). *Avibirnaviruses* affect avian hosts; the other *Birnaviridae* genera include *Aquabirnavirus*, which causes infectious pancreatic necrosis and affects aquatic species, as well as *Entomobirnavirus*, which includes the causative agent of *Drosophila X* virus and affects fruit flies (46, 221, 230). All members of the family *Birnaviridae* are non-enveloped, double stranded RNA (dsRNA) viruses with bisegmented genomes (25).

## **1.3 Capsid Structure**

IBDV is a single-shelled, non-enveloped virus with an icosahedral capsid symmetry based on a T=13 icosahedral lattice (135, 171) with primarily trimer clustered subunits, as determined by X-ray crystallography (19, 41). The capsid shell is

composed of 32 capsomeres and ranges from 55-65 nm in diameter (41, 85, 164, 171). Two hundred sixty viral protein 2 (VP2) clusters and two hundred viral protein 3 (VP3) clusters make up the capsid (19, 32). Outer trimers correspond to VP2, while inner trimers correspond to VP3, which acts as a scaffolding protein due to a basic carboxy-terminal that interacts with viral RNA (19, 41). The IBDV capsid is decidedly non-spherical in shape, due to the larger radius from the center of subunits near the five-fold axis compared to the smaller radius of the two- and three-fold axes (19).

#### **1.4 IBDV Genome**

The IBDV genome is composed of two linear segments: segment A, which is 3,261 bp, and segment B, which is 2,800 bp (8, 215). The two segments of the genome were identified using polyacrylamide gel electrophoreses (49, 152). The nucleic acid make-up of the IBDV genome consists of a purine/pyrimidine ratio of ~1, with a guanine and cytosine content of 55.3% (49). Viral RNA has a sedimentation rate of 14S in sucrose gradients and a buoyant density of 1.62 g/mL in cesium sulphate (49, 91, 215). IBDV and other birnaviruses lack a transcriptionally active core and organize their genome in ribonucleoprotein complexes involving viral RNA, VP1 and VP3 (77).

The longer genome segment A contains two partially overlapping open reading frames (ORFs). The smaller ORF, designated ORF-A-1 encodes the 17 kDa viral protein 5 (VP5). ORF-A-2 encodes a 110 kD polyprotein (NH3-pVP2-VP4-VP3-

COOH), which is cleaved autoproteolytically by *cis*-acting viral protease VP4 to create the viral protein 2 (VP2) precursor, which is known as VPX or pVP2, viral protein 3 (VP3) and viral protein 4 (VP4) (16, 91, 116, 154, 203). At least three pVP2 alanine-alanine bounds are cleaved near the protein precursor carboxy-terminus to form the mature VP2 protein and four additional peptides, which remain associated with the virion (35, 51). These additional peptides are involved in the perforation of host cell endosomes during viral infection (44, 72). Segment B encodes viral protein 1 (VP1) (19, 158). The IBDV genome is circularized by VP1, which amongst other roles in replication acts as a viral genome-linked protein (51, 154).

It is believed that the non-coding regions (NCRs) at the 5' and 3' termini of the IBDV genome have important roles in viral function and genome packaging. The 5' NCRs of both segments of the IBDV genome are made up of 32-nucleotide sequences, which are highly conserved between segments and serotypes, although the predicted secondary structure differs between serotypes. The 3' NCRs are conserved within segments but differ between A and B (159).

## **1.5 Viral Proteins of IBDV**

The IBDV genome encodes five viral proteins, as determined by SDS-PAGE analysis, these proteins have roles in viral structure, pathogenesis, antigenicity and replication (8, 66, 158, 160).

VP1 (97 kDa) is important in the replication of the viral genome, acting as an RNA-dependent-RNA-polymerase (RdRp) (138, 232). VP1 is present in the virion in both free polypeptide form and in a bound form attached covalently to the 5' end of the viral RNA, as previously referenced in section 2.4 (108, 154). The coding region of VP1 contains four motifs common to positive-sense single-stranded RNA virus RdRp, suggesting the RdRp role of VP1 (79, 151). Similar to other polymerases, VP1 RdRp activity requires metal-ion cooperation ( $Mg^{2+}$ ) for maximum activity levels (204, 232). VP1 RNA synthesis follows a copy-back mechanism; the template 3' end primes the synthesis of an antisense strand, which then forms an RNA hairpin (232). VP1 plays a role in *in vivo* virulence, as demonstrated through measurement of virulence levels of reassortment viruses encoding for variable VP1 regions from IBDV isolates of different virulence levels expressed in chicken embryo fibroblast (CEF) and Vero cell culture (125). It has been suggested that the impact of VP1 on virulence results from an ability to alter polymerase function and affect the morphogenesis of IBDV viroids (234).

The most prevalent viral protein in serotype 1 viruses is VP2 (441 aa), which is a structural protein, and the primary IBDV capsid protein, which makes up 51% of IBDV viral proteins (50). X-ray chromatography of VP2 revealed its structure contains three distinct domains, a designated base (B), shell (S), and projection (P) (41, 73, 115, 117, 230). The B and S domains make up the carboxy-terminus and amino-terminus of VP2 and are highly conserved, while the P domain (aa 206-350) is highly variable (12, 117). The P domain is located in a loop with S domain  $\beta$  strands

on either side, which are surrounded on the N and C termini by B domain  $\alpha$  helices (41). The outermost section of the P domain contains two hydrophilic loops  $P_{BC}$ , also known as major hydrophilic peak A (aa 212-224), and  $P_{HI}$ , also known as major hydrophilic peak B (aa 314-325) which are associated with antigenic variability (9, 117). VP2 is the only IBDV protein recognized by host neutralizing antibody, demonstrating that this protein is the viral epitope. Deletion studies suggest that the  $P_{BC}$  and  $P_{HI}$  loops of VP2 may contain an epitope (13, 21, 66, 117, 41). There are also two additional minor loops in the P domain of VP2,  $P_{DE}$  (aa 253) and  $P_{FG}$  (aa284), which are important in IBDV cell culture replication and pathogenesis in a chicken host (117, 121, 228). Mutations in the VP2 P domain result in differential levels of monoclonal antibody interactions (117, 195). Multiple studies have demonstrated the presence of IBD virulence, and pathogenicity phenotype markers on VP2 by observing the impact of single nucleotide point mutations (SNPs) (22, 117, 161), although all amino acid markers have not been established.

As the other structural protein of IBDV, VP3 is the second most prevalent viral protein, making up 40% of IBDV protein (50). VP3 contains epitopes that elicit non-neutralizing antibody response and non-protective antibodies (13, 31, 139). In addition, VP3 has a role in host evasion, preserving IBDV replication ability by preventing host programmed cell death (28). It has been shown that VP2 expression in cells triggers the double-stranded RNA (dsRNA)-dependent protein kinase response, which triggers phosphorylation of the eukaryotic initiation factor  $2\alpha$  (eIF2 $\alpha$ ), resulting in blockage of protein synthesis and activation of apoptosis- thus thwarting effective

viral replication. VP3 has been demonstrated to bind IBDV dsRNA, which precludes activation of PKR in the presence of VP2 (28). VP3 is a multifunctional protein, which interacts with VP1 and VP2 to assist in the completion of multiple viral functions. VP3 is vital for IBDV morphogenesis and capsid assembly; it acts as a scaffolding protein through interaction with the carboxy-terminus of the pVP2 protein precursor and genomic dsRNA, to form a disordered layer inside of the outer VP2 capsid (10, 36, 41, 142, 169). VP3 is involved in viral replication through binding of the last ten residues of VP3 with VP1 to form a VP3-VP1 complex (10, 211). VP3 also binds to genomic RNA, and it has been suggested that VP3 may have a role in transcriptional regulation and VP1 polymerase activation (31, 10, 74, 211).

VP4 is a non-structural protein involved in proteolysis of the 110 kDa polyprotein encoded by IBDV genome segment A through a Ser/Lys catalytic dyad mechanism (184, 192, 233). The proteolytic functions of VP4 are dependent on amino acid residue phosphorylation, demonstrating that VP4 is a phosphoprotein (233). VP4 is found in intracytoplasmic type II tubules in IBDV infected cells, possibly through interaction with host cell cytoskeletal actin (80, 233). These type II tubules destroy host cytoskeletal elements, resulting in eventual lysis of the infected host cell and release of IBDV virions (233). VP4 also has a role in viral host evasion, through suppressing type I interferon (IFN) response by interacting with host glucocorticoid-induced leucine zipper, which is an important protein in immunosuppressive and anti-inflammatory functions (7, 119).

VP5 is a highly basic, cysteine-rich, non-structural protein involved in viral pathogenesis (253, 254). Immunofluorescence (IF) analyses indicate that VP5 functions as a class II transmembrane protein in affected cells (128), and likely acts as a death protein, causing altered host cell membrane permeability and eventual cell lysis (2, 128, 163, 216, 227). An experimental IBDV mutant with VP5 knockout had reduced apoptotic effect in cell culture, demonstrating the protein's role in apoptosis (253, 254). VP5-mediated apoptosis results in the release of viral progeny from host cells, thus illustrating the importance of VP5 in IBDV pathogenesis and dissemination (254, 128). Although VP5 causes apoptosis during the later stages of viral infection, a VP5 knockout mutant demonstrated inhibition of apoptosis during early infection (126).

## **1.6 Antigenic Variation**

Similar to other RNA viruses, the IBDV genome is prone to mutations, which may affect the antigenic profile of the virus. RNA virus polymerase lacks the 3' exonuclease proofreading activity, and therefore there is a greater likelihood for errors in the transcription of the genome of RNA viruses compared to DNA genomes (183, 206). Vaccine-escape antigenic variant mutants may develop and proliferate in the field, leading to virus strains that are not effectively neutralized by vaccine virus antibodies (54, 55, 56). Previous IBDV mutations have impacted antigenicity and virulence leading to a variety of IBDV subtypes and serotypes (22, 26, 41, 66, 82, 99,

117, 195, 220, 228, 258). Due to the capacity of IBDV to generate escape mutant antigenic variants with altered pathogenicity, the performance of routine bursal surveys is necessary to quickly identify new antigenic variants and pathotypes in the field (20).

The structural protein VP2 is the major host immunogen due to the location of the only neutralizing antibody epitope on this protein. VP2 is the prominent area of focus for research regarding IBDV antigenicity (9, 13, 12, 67, 170, 223, 225). Studies modifying the VP2 P domain (aa 206-350) have demonstrated that the region represents a neutralizing, conformation-dependent antigenic domain (9, 61). This location is referred to as the VP2 hypervariable region or variable domain, since most changes between IBDV strains are grouped at this region (12, 61). The VP2 hypervariable region is composed of a hydrophobic area, flanked by the two hydrophilic peaks, A (aa212-224) and B (aa314-325), which constitute loops P<sub>BC</sub> and P<sub>HI</sub> respectively, as well as two smaller hydrophilic regions that make up loops P<sub>DE</sub> (aa253) and P<sub>FG</sub> (aa284) (9, 12, 82, 117). Variations in antigenicity have been associated with mutations located within the hydrophilic regions of the hypervariable region (12, 223).

Changes in antigenicity associated with single nucleotide polymorphisms (SNPs) in the hypervariable region nucleotide sequence have been previously characterized. Point mutations in the first codon location of nucleotides sequences of amino acids 222 and 254 of the hypervariable region are both consistently identified in variant IBDVs and have been associated with antigenic drift (98).

The emergence of antigenic variants in the United States in the mid-1980s led to investigation of antigenic variation of IBDV in the field. Neutralizing monoclonal antibodies (MCAs) R63 and B69 were used on antigen-capture enzyme immunoassays to compare classical or standard type IBDV to antigenic variants, such as Delaware Variant E. While the R63 MCA antigenic neutralization site was present on both classical and variant strains, the B69 MCA antigenic neutralization site was only identified on classical type IBDV strains, providing evidence for the shift in antigenicity between classical and variant types of IBDV (201). In addition to MCA testing, virus neutralization assays and *in vivo* cross-protectivity trials have been utilized to distinguish between antigenic subtypes of IBDV and determine levels of cross-reactivity between isolates (20, 94, 185, 186).

The vvIBDV antigenic region is similar to that of classical type virus; the SNP amino acid changes between classical and vvIBDV are found throughout all viral proteins, although the majority of these SNPs are located in the VP2 hypervariable region (18, 27, 174). Although vvIBDV strains appear similar to classical strains, vvIBDV shows greatly increased pathogenicity compared to classical type virus (24, 60, 78, 223, 224, 226). The antigenic region has been highly conserved among vvIBDV from different regions (60, 129, 224, 225, 226), however VP2 hypervariable region analysis of multiple vvIBDV isolates from China has demonstrated differences between hypervariable regions, suggesting that vvIBDV may be evolving (120).

Australian serotype 1 IBDV strains resemble U.S.A. classical and variant type virus, with no vvIBDV presence at this time. The Australian type IBDV virus is genetically distinct from viruses found in other continents (193).

In addition to antigenic subtypes within serotype 1, there are differences between the antigenic regions serotype 1 and serotype 2 IBDV (147). Serotype 2 isolates, which were isolated from turkeys affected with sub-acute IBD, demonstrated antigenic homogeneity (146, 147, 149). Neutralization testing demonstrated low levels of cross reactivity (cross-protectivity, cross-neutralization) between serotype 1 and serotype 2, indicating significant antigenic variability between serotypes (146).

## **1.7 Viral Replication and Pathogenesis**

Virus entry is a multi-dimensional process involving attachment, signaling, endocytosis, penetration into the host cell, and uncoating of the viral genome (252). Viral receptors are important in determining cell tropism. IBDV attachment to host receptors is still being researched, although it has been determined that the receptor is an N-glycosylated protein associated with IgM+ B-cells (140, 168). Attachment has shown to involve signaling between host chicken heat shock protein 90 and  $\alpha 4\beta 1$  integrin with the IBDV VP2  $\alpha 4\beta 1$  integrin binding motif (45, 76, 123). IBDV internalization was analyzed using immunofluorescence and Western blot VP3 detection (12 h. PI) and VP2 localization (1 h. PI) (76). IBDV internalization was compared to well documented ligands to determine the method by which host cells

take up the virus. It was determined that IBDV endocytosis is primarily accomplished through macropinocytosis (76). In addition, evidence has been presented that Rab5-a regulatory GTPase involved with endosomal sorting actin, and caveolin/lipid rafts in the cellular membrane are important for viral entry (76, 245). The method by which viral proteins interact with cellular machinery has been demonstrated through mutagenesis experiments showing that the VP3 domain PATCH 2 (P2) then interacts with host cell endosomal membranes, affecting virus replication levels (77). Many elements of IBDV replication mechanism and function have yet to be understood (77).

IBDV is transmitted through the fecal-oral route, with virus particles orally ingested by the host (76, 150). Classical type serotype 1 virus disseminates rapidly through the host. At four hours PI, virus is detectable through immunofluorescence in the digestive tract, primarily in macrophages in the blind ends of the cecum. By six hours PI, first transient viremia has been initiated, and virus has spread throughout the gut-associated lymphatic tissue (GALT) and replicates in macrophage and lymphoid cells. At this timepoint, virus is also present in liver macrophages (Kupffer cells). At eleven hours PI, virus is found in tubular epithelial cells in the kidneys. In regard to viral replication in the target organ, the bursa of Fabricius, virus initially becomes measurable approximately six hours PI. Bursal presence is irregular from six to nine hours PI, at which point the virus begins to rapidly spread throughout the organ and can be found in large amounts after eleven hours at which point a second replication cycle has occurred (141, 214, 109, 153). At sixteen hours PI, a second stage of viremia begins, where B-lymphocytes in multiple lymphoid organs including the spleen,

thymus, Harderian gland, and cecal tonsils are targeted for viral replication (83, 109, 196, 205).

The bursa of Fabricius is the primary target organ for IBDV replication, as demonstrated by inoculation of bursectomized chickens. Bursectomized birds present no clinical signs and upon necropsy only the spleen is infected with moderate hyperplasia (106). This phenomenon is associated with the determination of the primary target cell for viral infection as the B-lymphocyte, even though IBDV can also replicate in macrophages (106, 153). IBDV infects and causes apoptosis of dividing IgM+ B lymphocytes located in the bursa (17, 52, 86, 136, 143, 257). Classical IBDV causes the most severe clinical signs in birds between three and six weeks of age, which may be partially attributed to IgM+ B-lymphocyte quantities; from zero to two weeks of age quantities are very low, although IgM+ B-lymphocyte levels rise rapidly between three and six weeks, before decreasing again at eight weeks of age (257).

## **1.8 Immunological Impact**

IBDV infection occurring in young birds (<7 days of age) can cause long-lasting, severe immunosuppression in recovered birds. Immunosuppression is related to decreased humoral immunity associated with suppression of antibody production, as well as decreased mucosal immunity due to Harderian gland infection (219).

Significant economic impact of IBDV in the United States results from secondary

infections and poor vaccine response in immunosuppressed birds (64, 84, 172, 188, 189, 246, 255).

The bursa of Fabricius is a gut-associated primary lymphoid organ, which acts as the site of B-cell differentiation in avian species. The structure of the bursa is follicular; a mature bursa contains  $\sim 10^5$  follicles, each with  $10^4$  replicating lymphocytes, which develop a diverse immunoglobulin repertoire through gene conversion (177, 180). Early in embryonic development, bursal cells divide and form follicles for B-lymphocyte proliferation. At approximately day eighteen of embryonic development, B-cells begin emigrating from the bursa to peripheral lymphoid tissue; this process continues, expanding the pool of peripheral B-cells, until the bird reaches maturity at approximately six weeks of age. IBDV infects and kills B-cells in the bursa, preventing proliferation and emigration. Therefore, IBDV infection of young birds results in the highest degree of immunosuppression (194, 243).

IBDV infection causes severe depletion of B-lymphocytes due to virus induced apoptosis. Although birds do show partial recovery of lymphoid B-cells post infection, chicken humoral immune response remains severely suppressed (5, 29, 68, 110, 229, 242, 243). The severe bursal atrophy associated with IBD causes functional damage resultant from structural damage to the organ. The bursas of recovered birds contain large, reconstituted follicles and small follicles with altered functionality. The extent of immunosuppression is believed to be linked to the extent of small, non-functional follicles in the recovered bird (5).

During the acute stage of infection CD4+, CD8+ and KUL01+ cells are present in the bursa, signifying the influx of T cells and macrophages as the host immune response to IBDV infection (178, 243). The cell-mediated CD4+ and CD8+ T-cell response is important for controlling viral replication in the first five days post-infection, possibly through optimization of macrophage and B-cell activities (127, 178). Although T-cells are important in virus clearance, uninfected bystander cells are also killed possibly through the release of cytokines and cytotoxic effect, thus causing further damage to the bursa (71, 178, 199). KUL01+ macrophage response is involved in clearance of remnants of lysed cells that had been involved in viral replication (161, 236). After five days, antibody-dependent cytotoxicity is the primary method of viral clearance (6, 47, 178).

## **1.9 Transmission**

IBD is highly transmissible and can be spread between infected susceptible flocks. The primary method of transmission is the fecal-oral route (14, 256). Infected birds start shedding virus in feces five days post-infection (PI) and continue to shed until twelve days PI (256).

IBDV is very persistent in the environment, even after cleaning and disinfection (14, 112); viable virus was found to persist in the environment, including feed and litter, for up to 122 days after removal of infected chickens (14). Therefore, it

is common for IBDV to be present in successive flocks placed within the same house (40) and virus can be spread between houses through fomites contamination (70, 209).

Virus has been isolated from adult lesser mealworms (darkling beetles), which are prevalent in poultry houses, up to fourteen days post-consumption of IBD inoculated chicken feed (145). In addition, susceptible chickens fed homogenates of adult lesser mealworms from IBD become infected with IBDV (200). It is thus believed that the lesser mealworms can serve as a viral reservoir of IBDV (145, 200).

Chickens are most susceptible to infection during three to six weeks of age, during maximum bursal development (62, 84, 103). Birds younger than three weeks of age should be protected by maternal antibody (75), while mature birds older than six weeks of age are resistant to IBDV infection (89, 103, 118).

### **1.10 Clinical Signs**

Birds affected with serotype 1 classical type IBDV in the field present with head and body tremors approximately two days before outbreaks become fully evident. Early signs also include diarrhea, vent picking, and soiled vent feathers (38, 40, 240, 241). Birds in the early stages of disease also show slightly elevated body temperatures, which drop significantly before mortality. Peak mortality occurs between four and six days PI. Experimentally infected birds show signs of acute illness between one and four days PI (incubation period). Affected birds appear depressed, anorexic, and ruffled with thick, yellowish, and mucoid feces during the

early acute phase of infection (38, 40). Feces appear whitish and mucoid by the third day PI. Temperatures are slightly elevated at two days PI and sub-normal at five days PI. Birds typically recover between six and ten days PI (38). In the field, mortality ranges from 1-50% depending on breed of bird, virulence of strain, and age of infection (38, 156). Variant strains of IBDV typically cause subclinical infection. However, variant IBD may cause huddling and mild diarrhea. In addition, long-lasting immunosuppression and bursal lesions are still induced by sub-clinical disease, with associated secondary diseases causing economic losses (57, 90).

vvIBDV results in a severe form of infection, causing 90-100% mortality in SPF birds, and variable high mortality rates in the field (101, 225). Clinical signs associated with vvIBDV include depression, reluctance to move, prostration, and diarrhea. In one experimental trial, 50% mortality was seen in SPF birds within three - days of infection (101). The range of age-susceptibility is increased in vvIBDV compared to standard or variant type strains, covering the entire grow out period for broilers (34, 165, 217, 222).

### **1.11 Gross Lesions**

Serotype 1 IBD causes gross lesions and clinical signs in chickens. Serotype 2 IBD does not cause clinical disease in chickens. Only a limited number of serotype 2 strains cause slight lymphocyte depletion in turkeys, without any accompanying gross lesions in affected birds (1, 39, 95, 102).

Upon necropsy, birds that are affected with acute phase standard type IBD present with hypertrophied bursas, skeletal muscle hemorrhaging, kidney urates, and enlarged or atrophied spleens (38, 58). Bursas begin to hypertrophy approximately 36 hours PI and are often yellow and striated. Occasionally, bursas will become hemorrhagic (38, 240). After 96 hours, the bursa begins to atrophy, reaching a maximum atrophied state at 120 hours PI and thereafter (38). The primary gross difference between classical and variant type IBDV regards the early impact of virus on the bursa. Variant strains cause early bursal atrophy without the hypertrophic stage characterizing standard type virus (57).

vvIBDV causes a more severe degree of lesions seen in classical type infection, and the exact cause of the more acute disease and higher rates of mortality seen in vvIBDV infection is unknown. In most cases of vvIBDV mortality, the bursa is turgid, edematous, and sometimes hemorrhagic (92). Swollen, nephrotic kidneys and atrophied thymus are also common in vvIBDV (63, 92, 190, 197).

### **1.12 Microscopic Lesions**

Most microscopic lesions caused by IBDV are found in the bursa of Fabricius, although lesions are also visible in other lymphoid structures (132). Lymphocyte degeneration and necrosis are observed in the bursal medulla during the early stages of infection (1 day post-infection), which spread until the entire bursa is affected at 3-4 days post-infection (95). During infection with standard type IBDV, a significant

inflammatory response occurs in the bursa. High numbers of heterophils are evident in the bursa, causing bursal swelling, edema, and increased bursal weight. In addition to heterophils, pyknotic debris and hyperplastic reticuloendothelial cells are also detected in infected bursas. Apoptosis of infected cells due to virus replication, and cytotoxicity from the host immune response triggers phagocytosis by heterophils and plasma cells. Apoptosis and subsequent phagocytosis results in formation of cystic cavities in the bursal medulla. Infection with variant IBDV causes extensive lesions in the bursa, but lacks inflammatory response (132, 196). Fibroplasia occurs during bursal atrophy, and proliferative bursal epithelium forms glandular structures containing globules of mucin (41, 95, 132, 162, 243). Scattered foci of recovering lymphocytes appear after the acute phase of infection. Post-infection, large, reconstituted follicles with the ability to produce lymphocytes, and small, non-functional, follicles lacking a cortico-medullar boundary are present in the bursa (243).

In addition to the bursa, other lymphoid organs are also affected by IBD. During infection, microscopic lesions are visible in the spleen in the form of lymphoid necrosis in the germinal centers and periarteriolar sheath. Macrophages are present in the spleen to phagocytize remnants of necrotic cells. Lymphoid recovery of the spleen begins at nine days post-infection. Lymphoid necrosis and hyperplasia are visible in the reticular and epithelial aspects of the medulla of the thymus at four days post-infection. Scattered foci of necrosis were observed in the cortex of the thymus. Cortical thymus lymphocytes recover by twelve days post-infection (41). Lymphoid cells in germinal centers of the cecal tonsils are seen in combination with increased

macrophage and heterophil presence (213). IBD infection causes a marked depletion of plasma cells in the Harderian gland, although recovery occurs within fourteen days of exposure (53, 208).

### **1.13 Prevention and Control**

IBDV is ubiquitous and persistent in the environment, with a wide range of pH and disinfectant resistance, therefore proper biosecurity is an important preventative measure in the field (3, 4, 15, 30, 38, 81, 142). The virus does not show loss of efficacy at a pH range of 2 to 10 (176). IBDV is heat-resistant and loses less than half a log at 56 C° for three hours (15). In bursal homogenate supernatant, 1 log<sub>10</sub> is lost in 18.8 minutes at 70 C, 11.4 minutes at 75 C and 3.0 minutes at 80 C (3). IBDV becomes unstable above 75 C (176).

Maternally-derived antibodies (MDAs) against IBDV neutralize live vaccine virus, therefore non-SPF chicks cannot be effectively protected by administration of live vaccine while MDAs are still circulating in the chicks. Due to this complication in protecting young chicks through active immunization processes, protection against IBDV infection is afforded through hyperimmunization of breeder flocks with an inactivated vaccine, which results in vertical passage of MDAs (43, 247, 222, 269). Passive immunity is transferred from hen to chicken through immunoglobulins in the yolk sac, which is subsequently absorbed by the chick (43). The half-life of IBD

antibody is three to eight days (65, 75, 244, 247), and it persists in chicks until approximately twenty days-of-age.

Immunization of breeder flocks and resultant passive immunity is the primary control method utilized for IBDV. Maternal antibodies usually protect birds for up to three weeks post-hatch. Early protection is vital due to severe immunosuppression caused by infection of young birds. Breeder flocks are initially vaccinated with live vaccine (132) and are subsequently primed with oil emulsion inactivated vaccine to bolster antibody response in breeder birds (132, 148, 196, 231, 248). Due to variation in maternally-derived antibody titers in broiler chicks, many vaccination programs include administration of vaccine to broilers (41).

Both live attenuated and inactivated vaccine are utilized in the control of IBD outbreaks. “Mild” vaccines, which are highly attenuated, are not effective when maternal antibody is present or against vvIBDV strains. Vaccines which are “intermediate” (moderate attenuation) or “hot” (low attenuation) are more effective in the presence of maternal antibody but cause bursal lesions. Since live vaccines allow viral replication in host cells, bursal atrophy and accompanying immunosuppression may result from immunization with minimally attenuated live vaccines (144, 157, 111, 179, 218).

Inactivated vaccines use non-replicating killed whole virus, viral subunits, or recombinant viral antigens in combination with adjuvants to induce the host immune response. Repeated administrations are often required for effective immunogenicity,

therefore inactivated vaccines are used primarily in breeder flocks to produce enhanced antibody response for passive immunity in offspring (157).

Vaccines including variant IBDVs such as Delaware Variant E are protective against both classic and variant IBDV and are therefore found in all vaccination programs in the United States. Standard type live vaccines show low efficacy against infection with antigenic variants, although they afford protection against vvIBDV strains (157, 179). Autogenous inactivated vaccines with optimized antigenic content are utilized to protect against emerging antigenic variant viruses (154, 157, 187).

#### **1.14 Diagnosis**

Clinical signs in the field are the first indications of an IBD outbreak. In addition, characteristic gross and microscopic lesions observed in disease mortality or euthanized birds can be used for preliminary diagnosis (191). Bursal lesions are the primary lesions observed for differential diagnosis. Marek's disease does affect the bursa, but is accompanied with distinctive, characteristic tumors not found in IBD (106, 182, 191). Some non-infectious causes of disease, such as mycotoxins, aflatoxins, steroidal anti-inflammatory drugs, poor nutrition, and stress can affect the bursa, therefore laboratory testing is necessary to confirm IBDV infection (69, 180, 191, 210). Histopathological analysis of the bursa of Fabricius can give a definitive diagnosis of IBD. Histopathological lesions include lymphocyte necrosis, lymphocytic follicular depletion, and bursal atrophy (100, 102).

IBDV can be isolated using bursal tissue homogenates in SPF eggs. Most field isolates cannot be adapted to grow in primary cell culture. Pathogenic IBDVs are rapidly attenuated when passed in cell culture. This phenomenon is accompanied by an amino acid change in the hypervariable region of VP2 at position 253. Cell culture adapted strains can grow and demonstrate cytopathic effects in avian and mammalian continuous cell lines. Observable cytopathic effects include small, round, refractive cells (42, 97, 107, 114, 130, 147, 173, 191). Virus isolation in chicken embryos can be performed through inoculating ten-day old SPF eggs via the chorioallantoic membrane (CAM) route (191, 210). Embryo lesions can differentiate between classical and variant strains. Characteristic lesions seen in standard type IBDV-affected embryos include stunting, hemorrhagic embryos, yellowish “parboil” livers, and pale spleens. Variant type virus induces cream colored, edematous embryos with liver necrosis, splenomegaly, and bile stasis (184). vvIBDV causes high mortality in embryos (189, 212).

The agar gel immunodiffusion (AGID) serological test can be utilized to detect antibodies in serum, and virus or antibody in bursal tissue. Minced bursal tissue is placed in wells of an AGID plate against known positive IBD serum. Clear precipitin lines form between positive samples and sera. AGID is simple but less sensitive than other testing procedures (166, 191). Agar gel precipitation testing (AGPT) is another insensitive method of detecting IBDV antibody, using known antigen and test serum in wells (191, 235). Another variety of serological test is the enzyme linked immunosorbent assay (ELISA) test. ELISA testing is utilized to quantify antibody titer

and can therefore be used to determine vaccination status of breeder flocks (134, 191). ELISA tests are very sensitive, but cannot differentiate between strains of virus, and therefore it cannot be used to determine if positive results are caused by pathogenic virus or vaccine induced antibody (48, 88, 124, 152, 191, 207).

The molecular technique of reverse transcriptase PCR (RT-PCR) can also be used in diagnosis of IBD through detection of the viral genome. RT-PCR can detect viable or non-viable virus in tissue sample homogenates and cell culture (122, 167, 191, 225). Primers for conserved regions of the viral genome are used to determine target areas for amplification (114).

## **Chapter 2**

### **ANTIGENIC CHARACTERIZATION OF IBDV FIELD ISOLATES**

#### **2.1 Introduction**

Routine bursal surveys have identified commercial broiler flocks infected with IBDV at ages when they should be protected by maternally-derived antibodies. The goal of this study is to serologically characterize contemporary IBDV field isolates harvested from flocks presenting histological signs of disease in birds younger than twenty-four days-of-age. Preliminary virus neutralizations were performed running field isolates against Delaware Variant E antiserum, demonstrating showed poor neutralization of field isolates. Field IBDVs will isolated and evaluated using cross-neutralization assays to evaluate cross-protective potential. This may lead to identification of vaccine candidates.

#### **2.2 Materials and Methods**

##### **2.2.1 Selection of Field IBDVs**

Field virus was selected from commercial flocks using a variety of criteria. Bursas were collected from commercial broiler flocks through routine survey, and

histopathological evaluation of bursas identified flocks affected with IBD before 24 days of age. Bursal homogenates from affected flocks were tested via preliminary virus neutralization assays using Delaware Variant E antiserum. Field IBDVs from flocks affected with IBDV at younger than twenty-four days of age, which were moderately to poorly neutralized by DE Variant E antiserum, were considered for selection for further evaluation.

Additional criteria considered for the selection of isolates included PCR testing of field IBDVs with IBDV A and IBDV B primer sets, which bind to different regions of VP2 (Table 2.2). Some contemporary field isolates were positive for one primer set but not the other, which is indicative of mutations in these regions (Table 2.3). VP2 hypervariable region sequencing was performed by Dr. Daral Jackwood (The Ohio State University) to identify different IBDV “genotypes”.

Field isolates were selected from different companies and geographic locations across the United States (Table 2.1).

### **2.2.2 Preparation of IBDV Seed Stocks**

Five-week-old specific pathogen free (SPF) leghorn chickens obtained from SPAFAS Charles River Laboratories were separated into groups of eight birds each. Birds were housed and raised in accordance with University of Delaware Institutional Animal Care and Use guidelines under an approved Animal Use Protocol (IACUC #56R-2018-2). All groups were inoculated intraocularly and intranasally with 0.2 mL

of diluted bursal homogenates obtained from select commercial flocks affected with IBDV at younger than twenty-four days-of-age. Bursal tissues were processed by using a 20% weight to volume ratio (w/v) in an equal mix of brain heart infusion and antibiotic/antimycotic (10,000 penicillin and 10,000 bistreptomycin sulfate per ml). One control group was sham inoculated with sterile media. After inoculation, each group of eight birds were kept in modified Horsfall isolation units; feed and water were offered *ad libitum*.

Four days post-inoculation (PI), four birds were removed from each group and euthanized via cervical dislocation. Birds were then necropsied, and visible lesions recorded. Bursas were aseptically harvested with autoclaved scissors and forceps into sterile twelve-well plates. Bursas were then weighed and held on ice prior to homogenization. In a laminar flow hood, bursas were combined with 20% w/v with equal mix of brain heart infusion media and antibiotic/antimycotic (10,000 penicillin and 10,000 bistreptomycin sulfate per ml) per gram of tissue and manually homogenized. Homogenized bursas were frozen and thawed three cycles to release virus before being centrifuged at 500 xg for 10 minutes. Supernatants were aliquoted into sterile cryovials and stored at -80 C.

Prior to use in testing, virus stocks were tested for adventitious agents. Virus stock purity was confirmed via RT-PCR (Qiagen OneStep) with reovirus, astrovirus, and adenovirus specific primers (Appendix A). Virus stocks were inoculated into primary chicken embryo fibroblast cell culture, and cells were evaluated for cytopathic effects to confirm purity from reovirus presence. Sera was tested for purity through an

enzyme-linked immunosorbent assay (ELISA) test with IDEXX Avian Reovirus Ab test kit (serial #99-09264).

### **2.2.3 Isolation of Viral RNA and Polymerase Chain Reaction Testing**

Virus stocks composed of 20% bursal homogenate in brain-heart infusion and antibiotic antimycotic (10,000 penicillin and 10,000 bistreptomycin sulfate per ml) were filtered before RNA isolation was performed. TRI Reagent-LS (Molecular Research Center) was used according to product specification. Bromochloropropane (BCP) was used as a substitute for chloroform. Isopropanol was added to aqueous RNA to precipitate RNA prior to resuspension in molecular grade water. RNA concentration and quantity were verified using a Nanodrop spectrophotometer. IBDV presence was confirmed via RT-PCR with IBDV A and B primer sets (Table 2.2). This procedure was performed following the manufacturer's guidelines in the Qiagen One Step RT-PCR kit Handbook. Primer sets for adenovirus, astrovirus, and reovirus were also used to test for purity of virus stocks. The amplified products were tested by gel electrophoresis.

### **2.2.4 Preparation of Antiserum**

Groups of SPF leghorns, with four birds in each group, were removed from modified Horsfall isolation units two weeks post-inoculation with bursal homogenate from commercial flocks affected with IBDV. A control group of four birds, which had

been mock-inoculated with equal mix of BHI media and antibiotic/antimycotic (10,000 penicillin and 10,000 bistreptomycin sulfate per ml), were also removed from isolators. Ten mL of whole blood were collected from each bird via cardiac puncture. After blood was collected, birds were euthanized by cervical dislocation. Birds were then necropsied, and visible lesions recorded.

Blood was allowed to clot, serum was collected aseptically, heat inactivated at 56 C for 30 minutes. Individual sera were tested for IBDV antibody titer of each bird through an enzyme-linked immunosorbent assay (ELISA) test with IDEXX IBD Ab test kit (Serial #99-09260). Samples without adequate antibody titer were removed. Individual sera samples from birds from the same treatment group were pooled, mixed, and aliquoted into sterile 2 mL cryovials for storage at -80 C.

### **2.2.5 Inoculation of Embryonated SPF Chicken Eggs**

At ten days of incubation, embryonated specific-pathogen-free (SPF) chicken eggs were disinfected with 70% alcohol. A small hole was made in the top of the egg in the center of the air sac. Each egg was then turned on its side, and a small hole was punched in the side of the egg approximately halfway from the top, avoiding any major blood vessels. A rubber pipet bulb was depressed, and the suction tip was held over the hole at the top of the egg. The pipet bulb was then slowly released to create a vacuum to pull the membrane away from the shell under the hole on the side. Eggs

were then placed in an egg flat on their sides, with the side hole facing up. Eggs were inoculated with 0.1 mL inoculum using a tuberculin syringe with a 26-gauge needle.

### **2.2.6 Titration of Virus Stocks**

Each virus stock was serially diluted  $10^{-1}$  to  $10^{-6}$  in BHI media and antibiotic/antimycotic media in a laminar flow hood. Ten-day-old embryonated SPF chicken eggs were placed into six groups of four eggs per virus stock. Four eggs were inoculated with each dilution ( $10^{-1}$  to  $10^{-6}$ ). 0.1 mL of diluted virus stock was inoculated into each egg via the CAM route described earlier.

Eggs were incubated and candled daily to check for embryo mortality; any mortality was recorded, and dead eggs were removed. First day mortality was considered nonspecific and not used in subsequent analysis. Six days post inoculation, all eggs were removed from incubators and embryos were euthanized via refrigeration at 4 C overnight. Eggs were then opened with forceps and embryos were evaluated for gross lesions.

### **2.2.7 Virus Neutralization Assays**

Each virus stock was serially diluted  $10^{-1}$  to  $10^{-3}$  in BHI media and antibiotic/antimycotic media in a laminar flow hood. Antiserum was diluted 1:5 with media, other than Delaware Variant E, which was diluted 1:8 with media. Diluted antiserum was combined in a 1:1 ratio with  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  dilutions of each virus

stock. Combined antiserum and virus stock were mixed and incubated at 37°C for 45 minutes.

Ten-day-old embryonated SPF chicken eggs were placed into three groups of five eggs per dilution. Five eggs were inoculated for each dilution using the CAM route. Eggs were incubated and candled daily to check for embryo mortality; any mortality was recorded, and dead eggs were removed. First day mortality was considered nonspecific and not used in subsequent analysis. Six days post-inoculation, all eggs were removed from incubators and embryos were euthanized via refrigeration at 4 C overnight. Eggs were then opened with forceps and embryos were evaluated for gross lesions.

### **2.2.8 Quantitative Analysis of Titrations of Virus Neutralizations**

The Reed and Muench method (210) was utilized for quantitative analysis to determine the 50% embryo infectious dose (EID<sub>50</sub>) and the 50% embryo lethal dose (ELD<sub>50</sub>). Virus titers were calculated using EID<sub>50</sub>. Virus neutralization indices were determined through subtracting a virus neutralization assay titer obtained by evaluating embryos from eggs inoculated with combined antiserum-virus stock from the evaluated IBDV titer. A neutralization index of  $\geq 3.50$  was considered neutralizing.

## **2.3 Results**

### **2.3.1 Clinical Presentation and Gross Lesions of IBDV Infected Chickens**

Virus stocks of field IBDVs and conventional strains were produced in specific-pathogen-free leghorns. Birds inoculated with STC appeared depressed and ruffled within the first seven days PI. Most birds were asymptomatic, indicating that current commercial field isolates induce subclinical disease, resembling DE Variant E rather than classical (STC) type infection.

Most gross lesions were observed in the spleen and bursa. The majority of field isolates induced splenomegaly and atrophied bursas at four days post-infection (Figure 2.1). Typical presentation of bursas infected with Delaware Variant E IBDV is atrophy, while classical infection causes bursas to become enlarged, striated, straw-colored, and edematous. Most of the bursas harvested from birds inoculated with field IBDV isolates presented as atrophied, although some were also straw-colored or striated (Table 2.4).

### **2.3.2 PCR Primer Sets IBDV A and B**

IBDV field isolates were tested using PCR with primers IBDV-A and IBDV-B, which anneal to different locations on VP2 (Table 2.2). Part of primer IBDV-A overlaps with the hypervariable region of VP2. While many field isolates tested positive for both primers, some isolates tested positive for IBDV-A, but not IBDV-B or vice versa (Table 2.3).

### **2.3.3 Embryo Lesions Observed in Titrations and Virus Neutralization Assays**

Conventional IBDV isolates DE Variant E and STC induce distinct lesions when inoculated in SPF embryos via the CAM route. Inoculation with variant IBDV DE Variant E results in cream-colored embryos with bile stasis, liver necrosis, and splenomegaly. Inoculation with classical IBDV STC results in hemorrhagic embryos with parboiled liver, pale spleen, and spleen necrosis.

Most contemporary IBDVs isolated from commercial flocks caused a combination of classic and variant-type lesions in embryos (Figure 2.2). Field isolates caused mixed presentations of classic and variant lesions in embryos, in some cases a combination of classic and variant type lesions were observed in the same embryo.

#### **2.3.4 Titrations of Virus Stocks**

Virus stocks were titrated in embryonated SPF chicken eggs. EID<sub>50</sub> (50% Embryo Infectious Dose) and ELD<sub>50</sub> (50% Embryo Lethal Dose) were calculated using the Reed and Muench method. The amount of virus per mL of bursal homogenate supernatant for EID<sub>50</sub> was established as viral titer.

Thirty-eight isolates were titrated (Table 2.5), and four of these isolates (STC, AVS-DX, AVS-PP, and AVS-DY) had a small difference between ELD<sub>50</sub> and EID<sub>50</sub> (<1.0 logs/mL). All other isolates showed greater difference between ELD<sub>50</sub> and EID<sub>50</sub>.

Most titers ranged from 5.5 logs/mL to 7.0 logs/mL. The only isolates with lower titers were AVS-JS (5.3 logs/mL) and AVS-PA (4.2 logs/mL)- neither of these

isolates were utilized for virus neutralizations. AVS-TV was the only isolate with a titer greater than 7.0 EID<sub>50</sub>.

### **2.3.5 Performance of Virus Neutralization Assays**

Virus neutralization assays were performed to evaluate antigenicity of isolates. Antiserum elicited by historically significant IBDVs Delaware Variant E, AL-2, as well as recent field isolates AVS-VN, AVS-PP, AVS-RA, AVS-GR, AVS-DY, AVS-BM, AVS-JP, AVS-TY, AVS-SCH, AVS-RRV, AVS-TV, and AVS-MB were tested against ten challenge IBDVs (Table 2.6) to analyze cross-protective potential of antisera.

Virus neutralization indices were calculated using the Reed and Muench method. A neutralization index of greater than 3.50 was considered effective neutralization. Isolates AVS-MB and AVS-TV were highly cross-protective, and neutralized 10/10 and 9/10 challenge viruses respectively. Isolates AVS-RRV and AVS-SCH did not effectively neutralize any evaluated viruses. Homologous virus neutralizations showed that the antiserum created against AVS-SCH and AVS-RRV did neutralize homologous virus stocks (Table 2.6).

Other contemporary isolates neutralized between 2/10 and 8/10 challenge isolates. The inconsistent level of neutralization produced by field isolates indicates that these isolates differ not only from previously characterized isolates but also from each other. Delaware Variant E only neutralized previously characterized isolate AL-

2, and was not effective against contemporary isolates, confirming preliminary test results that demonstrated antigenic variance of field IBDVs.

Since AVS-MB demonstrated the ability to elicit highly cross-protective antibodies, it was further evaluated against poorly cross-protective isolates AVS-SCH and AVS-RRV (Table 2.7). AVS-RRV and AVS-SCH were also tested against each other. AVS-MB was less effective in neutralizing Red River Valley and AVS-SCH than it had been against all other challenge viruses, with neutralization indices of 2.91 and 3.33 respectively. AVS-RRV and AVS-SCH antiserum presented further poor neutralization capability. AVS-RRV had neutralization indices of 2.15 against AVS-SCH, and 2.50 against AVS-MB. AVS-SCH had neutralization indices of 2.50 against AVS-RRV and 1.50 against AVS-MB.

## **2.4 Discussion**

Preliminary testing of field isolates included evaluating neutralization potential of Delaware Variant E antisera on field IBDVs to confirm antigenic variation. Since recent field isolates are escaping vaccine protection, there have been outbreaks in young commercial broiler flocks, which may have severe immunosuppressive impact. Therefore, it is necessary to find a new virus candidate for use in widespread industry vaccines to protect against the newly arising antigenically distinct field IBDVs. Virus neutralization assays were utilized to evaluate cross-protective potential of antibodies

elicited against IBDV field isolates from flocks showing histologic signs of infection younger than twenty-four days-of-age.

All virus stocks were titrated in SPF embryonated eggs and EID<sub>50</sub> (50% embryo infectious dose) and ELD<sub>50</sub> (50% embryo lethal dose) were calculated. Classic IBDVs are highly embryo lethal due to increased inflammatory response, while variant IBDVs typically cause lower levels of mortality. As such, there is typically a smaller difference between EID<sub>50</sub> and ELD<sub>50</sub> for classic type IBDVs (<1.0 logs/mL) and larger difference for DE Variant E-like viruses. Thirty-eight isolates were titrated, four of these isolates (STC, AVS-DX, AVS-PP, and AVS-DY) had a small difference between ELD<sub>50</sub> and EID<sub>50</sub> (<1.0 logs/mL). STC, AVS-PP, and AVS-DY caused classic-like lesions in embryos. AVS-DX induced classic and variant-like lesions. The only other isolate causing STC-like lesions without variant-like lesions in embryos was AVS-GN. This supports the supposition that most of the contemporary field isolates more closely resemble variant-type than classic-type IBDVs.

Cross neutralizations were performed to evaluate cross-neutralizing potential of field IBDV antisera against a number of IBDV field isolates. DE Variant E antisera effectively neutralized historically significant isolates STC and AL-2, supporting previous research (185, 186, 220). However, DE Variant E was not effective at neutralizing most of the contemporary field IBDVs evaluated here, thus confirming preliminary testing regarding antigenic variation.

Isolate AVS-RRV did not effectively neutralize any challenge virus. However, this is possibly related to the different background of AVS-RRV. Unlike all other

isolates used for testing, AVS-RRV was collected from a layer flock. One evaluated field isolate, AVS-MB, was moderately protective against AVS-RRV (Neutralization Index=3.33) (Table 2.7). This one-way neutralization mirrors the relationship between STC and DE Variant E. DE Variant E is protective against STC, however STC does not effectively neutralize DE Variant E (185, 186, 220).

The field isolate AVS-SCH antiserum also did not effectively protect against any of the ten challenge viruses it was run against. AVS-SCH was chosen for testing after evaluation of the VP2 hypervariable region sequencing performed by Dr. Daral Jackwood. The AVS-SCH VP2 hypervariable region had unique amino acid changes that will be discussed later.

Isolates AVS-MB and AVS-TV elicit highly cross-neutralizing antibodies. AVS-MB protected against ten out of twelve challenge viruses with a neutralization index of 3.50 or greater. The only isolates that AVS-MB was not highly protective against were AVS-SCH and AVS-RRV, both of which appear to be antigenically distinct from other isolates tested. However, AVS-MB was still moderately protective, with a 2.91 neutralization index when run against AVS-SCH and 3.33 neutralization index when run against AVS-RRV. AVS-TV antisera was demonstrated to neutralize against nine out of ten challenge viruses, only performing poorly when run against STC. Isolates AVS-MB and AVS-TV should be evaluated further as potential vaccine candidates.

**Table 2.1** Initial selection of field isolates for characterization. Criteria for selection included farm location, company, histological evaluation and age of flock. The variety of locations, companies, and dates when isolates were harvested indicates widespread IBDV vaccine escape mutants which are antigenically distinct from current vaccine viruses.

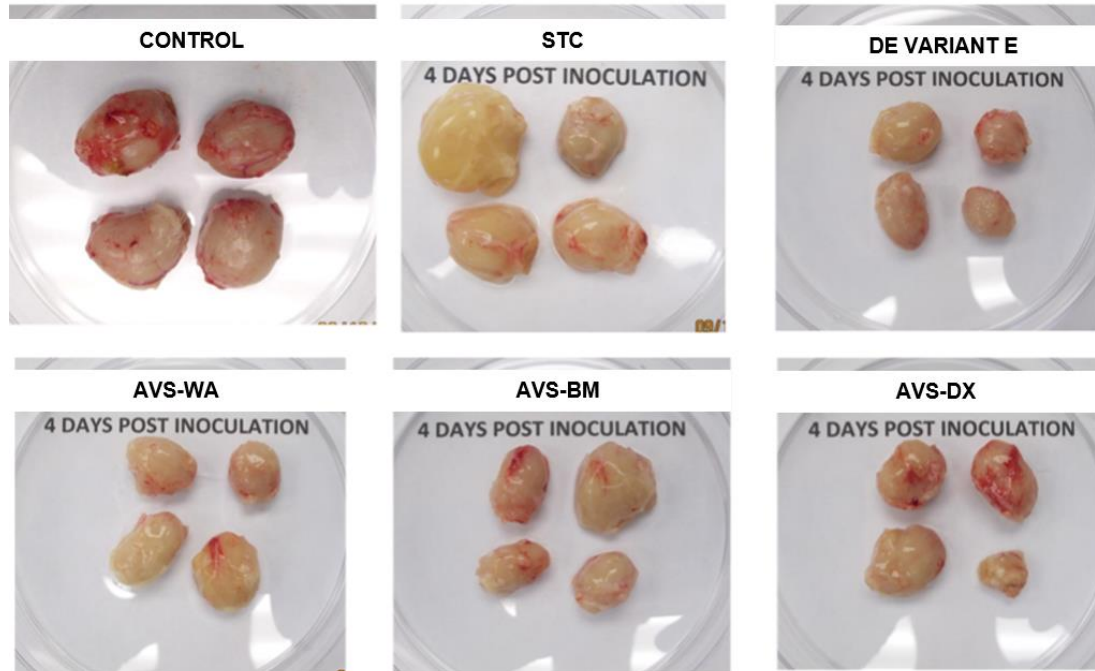
Isolate	Isolation Date	Flock Age	Location	Company
AVS-JH	2013	15D	Georgia	Company 1
AVS-CAL	06/05/17	17D	Georgia	Company 1
AVS-SS	02/14/18	18D	Georgia	Company 1
AVS-SCH	04/28/18	19D	Wisconsin	Company 1
AVS-JG	03/20/18	19D	Georgia	Company 1
AVS-FL	02/14/18	19D	Georgia	Company 1
AVS-WA	02/28/17	20D	Georgia	Company 1
AVS_DB	03/20/18	20D	Kentucky	Company 1
AVS-MS	01/03/18	20D	Virginia	Company 1
AVS-EAC	06/11/18	22D	West Virginia	Company 1
AVS-CHA	11/29/17	22D	Tennessee	Company 1
AVS-GN	11/29/17	24D	Tennessee	Company 1
AVS-CAN	2016	15D	Alabama	Company 2
AVS-TV	10/30/2018	16D	Alabama	Company 2
AVS-DX	2016	17D	Mississippi	Company 2
AVS-WF	08/29/18	17D	Alabama	Company 2
AVS-TA	08/29/18	17D	Mississippi	Company 2
AVS-VA	08/27/18	19D	Georgia	Company 2
AVS-RO	2016	20D	Georgia	Company 2
AVS-BM	2016	20D	Georgia	Company 2
AVS-SA	08/27/18	20D	Georgia	Company 2
AVS-DL	08/27/18	20D	Georgia	Company 2
AVS-PA	08/29/18	21D	Alabama	Company 2
AVS-RA	08/27/18	22D	Georgia	Company 2
AVS-JS	09/27/18	17D	Alabama	Company 3
AVS-RP	06/13/18	19D	Georgia	Company 3
AVS-RR	03/01/18	21D	Tennessee	Company 3
AVS-DA	06/13/18	21D	Georgia	Company 3
AVS-SAN	09/24/18	24D	Mississippi	Company 3
AVS-JP	04/17/18	18D	Texas	Company 4
AVS-DY	04/25/18	18D	Mississippi	Company 4
AVS-PP	04/17/18	22D	Texas	Company 4
AVS-RRV	11/08/18	44D	Texas	Laying Hens
AL-2	2005	20D	North Carolina	Reference
DE Variant E	1985	Sentinel	Sentinels DMV	Reference
AVS-MB	2005	Sentinel	Georgia	Reference
AVS-BH	2005	Sentinel	DMV	Reference
STC	Unknown	Unknown	USDA APHIS	Reference

**Table 2.2** RT-PCR primers for IBDV A and IBDV B. These primers identify sequences in two different locations on IBDV Viral Protein 2.

	<b>Primer Name</b>	<b>Target Gene</b>	<b>Nucleotide Location</b>	<b>Product Size</b>
IBDV-A Forward Primer	<i>Mus-IBDV-A-For</i> 5'- GTAACGATCACACTGTTCTCAGC- 3'	VP2	746-768	247aa
IBDV-A Reverse Primer	<i>Mus-IBDV-A-Rev</i> 5'- CTCTATTGGGTCGGTCAATGTAG- 3'		971-993	
IBDV-B Forward Primer	<i>IBDV-B-For</i> 5'GCCCAGAGTCTACACCAT-3'	VP2	679-696	745aa
IBDV-B Reverse Primer	<i>IBDV-B-Rev</i> 5'-AGTTTCTGTATTAGGCC-3'		1404-1421	

**Table 2.3** RT-PCR Primers IBDV A and IBDV B results. Some isolates had negative results for IBDV A or IBDV B. Isolates which were negative for IBDV A or IBDV B are highlighted in yellow.

<b>Isolate</b>	<b>IBDV A</b>	<b>IBDV B</b>
DE Variant E	Positive	Positive
STC	Positive	Positive
AL-2	Positive	Positive
AVS-TV	Positive	Negative
AVS-RA	Negative	Positive
AVS-RO	Negative	Positive
AVS-BM	Negative	Positive
AVS-JH	Positive	Positive
AVS-CN	Positive	Positive
AVS-DX	Positive	Positive
AVS-SCH	Positive	Positive
AVS-EAC	Positive	Positive
AVS-JG	Positive	Positive
AVS-DB	Positive	Positive
AVS-MS	Positive	Positive
AVS-SS	Positive	Positive
AVS-FL	Positive	Positive
AVS-CAL	Positive	Positive
AVS-CHA	Positive	Positive
AVS-GN	Positive	Positive
AVS-RR	Positive	Positive
AVS-RP	Positive	Positive
AVS-DA	Positive	Positive
AVS-SAN	Positive	Positive
AVS-JS	Positive	Positive
AVS-SA	Positive	Positive
AVS-DL	Positive	Positive
AVS-WA	Positive	Positive
AVS-WF	Positive	Positive
AVS-PA	Positive	Positive
AVS-MB	Positive	Positive
AVS-RRV	Positive	Positive
AVS-TY	Positive	Positive
AVS-BH	Positive	Positive
AVS-PP	Positive	Positive
AVS-JP	Positive	Positive
AVS-VA	Positive	Positive
AVS-DY	Positive	Positive



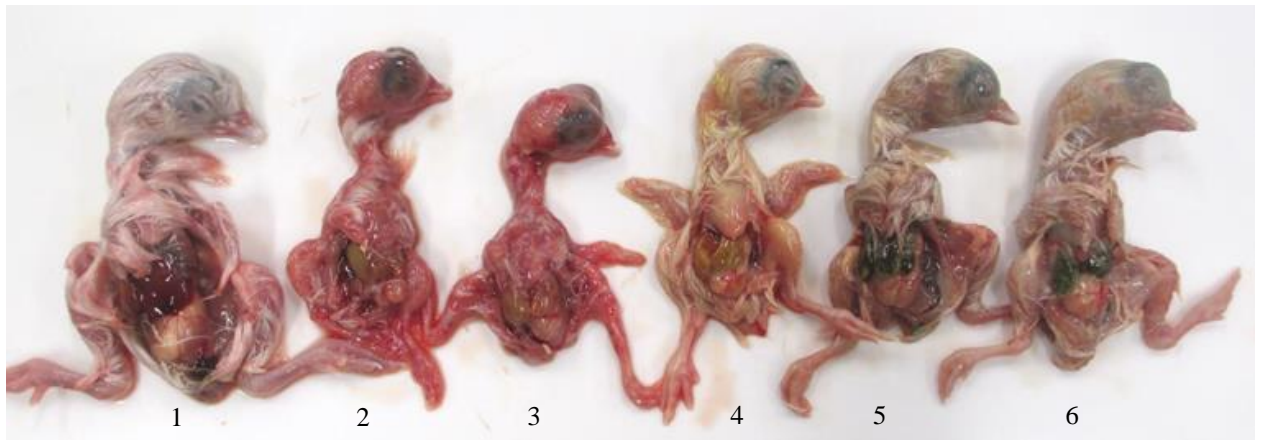
**Figure 2.1** Bursae of Fabricius from specific pathogen free leghorns harvested four days post-inoculation with contemporary IBDVs. A control group was mock inoculated with media and presented normal bursae. STC bursal homogenate inoculated birds had enlarged, edematous, and straw-colored bursae. Delaware Variant E bursal homogenate inoculated birds presented very atrophied bursa. Birds inoculated with AVS-WA and AVS-DX novel field variant bursal homogenate presented with atrophied bursae similar to Delaware Variant E inoculated leghorns. Of four leghorns necropsied four days post-inoculation with novel field variant AVS-BM bursal homogenate, three bursae were atrophied, and one bursa appeared enlarged and straw-colored, similar to STC inoculated leghorns.

**Table 2.4** Pathology of leghorns 4 days post-inoculation with IBDV. Combined and average bursal weight in grams, presence of splenomegaly, and bursal lesions from specific pathogen free leghorns necropsied four days post-inoculation. There were four birds from each group necropsied at this time point. One control group was mock inoculated with media, three groups were inoculated with historically significant bursal homogenate of IBDV strains STC, Delaware Variant E, and AL-2, and twenty-six groups were initially inoculated with isolates from commercial broiler flocks.

Inoculate	Bursal Lesions	Presence of Splenomegaly	Total Bursal Weight (g)	Average Bursal Weight (g)
Control	No Visible Lesions	No	9.8	2.45
DE Variant E	Bursal Atrophy	Yes	3.8	0.95
STC	Enlarged	Yes	9.3	2.325
AL-2	Enlarged	Yes	9.2	2.30
AVS-WA	Atrophied	Yes	4.7	1.175
AVS-RO	Enlarged	Yes	7.4	1.85
AVS_BM	No visible lesions	Yes	5.0	1.25
AVS-JH	Slightly Enlarged	Yes	6.5	1.625
AVS-CAN	Atrophied	Yes	4.0	1.00
AVS-DX	Slightly Enlarged	Yes	5.0	1.25
AVS-SA	Atrophied, gelatinous	Yes	8.01	2.00
AVS-DL	Atrophied	Yes	8.38	2.095
AVS-RA	Atrophied, gelatinous	Yes	7.21	1.80
AVS-WF	Atrophied	Yes	4.91	1.228
AVS-PA	Atrophied, gelatinous, edematous, striated	Yes	5.41	1.353
AVS-RR	Atrophied, striated	Yes	7.98	1.995
AVS-RP	Atrophied, striated	No	5.28	1.32
AVS-DA	Atrophied	Yes	4.93	1.2325
AVS-SAN	Atrophied	Yes	4.49	1.1225
AVS-JS	Atrophied	Yes	4.52	1.13
AVS-TV	Atrophied, yellow, striated, gelatinous	Yes	8.49	1.698
AVS-RRV	Atrophied, edematous, yellow, striated	Yes	6.01	1.50
AVS-DY	Atrophied	Yes	3.01	0.75
AVS-PP	Atrophied, enlarged	Yes	5.25	1.05
AVS-JP	Atrophied	No	2.73	0.683
AVS-VA	Edematous	No	5.85	1.46
AVS-TA	Atrophied	Yes	2.73	0.68
AVS-MB	Atrophied, yellow, gelatinous	Yes	5.94	1.188
AVS-SCH	Atrophied, gelatinous	Yes	3.53	0.883
AVS-EAC	Atrophied	Yes	4.31	1.078
AVS-JG	Atrophied	Yes	2.81	0.7025
AVS-DB	Atrophied, gelatinous	Yes	4.91	1.2275
AVS-MS	Atrophied	Yes	2.90	0.725
AVS-SS	Atrophied	Yes	2.82	0.705
AVS-FLK	Atrophied	Yes	2.95	0.735
AVS-CAL	Atrophied	Yes	4.25	1.0625
AVS-CHA	Atrophied	Yes	4.53	1.1325
AVS-GN	Atrophied	Yes	3.07	0.7675

**Table 2.5** Virus titrations and homologous neutralization indices. Virus stocks were titrated in specific pathogen free (SPF) eggs. 50% embryo lethal dose (ELD<sub>50</sub>)/mL and 50% egg infectious dose (EID<sub>50</sub>) were calculated. Embryos inoculated with different isolates presented either Delaware Variant E-like, Classical (STC)-like, or a combination of Delaware Variant E and STC-like lesions. Reference strains Delaware Variant E, STC, and AL-2, and thirty-five isolates from commercial flocks were evaluated.

Isolate	IBDV Lesions in Embryos	Log <sup>10</sup> ELD <sub>50</sub> /mL	Log <sup>10</sup> EID <sub>50</sub> /mL	Homologous Neutralization
DE Variant E	Variant E-like	5.7	7.0	5.50
STC	STC-like	6.3	6.7	6.00
AL-2	Variant E-like	4.3	6.4	5.40
AVS-WA	STC and Variant E-like	4.6	6.6	3.77
AVS-RO	STC and Variant E-like	5.5	5.6	3.33
AVS-BM	STC and Variant E-like	4.5	6.0	3.67
AVS-JH	STC and Variant E-like	5.2	6.4	4.50
AVS-CAN	STC and Variant E-like	4.7	5.7	2.87
AVS-DX	STC and Variant E-like	6.2	6.4	3.67
AVS-SCH	Variant E-like	3.6	5.8	3.50
AVS-EAC	Variant E-like	3.0	6.4	4.63
AVS-JG	STC and Variant E-like	<2.0	5.5	4.50
AVS-DB	Variant E-like	3.8	6.3	4.83
AVS-MS	STC and Variant E-like	5.4	6.8	3.98
AVS-SS	Variant E-like	4.2	6.7	4.17
AVS-FA	STC and Variant E-like	4.5	6.5	5.33
AVS-CAL	STC and Variant E-like	4.3	6.7	4.70
AVS-CHA	STC and Variant E-like	4.2	6.7	4.83
AVS-GN	STC-like	4.0	6.3	4.00
AVS-RR	STC and Variant E-like	5.0	6.7	4.50
AVS-RP	STC and Variant E-like	4.0	6.5	4.50
AVS-DA	STC and Variant E-like	5.2	6.2	4.83
AVS-SAN	Variant E-like	4.0	6.7	5.00
AVS-JS	STC and Variant E-like	4.2	5.3	2.23
AVS-SA	Variant E-like	4.8	6.3	3.85
AVS-DL	Variant E-like	5.2	6.2	3.14
AVS-RA	STC and Variant E-like	<2.0	5.5	3.33
AVS-WF	Variant E-like	4.2	5.5	4.05
AVS-PA	STC and Variant E-like	2.0	4.2	2.33
AVS-MB	Variant E-like	<2.0	6.5	3.53
AVS-TV	STC and Variant E-like	5.3	>7.0	5.00
AVS-RRV	STC and Variant E-like	<2.0	5.6	5.23
AVS-TA	STC and Variant E-like	4.0	6.4	>3.23
AVS-BH	Variant E-like	<2.0	6.0	>4.00
AVS-PP	STC-like	5.0	5.7	>5.00
AVS-JP	STC and Variant E-like	4.8	6.0	4.27
AVS-VA	STC and Variant E-like	<2.0	5.8	3.33
AVS-DY	STC-like	5.5	5.7	>4.50



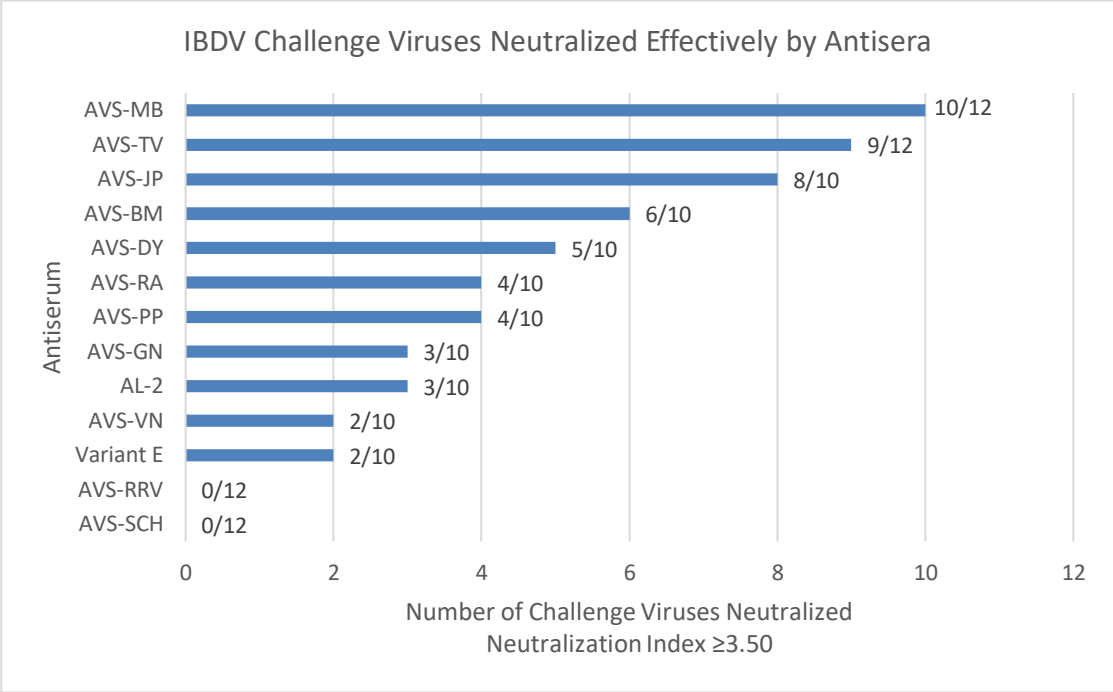
**Figure 2.2** Embryo lesions associated with IBDV infection. Many of the novel field isolates presented lesions typical for both classical and variant type infected embryos. All of the isolates depicted in this figure were inoculated with seed stock from isolate AVS-RO. Embryo 1 presented no visible lesions. Embryo 2 and 3 presented STC type lesions including parboil liver, pale spleen, and a hemorrhagic appearance. Embryo 3 presented a combination of classical and variant type lesions- it was cream colored, with a parboil liver. Embryos 4 and 5 presented DE Variant E type lesions- bile stasis, liver necrosis, splenomegaly, and creamy color.

**Table 2.6** Virus neutralization assays performed using select isolates. A neutralization index of 3.50 or higher was considered effective, and all virus neutralization indices greater than or equal to 3.50 are highlighted in yellow. The isolates AVS-MB and AVS-TV were most effective at neutralizing a range of challenge isolates, while isolates AVS-SCH and AVS-RRV did not effectively neutralize any challenge viruses despite moderate homologous neutralization. Since cross neutralization varied between antisera and challenge viruses, not only are novel field isolates different from historically significant isolates Delaware Variant E, AL-2, and STC, they are also different from each other.

IBDV Isolates	Isolate Antiserum													
	DE Variant E	AL-2	AVS-SCH	AVS-RRV	AVS-VN	AVS-PP	AVS-RA	AVS-GR	AVS-DY	AVS-BM	AVS-JP	AVS-TY	AVS-TV	AVS-MB
STC	3.00	2.67	≤1.57	2.06	3.33	≤2.00	2.37	≤2.00	2.50	3.71	2.33	≥4.00	2.92	≥4.54
DE Variant E	≥4.50	≤1.62	≤1.83	≤1.50	2.76	3.27	3.00	2.50	1.50	3.30	≥3.50	≥3.50	≥4.67	≥4.36
AL-2	4.00	4.36	≤1.93	≤2.17	3.50	≥4.50	3.31	3.90	4.00	4.45	3.50	≥4.50	4.50	≥4.85
AVS-WA	2.00	3.63	2.13	2.00	2.75	≥4.25	≥4.13	≥4.13	4.25	3.29	4.25	≥4.25	≥4.02	≥4.13
AVS-RO	≤2.16	2.33	≤2.00	≤2.00	2.67	2.34	≥4.83	2.83	1.67	4.13	≥3.57	3.17	≥4.00	≥4.50
AVS-DX	1.45	≤2.21	2.38	2.83	2.63	2.00	2.36	≤2.05	3.11	3.71	3.10	2.33	3.50	≥4.71
AVS-EAC	2.95	1.63	≤1.15	≤2.38	3.00	2.73	2.95	≤1.38	4.36	3.31	4.50	≥4.36	≥4.50	≥4.00
AVS-DB	2.86	3.85	2.96	≤1.73	2.73	≥4.50	≥5.13	≥4.50	3.50	≥4.80	4.09	≥4.50	≥4.00	≥5.13
AVS-FA	≤3.13	3.67	≤3.38	≤2.27	4.37	≤2.00	≤3.20	≤3.20	3.67	4.12	≥5.00	4.50	≥4.77	5.17
AVS-CAL	2.75	≤2.33	≤2.33	≤2.17	2.25	≥4.50	≥5.00	≥4.83	2.60	2.82	≥4.50	≥4.50	4.00	≥5.00
Homologous	≥4.50	4.35	3.50	≥3.77	≥3.33	≥5.00	3.33	4.50	≥4.50	≥4.00	4.27	≥3.23	5.00	3.58

**Table 2.7** Virus neutralizations of poorly and highly cross-protective isolates. Highly cross-protective isolate AVS-MB antiserum was run against challenge viruses AVS-SCH and AVS-RRV, which elicited poorly cross-protective antibodies. AVS-RRV and Suchla antiserum were also run against AVS-RRV, AVS-SCH, and AVS-MB challenge virus.

	Antiserum			
		AVS-MB	AVS-RRV	AVS-SCH
Challenge Virus	AVS-MB	3.53	<1.50	1.50
	AVS-RRV	3.33	5.23	2.50
	AVS-SCH	2.91	2.15	3.50



**Figure 2.3** The number of challenge viruses effectively neutralized varied by test antiserum. An effective neutralization index of  $\geq 3.50$  was considered effective. The two highest neutralizing antisera were from isolates AVS-MB (10 neutralized) and AVS-TV (9 neutralized), while the least neutralizing were AVS-SCH and AVS-RRV, neither of which neutralized any challenge viruses effectively. Homologous neutralization indexes were not included in this figure.

## Chapter 3

### GENOMIC CHARACTERIZATION OF SELECT IBDV ISOLATES

#### 3.1 Introduction

The IBDV genome is comprised of two dsRNA segments that encode five viral genes (17, 52, 86, 136, 143, 257). The two segments are designated segment A and segment B (8, 215). Segment B encodes VP1, an RNA-dependent-RNA-polymerase (RdRp). Segment A has two open reading frames (ORFs), a short frame ORF-A2 that encodes VP5, and a long frame ORF-A1, which terminally overlaps ORF-A2 in a +1-nucleotide frameshift and encodes polyprotein N-pVP2-VP4-VP3-C (16, 91, 116, 154, 203). This polyprotein is cleaved by viral protease VP4 (233). VP2 precursor pVP2 is cleaved multiple times at the carboxyl terminus to yield the mature VP2. VP2 fuses to the scaffolding, inner capsid protein VP3 and functions as the external capsid protein (19, 41). Due to roles in virus replication and function, VP1 and VP4 are highly conserved.

There are two distinct pathotypes of IBDV in the United States, classic and variant. Capsid protein VP2 has previously been associated with the variation of antigenicity between classic and variant pathotypes and has been the focus of most previous research (38). VP2 is highly conserved except for the hypervariable region (aa 206-305). The hypervariable region includes two hydrophobic peaks (A and B), which are designated P<sub>BC</sub> (aa 212-224) and P<sub>HI</sub> (aa 314-325) respectively. There are

two additional loops, P<sub>DE</sub> (amino acid 253) and P<sub>FG</sub> (amino acid 284). VP2 structure contains three distinct domains, a base, shell, and projection region (73, 115, 117). The base and shell domains make up the N and C termini of VP2 and are highly conserved, while the projection domain (aa 206-350), which is encoded by the hypervariable region, is less conserved (12, 117). Amino acid changes in the hypervariable region have previously been associated with virulence and antigenicity. Amino acid changes at positions 222 and 254 have previously been associated with antigenic variation (98), and a change at position 253 has been associated with IBDV attenuation and cell culture adaptation when pathogenic virus is passed through cell culture.

VP3 is a multi-functional protein, which has been primarily characterized by its structural function as a scaffold for VP2. VP3 is also important for host immune evasion by blocking the double-stranded RNA (dsRNA)-dependent protein kinase (PKR) response (10, 28, 36, 41, 142, 169). VP5 is a non-structural protein which has been experimentally associated with apoptotic effect and viral dissemination (126, 253, 254).

The genome of IBDV is highly conserved, and most of the amino acid changes associated with the altered antigenicity between classic and variant IBDVs are located in the hypervariable region of VP2. However, isolates with the same hypervariable region may differentially neutralize isolates, indicating that other areas of the viral genome are important in terms of antigenicity. Therefore, evaluation of the entire genome is important when evaluating sequenced isolates.

In this study, two highly cross-neutralizing and two poorly cross-neutralizing antigenic variants from commercial broiler flocks were sequenced and compared, along with previously characterized isolate sequences to gain insight into biological implications of amino acid changes, and their impact on protein function and viral antigenicity.

## **3.2 Materials and Methods**

### **3.2.1 Sequencing of IBDV Genomes**

Illumina high-throughput sequencing was used to determine the genome sequences of select IBDVs. Sequencing was done at the University of Delaware Sequencing Facility using the Illumina NextSeq 550 sequencer. IBDVs were sequenced using 151-bp paired-end reads on medium output.

### **3.2.2 Assembly of Sequences**

Illumina sequence results were analyzed by Dr. Shawn Polson at the Center for Bioinformatics and Computational Biology, University of Delaware. Trimming of raw Illumina reads was done using the CLC Genomics Workbench 20 (CLC Bio) with the following parameters (Quality limit: 0.05; Ambiguity Trim: 0 N's; Adapter Removal: Illumina 5' RNA adapter 1.0; Min length: 25). An iterative assembly process used CLC high-throughput de novo assembler and CLC high-throughput reference mapping

tools, followed by manual genome finishing. Genome finishing was assisted using reference sequence DE Variant E at 90% identity.

### **3.2.3 Generation of Predictive 3D Protein Structure**

The I-TASSER server (104, 105, 212) was used to create 3-D protein models based on algorithms for protein structure and function. The model with the highest C-Score was used for analysis. C-Score varied from 0.63 to 0.73 for models used in this analysis.

## **3.3 Results**

### **3.3.1 Selection of IBDVs for Sequencing**

IBDV isolates AVS-MB, AVS-TV, AVS-SCH and AVS-RRV were selected for sequencing and genome comparison after analysis of antigenic similarity of isolates, which was evaluated using virus neutralization assays. Two of the selected isolates, AVS-SCH and AVS-RRV, were poorly cross-protective, while isolates AVS-MB and AVS-TV elicited highly cross-protective antibodies (Table 2.6). By comparing isolates with different levels of cross-protectivity, similarities and differences between low and high cross-protective isolates may suggest associations between nucleotide and amino acid changes and antigenicity.

### **3.3.2 Evaluation of VP1 Genome**

IBDV RNA-dependent-RNA-polymerase (RdRp), VP1, is vital for viral replication. Among evaluated IBDV isolates amino acid sequences, it has a high percent identity, ranging from 98.30% to 99.89% (Figure 3.1). There was more variability in nucleotide sequences, with identities of 95.68% to 98.94% (Figure 3.2). Highly neutralizing IBDV field isolates, AVS-MB and AVS-TV, and poorly neutralizing isolate AVS-RRV have greater than 99% identity to each other for VP1 amino acid sequences. Isolate AVS-SCH VP1 is 99.09% similar to AVS-RRV, and 98.64% and 98.41% similar to AVS-MB and AVS-TV respectively. AVS-SCH VP1 is less than 99% similar to all other isolates.

VP1 amino acid percent identity was greater than 98% among all isolates. AVS-TV and AVS-MB, as well as other contemporary isolates AVS-MD and AVS-AX had changes at positions 13 and 158, and 879. AVS-CN shared the changes at positions 13 and 879. Testing of AVS-MD, AVS-AX, and AVS-CN could support if changes at these positions improve levels of cross-protective antibody elicited during infection.

### **3.3.3 Evaluation of VP2 Genome**

Most of VP2 is highly conserved, the majority of amino acid differences are located in the hypervariable region (amino acids 206-305). Decreased amino acid conservation in the hypervariable region is shown in a conservation map (Figure 3.6). Pairwise comparison of VP2 amino acid sequences shows percent identities ranging from 95.95% to 100% (Figure 3.4). Nucleotide identities ranged from 94.21% to

98.89% (Figure 3.5). Highly neutralizing isolates AVS-MB and AVS-TV amino acid sequences are 98.73% similar to each other and are both greater than 97% similar to prevalent vaccine IBDV, Delaware Variant E. Poorly neutralizing isolates AVS-RRV and AVS-SCH are most different from other isolates and each other. Percent identity of AVS-SCH VP2 ranges from 89.24% to 94.94%. It is most most similar to Delaware Variant E and most dissimilar to AVS-RRV. AVS-RRV VP2 percent identity ranges from 88.61% to 91.77%. It is most similar to classic IBDV STC and most dissimilar to AVS-SCH.

Outside of the hypervariable region, there are changes in amino acids 77-79. IBDVs AVS-CN, AVS-AX, AVS-MD, AVS-TV, AVS-MB, AVS-RO, and AVS-BM all share a change at position 77 when compared to Delaware Variant E and STC. AVS-SCH has a mutation from asparagine (D) to hydroxylic amino acid serine (S) at position 79.

### **3.3.4 Evaluation of the VP2 Hypervariable Region Genome**

The hypervariable region of VP2 contains hydrophilic peaks P<sub>BC</sub> (aa 212-224), P<sub>HI</sub> (aa 314-325), P<sub>DE</sub> (aa 253) and P<sub>FG</sub> (aa 284), which are more heterogenous than the remainder of VP2. This can be seen in a conservation map (Figure 3.9). The percent identity for VP2 hypervariable region amino acid sequences ranges from 89.24% to 100.0% (Figure 3.7). AVS-SCH, STC, and AVS-RRV are most dissimilar from all other sequences, with less than 95.0% identity to any other isolate. AVS-MB has 98.73% identity with AVS-TV. Nucleotide percent identities ranged from 90.06% to

97.84% (Figure 3.8). Nucleotide sequence for isolate AVS-DX was most dissimilar to other isolates, even more different than AVS-RRV and AVS-SCH, indicating a high number of synonymous mutations. The highest nucleotide identity, 97.85%, was shared between previously characterized isolates Delaware Variant E and AL-2.

The sequence locations of the peaks of loops P<sub>BC</sub> (aa 212-224), P<sub>HI</sub> (aa 314-325), P<sub>DE</sub> (aa 253) and P<sub>FG</sub> (aa 284) presented multiple amino acid positions with low conservation. VP2 loops P<sub>BC</sub> and P<sub>HI</sub> are regarded as important antigenic epitopes, therefore these changes may contribute to antigenic drift. Amino acid 222, which is located in the loop P<sub>BC</sub> has been previously implicated for antigenic variation and drift, particularly when comparing DE Variant E-like isolates to STC and other classic IB DVs (96, 98). STC has a proline (P) and Delaware Variant E has a threonine (T) at aa222. AVS-SCH possesses a glutamine (Q) at position 222. Interestingly, another contemporary isolate, AVS-BM, has an alanine (A) at this location.

Amino acid 254, which is positioned beside P<sub>de</sub> (aa253) demonstrated multiple changes shared between groups of isolates. AVS-MB, AVS-RO, AVS-BM, AVS-LA, AVS-BA, AVS-DX, and AVS-SCH all shared the same amino acid at this position, STC and AVS-RRV had a glycine (G), and AVS-BM, AVS-LA, AVS-BA and AVS-DX possessed an asparagine (N) at this position.

Many of the other changes fell between aa317 and 322, which are within the P<sub>HI</sub> loop. Isolates AL-2, AVS-CN, AVS-AX, AVS-MD, AVS-TV, AVS-MT, and AVS-MB, shared changes D318N and A321E when compared to Delaware Variant E. Delaware Variant E, AVS-SCH, AVS-LA and AVS-BN possesses an aspartic acid (D)

at 318 and an alanine (A) at 321, although AVS-LA and AVS-BN also possess a G314E mutation when compared to DE Variant E. AVS-DX shares this G314E change, although it has an asparagine (N) at position 318. STC and AVS-RRV are the same within loop P<sub>HI</sub>, and both have a glycine (G) at position 318 and an alanine (A) at position 318.

AVS-SCH has acid changes where all other isolates were conserved- these changes include V225A, S251N, T260N. AVS-RRV also has changes at otherwise conserved locations- G256A, G258D A279S, and E310K. AVS-RRV shared a T269K mutation with AVS-AX, AVS-RO, and AVS-BM. There are also changes which were shared by a combination of STC, AVS-RRV, and AVS-SCH. AVS-SCH, AVS-RRV, and AVS-SCH shared the change N213D, AVS-SCH and AVS-RRV both have a change A284T. STC and AVS-RRV share exclusive changes S254G, A270T, and N379D.

AVS-MB only had one change in the hypervariable region of VP2 which was not shared by any other isolates and must be evaluated further for impact on antigenicity since AVS-MB was the most neutralizing IBDV evaluated. AVS-TV VP2 hypervariable region amino acid sequence was the same as two other isolates, AVS-MT and AVS-MD. AVS-MB only had one change in the hypervariable region of VP2 which was not shared by any other isolates. AVS-TV VP2 hypervariable region amino acid sequence was the same as three other isolates. AVS-RRV, which was isolated from a layer flock in Texas, more closely resembles classic IBDV isolate STC than Delaware Variant E or any of the other isolates. AVS-RRV and AVS-SCH contained

the most mutations in the hypervariable region, many of which were in positions which were conserved among all other isolates. Any mix of these mutations could contribute to the poorly cross-protective antibody generated against AVS-SCH and AVS-RRV.

### **3.3.5 Evaluation of VP3 Genome**

VP3 amino acid sequences percent identity ranges from 97.87% to 100% (Figure 3.10). Nucleotide identity is lower, ranging from 94.35% to 99.44% (Figure 3.11). At amino acid position 60, isolates AVS-TV and AVS-MB, as well as AVS-LA, AVS-BA, and AVS-MD share the same missense mutation compared to STC, Delaware Variant E, and other isolates. Previously sequenced IBDV AVS-AX is distinct from all other sequences at this location with a T60D. At amino acid 44, AVS-SCH exhibits a change from basic amino acid histidine (H) to polar uncharged glutamine (Q).

### **3.3.6 Evaluation of VP4 Genome**

The viral protease VP4 is highly conserved amongst IBDVs, with an amino acid percent identity ranging from 97.77% to 100.0% (Figure 3.12). Nucleotide percent identity ranges from 94.66% to 98.22% for the sixteen IBDVs evaluated (Figure 3.13). AVS-RRV has one amino acid change at aa18, changing from basic amino acid histidine to hydrophobic aliphatic leucine. AVS-SCH amino acid changes

include Q75L and C227S. Additionally, AVS-AX (C227Y), AVS-LA (C227H), AVS-MT (C227S) and AVS-BA (C227H) also exhibit changes at this position. AVS-MB, AVS-TV, AVS-AX, AVS-CN, AL-2, and AVS-DX exhibit an amino acid change N94D.

### **3.3.7 Evaluation of VP5 Genome**

IBDV VP5 amino acid percent identity ranges from 90.41% to 100% (Figure 3.14), and nucleotide percent identity ranges from 94.66% to 98.49% (Figure 3.15). AVS-SCH exhibited changes S3N, C100S, S115P, and W127R. Changes in AVS-RRV sequence include A16D and K120E. The glutamic acid (E) at position 120 is shared with STC and contemporary isolates AVS-BM, AVS-RO, AVS-BA, and AVS-CN, while Delaware Variant E and the other remaining isolates contain a lysine (K).

AVS-TV has changes N8H, C100G, and K122E. There is an additional change shared between AVS-TV, AVS-MB, AVS-BM, AVS-RO, AVS-AX, AVS-MD, and AVS-CN at aa88. AVS-MB does not have any other changes to differentiate from Delaware Variant E in VP5.

### **3.3.10 Evaluation of VP2 3D Predictive Protein Models**

Predictive 3D protein modelling structure was used to evaluate the impact of certain mutations on the structure of VP2. First, models were created for STC and DE Variant E, which have antigenic differences. There were apparent structural changes between classical IBDV STC and variant IBDV Delaware Variant E, particularly in

the projection domain. These changes include more space between beta strands, and altered shape of hydrophilic loops, which are known antigenic epitopes (Figure 3.16). Next, the effects of amino acid changes D318N and A321E, which were shared amongst five of the contemporary isolates, were evaluated by inserting these changes into a Delaware Variant E sequence. A 3D protein model was created to observe potential impact of these changes on confirmation. The structure of the loop that contains these amino acids was altered to a much flatter confirmation, which may be relevant for antigenic variation of IBDVs with these mutations (Figure 3.17).

Models were also created for the VP2s of highly neutralizing IBDVs AVS-MB and AVS-TV as well as poorly neutralizing AVS-RRV and AVS-SCH to evaluate the impact of amino acid changes on the confirmation of regions associated with antigenicity. The projection domains of these models are different from each other, changes can be seen in the hydrophilic loops which have been associated with antigenicity. AVS-TV and AVS-MB share the mutation N69D that was only found in contemporary field IBDV sequences. This amino acid is located in the base domain. Since the base and shell domains are highly conserved in most IBDVs, this mutation outside of the projection domain may have some biological significance (Figure 3.18).

### **3.4 Discussion**

Contemporary IBDVs which elicit poorly or highly cross-protective antibodies were selected for sequencing and comparison with previously sequenced IBDVs.

The hypervariable region of VP2 peaks P<sub>BC</sub> (aa 212-224), P<sub>HI</sub> (aa 314-325), P<sub>DE</sub> (aa 253) and P<sub>FG</sub> (aa 284) had the highest rates of heterogeneity.

The least conserved amino acid positions of the hypervariable region of VP2 fell within previously characterized peaks P<sub>BC</sub>, P<sub>HI</sub>, P<sub>DE</sub>, and P<sub>FG</sub>. Amino acids 222, 254, and 318-322 showed the most changes.

Position 222 is considered important for the antigenic variance between STC and DE Variant E. Changes at position 222 have also been associated with DE Variant E escape mutants. Most contemporary isolates resembled Delaware Variant E at this location. STC, AVS-RRV, and isolate AVS-BM were the only evaluated isolates which were different at this location. Therefore, the antigenic variation of other field isolates were associated with different mutations.

All contemporary isolates differed from STC and Delaware Variant E at amino acid position 254, and there was a combination of mutations at positions 318 and 322 that were present in multiple recent field isolates, but not in Delaware Variant E and STC VP2.

Although the hypervariable region of VP2 has been the subject of many past studies regarding IBDV antigenicity, field isolates with the same hypervariable region may display distinct antigenicity. As such, other aspects of the IBDV genome must be considered when assessing antigenicity. There are some changes upstream from the hypervariable region of VP2 in modern isolates. IBDVs AVS-CN, AVS-AX, AVS-MD, AVS-TV, AVS-MB, AVS-RO, and AVS-BM all share a change at position 77 and AVS-SCH has a mutation at position 79. The VP2 sequences of previously

characterized isolates Delaware Variant E, STC, and AL-2 are all conserved outside of the hypervariable region, therefore these changes should be evaluated for potential impact on antigenicity and pathotype.

Along with VP2, VP3 also functions as a structural protein, by acting as a scaffold for VP2 in the capsid. Since VP3 is a capsid protein, it may be important in antigenicity. At amino acid position 60, isolates AVS-TV and AVS-MB, as well as AVS-LA, AVS-BA and AVS-MD share the same missense mutation which is different from STC, Delaware Variant E, and other isolates. Since VP3 is structural, it may be important in epitope recognition, therefore this change is potentially important for further evaluation.

Viral protease VP4 is vital for function of IBDV, since it cannot form new viroids without the ability to cleave the C-VP2-VP4-VP3-N polyprotein. As such, it was expected to be highly conserved among IBDVs with an amino acid percent identity ranging from 97.77% to 100%. AVS-RRV has one amino acid change at position H18L, AVS-SCH amino acid changes include Q75L and C227S. Since AVS-SCH and AVS-RRV tend to have more differences than other isolates, these changes may be associated differentially with creation of poorly neutralizing antibodies. Highly neutralizing isolates AVS-MB and AVS-TV, as well as contemporary isolates AVS-AX, AVS-DX, and AVS-CN, and previously characterized AL-2 exhibit one amino acid change at position N94D. Since Delaware Variant E neutralizes AL-2, this change alone likely does not influence IBDV escape from vaccine protection.

VP5, which is non-structural, not necessary for viral replication has been experimentally implicated in viral pathogenesis and dissemination (254). The confirmation of the overlapping ORFs of VP5 and IBDV polyprotein results in a greater number of missense single nucleotide polymorphisms in VP5. This pattern is related to the wobble hypothesis, which states that while the first two nucleotide positions in a codon are clearly distinguished, while the third codon position is not as concise. Several different codons code for the same amino acid. Codons which code for the same amino acid usually share the first two nucleotides but may vary in the third nucleotide position. Since the first codon position of VP5 is in the third position of VP2 due to the overlapping frameshift (Figure 2.1), more missense mutations will be expected in VP5 (16, 91, 116, 154, 203).

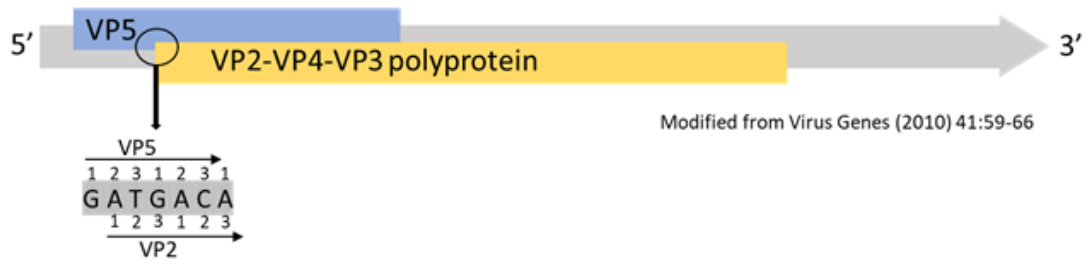
The amino acid identity for VP5 was 90.41% to 100.0%, while the nucleotide identity ranged from 96.58% to 98.89%. VP5 had an amino acid change shared by highly neutralizing isolates AVS-MB and AVS-TV. This change was the only difference between Delaware Variant E and AVS-MB. This change is also shared with less protective isolates AVS-BM, as well as other contemporary isolates AVS-RO, AVS-AX, AVS-MD, and AVS-CN.

Three-dimensional protein structure predictions were generated to evaluate the effect of specific aa changes. When a pair of mutations, D318N and A321E, which were found in multiple contemporary IBDVs were inserted into a Delaware Variant E sequence, the predicted protein structure presented an apparent change on one of the loops ( $P_{hi}$ ) of the projection region of VP2. Since these loops have been recognized as

important epitopes, these mutations may be important for antigenic variation of some isolates.

Three-dimensional predictive protein models were also created for highly neutralizing IBDVs AVS-MB, AVS-TV and poorly neutralizing IBDVs AVS-SCH and AVS-RRV. The projection domains of AVS-SCH and AVS-RRV appeared different from each other and from AVS-MB, AVS-TV, STC, and DE Variant E models. Changes in known antigenic epitopes of VP2 located in projection region may be associated with IBDVs that elicit poorly cross-neutralizing antibodies.

There were many amino acid changes of potential interest, however further analysis must be performed to determine biological significance. In previous studies, site directed mutagenesis has been used to test the biological impact of amino acid changes by targeting a single amino acid for alteration and testing. This method would be appropriate for advancing this research. In addition, since some isolates utilized for genome analysis have not been evaluated via virus neutralization assays, testing these isolates may be useful to support the importance of shared amino acid changes with highly or poorly cross-protective isolates.



**Figure 3.1** VP5 and VP2-VP4-VP3 polyprotein have an overlapping open reading frame. VP5 overlaps with part of the VP2 section of the polyprotein. VP5 has a +1-nucleotide frameshift, which results in a greater number of mutations in VP5 than in VP2.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
AVS-AX VP1	1		99.89	99.55	99.09	99.43	99.32	99.32	98.46	98.98	98.64	98.98	98.98	98.64	99.20	98.86	98.64	
AVS-MB VP1	2	99.89		99.55	99.09	99.43	99.32	99.32	98.64	98.98	98.64	98.98	98.98	98.64	99.20	98.86	98.64	
AVS-TV VP1	3	99.55	99.55		98.86	99.43	99.09	99.09	98.52	98.86	98.52	98.75	98.86	98.52	98.98	98.52	98.41	
AVS-DX VP1	4	99.09	99.09	98.86		98.75	99.55	99.55	99.09	99.20	99.09	99.20	99.20	98.75	98.75	99.20	98.86	
AVS-MD VP1	5	99.43	99.43	99.43	98.75		98.98	98.98	98.30	98.64	98.30	98.64	98.86	98.52	98.86	98.41	98.30	
AVS-MT VP1	6	99.32	99.32	99.09	99.55	98.98		99.77	99.09	99.43	99.09	99.43	99.43	98.98	98.98	99.20	99.09	
AVS-RRV VP1	7	99.32	99.32	99.09	99.55	98.98	99.77		99.09	99.43	99.09	99.43	99.43	98.98	98.98	99.20	99.09	
AVS-BM VP1	8	98.64	98.64	98.52	99.09	98.30	99.09	99.09		99.66	99.55	99.43	99.20	98.75	98.30	98.75	98.41	
Var E VP1	9	98.98	98.98	98.86	99.20	98.64	99.43	99.43	99.66		99.66	99.77	99.55	99.09	98.64	98.86	98.75	
AVS-RO VP1	10	98.64	98.64	98.52	99.09	98.30	99.09	99.09	99.55	99.66		99.43	99.20	98.75	98.30	98.75	98.41	
AVS-LA VP1	11	98.98	98.98	98.75	99.20	98.64	99.43	99.43	99.43	99.77	99.43		99.32	98.86	98.64	98.86	98.75	
STC VP1	12	98.98	98.98	98.86	99.20	98.86	99.43	99.43	99.20	99.55	99.20	99.32		99.32	94	98.86	98.75	
AVS-BA VP1	13	98.64	98.64	98.52	98.75	98.52	98.98	98.98	98.75	99.09	98.75	98.86	99.32		98.30	98.41	98.52	
AVS-CN VP1	14	99.20	99.20	98.98	98.75	98.86	98.98	98.98	98.30	98.64	98.30	98.64	98.64	98.30		98.52	98.30	
AL-2 VP1	15	98.86	98.86	98.52	99.20	98.41	99.20	99.20	98.75	98.86	98.75	98.86	98.86	98.86	98.41	98.52		98.52
AVS-SCH VP1	16	98.64	98.64	98.41	98.86	98.30	99.09	99.09	98.41	98.75	98.41	98.75	98.75	98.52	98.30	98.52		

**Figure 3.2** Pairwise comparison of IBDV RNA dependent RNA polymerase VP1 amino acid sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
STC VP1	1		98.48	97.61	97.46	97.27	98.37	98.60	98.07	97.35	97.69	97.73	97.39	97.16	97.16	97.39	97.05
Var E VP1	2	98.48		98.33	98.30	98.18	97.54	97.95	97.65	97.12	97.12	97.31	96.86	96.74	96.82	97.01	96.78
AVS-LA VP1	3	97.61	98.33		97.20	97.08	96.70	97.27	97.08	96.55	96.52	96.78	96.17	96.06	96.14	96.48	96.17
AVS-RO VP1	4	97.46	98.30	97.20		98.94	96.36	97.08	96.78	96.40	96.06	96.44	96.14	96.02	95.95	96.29	96.02
AVS-BM VP1	5	97.27	98.18	97.08	98.94		96.33	96.89	96.74	96.14	96.10	96.33	96.02	95.83	95.68	95.95	95.76
AVS-BA VP1	6	98.37	97.54	96.70	96.36	96.33		97.42	96.97	96.59	96.74	96.78	96.44	96.21	96.25	96.52	96.21
AVS-RRV VP1	7	98.60	97.95	97.27	97.08	96.89	97.42		97.88	97.46	97.65	97.84	97.39	97.20	97.05	97.12	97.08
AVS-MT VP1	8	98.07	97.65	97.08	96.78	96.74	96.97	97.88		97.08	97.35	97.61	96.97	96.97	96.78	96.78	96.74
AL-2 VP1	9	97.35	97.12	96.55	96.40	96.14	96.59	97.46	97.08		96.52	96.82	96.29	96.36	96.25	96.48	96.48
AVS-AX VP1	10	97.69	97.12	96.52	96.06	96.10	96.74	97.65	97.35	96.52		98.67	98.11	98.14	97.65	96.40	96.21
AVS-MB VP1	11	97.73	97.31	96.78	96.44	96.33	96.78	97.84	97.61	96.82	98.67		98.64	98.37	97.80	96.63	96.67
AVS-MD VP1	12	97.39	98.86	96.17	96.14	96.02	96.44	97.39	96.97	96.29	98.11	98.64		98.14	97.46	96.21	96.36
AVS-TV VP1	13	97.16	96.74	96.06	96.02	95.83	96.21	97.20	96.97	96.36	98.14	98.37	98.14		97.42	96.10	96.10
AVS-CN VP1	14	97.16	96.82	96.14	95.95	95.68	96.25	97.05	96.78	96.25	97.65	97.80	97.46	97.42		96.17	96.17
AVS-SCH VP1	15	97.39	97.01	96.48	96.29	95.95	96.52	97.12	96.78	96.48	96.40	96.63	96.21	96.10	96.17		96.21
AVS-DX VP1	16	97.05	96.78	96.17	96.02	95.76	96.21	97.08	96.74	96.48	96.21	96.67	96.36	96.10	96.17	96.21	

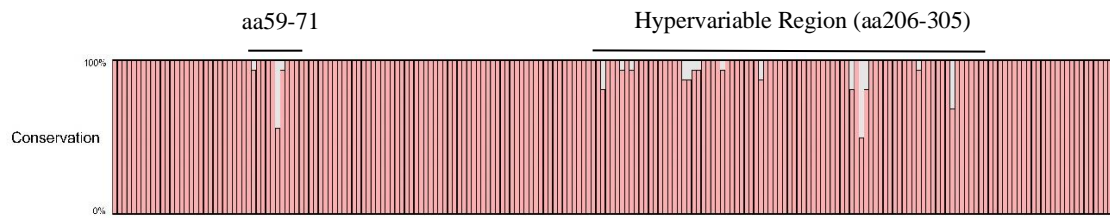
**Figure 3.3** Pairwise comparison of IBDV RNA dependent RNA polymerase VP1 nucleotide sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AVS-AX VP2	1		99.55	99.77	99.77	99.77	99.55	99.10	99.10	99.32	98.87	98.42	97.97	98.20	97.30	97.07	96.62
AVS-CN VP2	2	99.55		99.77	99.77	99.32	99.55	98.65	98.65	99.32	98.87	98.42	97.97	98.20	97.52	97.07	96.17
AVS-MD VP2	3	99.77	99.77		100.00	99.55	99.77	98.87	98.87	99.55	99.10	98.65	98.20	98.42	97.52	97.30	96.40
AVS-TV VP2	4	99.77	99.77	100.00		99.55	99.77	98.87	98.87	99.55	99.10	98.65	98.20	98.42	97.52	97.30	96.40
AL-2 VP2	5	99.77	99.32	99.55	99.55		99.77	98.87	98.87	99.10	99.10	98.65	98.20	98.42	97.52	97.30	96.85
AVS-MT VP2	6	99.55	99.55	99.77	99.77	99.77		98.65	98.65	99.32	99.32	98.87	98.42	98.65	97.75	97.52	96.62
AVS-BM VP2	7	99.10	98.65	98.87	98.87	98.87	98.65		99.55	98.42	98.65	98.20	97.75	97.30	96.85	97.07	96.40
AVS-RO VP2	8	99.10	98.65	98.87	98.87	98.87	98.65	99.55		98.42	98.65	98.20	97.75	97.30	96.62	96.85	96.85
AVS-MB VP2	9	99.32	99.32	99.55	99.55	99.10	99.32	98.42	98.42		98.87	98.42	97.97	98.20	97.30	97.07	96.17
Var E VP2	10	98.87	98.87	99.10	99.10	99.10	99.32	98.65	98.65	98.87		99.10	98.65	98.42	97.97	97.52	96.62
AVS-L VP2	11	98.42	98.42	98.65	98.65	98.65	98.87	98.20	98.20	98.42	99.10		99.55	98.65	97.52	97.30	96.40
AVS-BA VP2	12	97.97	97.97	98.20	98.20	98.20	98.42	97.75	97.75	97.97	98.65	99.55		98.20	97.07	96.85	95.95
AVS-DX VP2	13	98.20	98.20	98.42	98.42	98.42	98.85	97.30	97.30	98.20	98.42	98.65	98.20		96.85	96.85	95.95
AVS-SCH VP2	14	97.30	97.52	97.52	97.52	97.52	97.75	96.85	96.62	97.30	97.97	97.52	97.07	96.85		96.62	95.95
STC VP2	15	97.07	97.07	97.30	97.30	97.30	97.52	97.07	96.85	97.07	97.52	97.30	96.85	96.85	96.62		97.07
AVS-RRV VP2	16	96.62	96.17	96.40	96.40	96.85	96.62	96.40	96.85	96.17	96.62	96.40	95.95	95.95	95.95	97.07	

**Figure 3.4** Pairwise comparison of IBDV capsid protein VP2 amino acid sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AVS-MB VP2	1		98.50	97.33	98.11	97.92	96.36	97.85	97.66	95.77	97.14	95.90	96.75	96.75	96.36	96.88	95.44
AVS-TV VP2	2	98.50		97.33	98.11	98.18	96.16	97.98	97.27	95.64	96.88	95.90	96.49	96.55	96.23	96.88	95.31
AVS-MT VP2	3	97.33	97.33		98.37	96.94	97.20	96.81	97.72	96.42	96.81	96.39	96.29	96.49	96.62	96.88	96.10
Var E VP2	4	98.11	98.11	98.37		97.53	97.40	97.59	98.31	96.61	97.40	97.14	97.20	97.33	97.20	97.27	96.29
AVS-MD VP2	5	97.92	98.18	96.94	97.53		95.97	97.46	97.01	95.58	96.62	95.58	95.84	95.84	95.64	96.49	94.93
STC VP2	6	96.36	96.16	97.20	97.40	95.97		95.64	96.68	97.46	95.71	95.71	95.97	95.90	95.71	95.71	95.71
AVS-AX VP2	7	97.85	97.98	96.81	97.59	97.46	95.64		97.20	95.31	96.94	95.58	96.03	95.90	95.77	96.62	94.60
AL-2 VP2	8	97.66	97.27	97.72	98.31	97.01	96.68	97.20		96.09	96.88	96.16	96.49	96.62	96.36	96.88	95.57
AVS-RRV VP2	9	95.77	95.64	96.42	96.61	95.58	97.46	95.31	96.09		95.12	94.73	95.12	95.25	94.73	94.99	94.99
AVS-DX VP2	10	97.14	96.88	96.81	97.40	96.62	95.71	96.94	96.88	95.12		95.71	95.64	95.64	95.90	96.23	94.66
AVS-BA VP2	11	95.90	95.90	96.36	97.14	95.58	95.71	95.58	96.16	94.73	95.71		95.84	96.10	96.68	96.16	95.12
AVS-BM VP2	12	96.75	96.49	96.29	97.20	95.84	95.97	96.03	96.49	95.12	95.64	95.84		98.89	95.71	96.03	94.73
AVS-RO VP2	13	96.75	96.55	96.49	97.33	95.84	95.90	95.90	96.62	95.25	95.64	96.10	98.89		95.71	96.23	94.86
AVS-LA VP2	14	96.36	96.23	96.62	97.20	95.64	95.71	96.77	96.36	94.73	95.90	96.68	95.71	95.71		95.77	94.73
AVS-CN VP2	15	96.88	96.88	96.88	97.27	96.49	95.71	96.62	96.88	94.99	96.23	96.16	96.03	96.23	95.77		94.21
AVS-SCH VP2	16	95.44	95.31	96.10	96.29	94.93	95.71	94.60	95.57	94.99	94.66	95.12	94.73	94.86	94.73	94.21	

**Figure 3.5** Pairwise comparison of IBDV capsid protein VP2 nucleotide sequence percent identities.



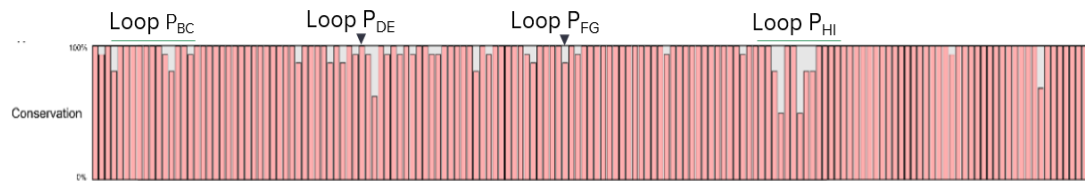
**Figure 3.6** Conservation of IBDV VP2 amino acid sequences. Most of VP2 is 100% conserved between sequences, with the majority of amino acid changes located in the hypervariable region (amino acid 206-305). Additional changes are present between amino acid 59 and 71.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AVS-CN VP2 HV	1		99.37	99.37	99.37	98.73	98.73	98.10	97.47	95.57	96.20	95.57	96.20	95.57	94.30	92.41	89.87
AVS-MT VP2 HV	2	99.37		100.00	100.00	99.37	99.37	99.73	98.10	96.20	96.84	96.20	96.84	96.20	94.30	93.04	90.51
AVS-MD VP2 HV	3	99.37	100.00		100.00	99.37	99.37	99.73	98.10	96.20	96.84	96.20	96.84	96.20	94.30	93.04	90.51
AVS-TV VP2 HV	4	99.37	100.00	100.00		99.37	99.37	99.73	98.10	96.20	96.84	96.20	96.84	96.20	94.30	93.04	90.51
AVS-AX VP2 HV	5	98.73	99.37	99.37	99.37		100.00	98.10	97.47	96.84	97.47	95.57	96.20	95.57	93.67	92.41	91.14
AL-2 VP2 HV	6	98.73	99.37	99.37	99.37	100.00		98.10	97.47	96.84	97.47	95.57	96.20	95.57	93.67	92.41	91.14
AVS-MB VP2 HV	7	98.10	98.73	98.73	98.73	98.10	98.10		97.47	94.94	95.57	95.57	96.20	95.57	92.67	92.41	89.87
Var E VP2 HV	8	97.47	98.10	98.10	98.10	97.47	97.47	97.47		96.10	96.84	96.84	97.47	95.57	94.94	93.04	90.51
AVS-BM VP2 HV	9	95.57	96.20	96.20	96.20	95.84	96.84	94.94	96.20		98.10	94.30	94.94	92.41	91.77	91.77	89.87
AVS-RO VP2 HV	10	96.20	96.84	96.84	96.84	97.47	97.47	95.57	96.84	98.10		94.94	95.57	93.04	91.77	91.77	91.77
AVS-BA VP2 HV	11	95.57	96.20	96.20	96.20	95.57	95.57	95.57	96.84	94.30	94.94		99.37	95.57	93.04	91.77	89.24
AVS-LA VP2 HV	12	96.20	96.84	95.84	96.84	96.20	96.20	96.20	97.47	94.94	95.57	99.37		96.20	93.67	92.41	89.87
AVS-DX VP2 HV	13	95.57	96.20	96.20	96.20	95.57	95.57	95.57	95.57	92.41	93.04	95.57	96.20		91.77	91.14	88.61
AVS-SCH VP2 HV	14	94.30	94.30	94.30	94.30	93.67	93.67	93.67	94.94	91.77	91.77	93.04	93.67	91.77		91.14	89.24
STC VP2 HV	15	92.41	93.04	93.04	93.04	92.41	92.41	92.41	93.04	91.77	91.77	91.77	92.41	91.14	91.14		91.77
AVS-RRV VP2 HV	16	89.87	90.51	90.51	90.51	91.14	91.14	89.87	90.51	89.87	91.77	89.24	89.87	88.61	89.24	91.77	

**Figure 3.7** Pairwise comparison of IBDV capsid protein VP2 hypervariable region amino acid sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Var E VP2 HV	1		97.89	97.04	97.46	97.25	97.04	98.63	96.19	97.25	95.56	96.19	95.35	95.98	94.08	94.29	95.14
AL-2 VP2 HV	2	97.89		95.14	97.04	96.62	95.98	95.98	96.62	96.83	93.66	95.14	94.29	94.93	92.81	92.39	94.71
AVS-BA VP2 HV	3	97.04	95.14		94.71	94.71	94.50	94.08	93.87	94.93	93.02	95.77	93.87	94.50	91.33	92.81	93.02
AVS-MT VP2 HV	4	97.46	97.04	94.71		96.19	95.56	95.98	95.35	96.41	94.93	94.71	93.45	94.08	93.66	94.08	93.87
AVS-MB VP2 HV	5	97.25	96.62	94.71	96.19		97.67	96.83	96.62	95.98	93.23	94.50	93.87	94.50	91.97	92.39	94.71
AVS-TV VP2 HV	6	97.04	95.98	94.50	95.56	97.67		97.46	96.83	96.19	93.23	94.08	93.45	93.87	91.75	92.18	94.08
AVS-MD VP2 HV	7	96.83	95.98	94.08	95.98	96.83	97.46		96.41	95.77	93.02	93.45	92.81	93.45	92.18	92.60	94.08
AVS-AX VP2 HV	8	96.19	96.62	93.87	95.35	96.62	96.83	96.41		95.77	92.39	93.45	93.45	93.66	91.54	91.12	94.29
AVS-CN VP2 HV	9	97.25	96.83	94.93	96.41	95.98	96.19	95.77	95.77		93.45	94.50	94.08	94.50	92.18	91.97	93.66
STC VP2 HV	10	95.56	93.66	93.02	94.93	93.23	93.23	93.02	92.39	93.45		93.02	91.97	92.39	94.93	93.23	91.33
AVS-LA VP2 HV	11	96.19	95.14	95.77	94.71	94.50	94.08	93.45	93.45	94.50	93.02		92.60	93.23	90.91	91.75	92.39
AVS-BM VP2 HV	12	95.35	94.29	93.87	93.45	93.87	93.45	92.81	93.45	94.08	91.97	92.60		98.31	90.70	90.06	91.33
AVS-RO VP2 HV	13	95.98	94.93	94.50	94.08	94.50	93.87	93.45	93.66	94.50	92.39	93.23	98.31		91.75	90.91	91.97
AVS-RRV VP2 HV	14	94.08	92.81	91.33	93.66	91.97	91.75	92.18	91.54	92.18	94.93	90.91	90.70	91.75		91.75	90.49
AVS-SCH VP2 HV	15	94.29	92.39	92.81	94.08	92.39	92.18	92.60	91.12	91.97	93.23	91.75	90.06	90.91	91.75		90.91
AVS-DX VP2 HV	16	95.14	94.71	93.02	93.87	94.71	94.08	94.08	94.29	93.66	91.33	92.39	91.33	91.97	90.49	90.91	

**Figure 3.8** Pairwise comparison of IBDV capsid protein VP2 hypervariable region nucleotide sequence percent identities.



**Figure 3.9** Conservation of IBDV VP2 hypervariable region amino acid sequences. The hypervariable region of VP2 includes two hydrophobic peaks, A and B, which are designated P<sub>BC</sub> (amino acids 212-224) and P<sub>HI</sub> (amino acids 314-325) respectively. There are two additional loops, P<sub>DE</sub> (amino acid 253) and P<sub>FG</sub> (amino acid 284).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AVS-LA VP3	1		100.00	100.00	100.00	99.57	99.15	99.57	99.57	99.57	99.57	99.15	98.72	99.15	98.72	99.15	99.72
AVS-MB VP3	2	100.00		100.00	100.00	99.57	99.15	99.57	99.57	99.57	99.57	99.15	98.72	99.15	98.72	99.15	99.72
AVS-TV VP3	3	100.00	100.00		100.00	99.57	99.15	99.57	99.57	99.57	99.57	99.15	98.72	99.15	98.72	99.15	99.72
AVS-BA VP3	4	100.00	100.00	100.00		99.57	99.15	99.57	99.57	99.57	99.57	99.15	98.72	99.15	98.72	99.15	99.72
AVS-AX VP3	5	99.57	99.57	99.57	99.57		99.15	99.15	99.57	99.57	99.57	99.15	98.72	99.15	98.72	98.72	98.72
AVS-CN VP3	6	99.15	99.15	99.15	99.15	99.15		98.72	99.57	99.57	99.57	99.15	98.72	99.15	98.72	98.30	98.72
AVS-MT VP3	7	99.57	99.57	99.57	99.57	99.15	98.72		99.15	99.15	99.15	98.72	98.30	98.72	98.30	98.72	98.30
Var E VP3	8	99.57	99.57	99.57	99.57	99.57	99.57	99.15		100.00	100.00	99.57	99.15	99.57	99.15	98.72	99.15
AVS-DX VP3	9	99.57	99.57	99.57	99.57	99.57	99.57	99.15	100.00		100.00	99.57	99.15	99.57	99.15	98.72	99.15
AL-2 VP3	10	99.57	99.57	99.57	99.57	99.57	99.57	99.15	100.00	100.00		99.57	99.15	99.57	99.15	98.72	99.15
AVS-RRV VP3	11	99.15	99.15	99.15	99.15	99.15	99.15	98.72	99.57	99.57	99.57		98.72	99.15	98.72	98.30	98.72
AVS-BM VP3	12	98.72	98.72	98.72	98.72	98.72	98.72	98.30	99.15	99.15	99.15	98.72		99.57	98.30	97.87	98.30
AVS-RO VP3	13	99.15	99.15	99.15	99.15	99.15	99.15	98.72	99.57	99.57	99.57	99.15	99.57		98.72	98.30	98.72
STC VP3	14	98.72	98.72	98.72	98.72	98.72	98.72	98.30	99.15	99.15	99.15	98.72	98.30	98.72		97.87	98.30
AVS-MD VP3	15	99.15	99.15	99.15	99.15	98.72	98.30	98.72	98.72	98.72	98.72	98.30	98.87	98.30	97.87		97.87
AVS-SCH VP3	16	98.72	98.72	98.72	98.72	98.72	98.72	98.30	99.15	99.15	99.15	98.72	98.30	98.72	98.30	97.87	

**Figure 3.10** Pairwise comparison of IBDV structural, scaffolding protein VP3 amino acid sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AL-2 VP3	1		99.35	98.44	98.06	97.67	97.80	98.44	97.54	97.54	97.93	97.67	97.80	96.64	96.64	97.15	96.63
Var E VP3	2	99.35		98.31	98.19	97.54	97.67	98.31	97.41	97.41	97.80	97.54	97.67	96.51	96.64	97.25	96.37
AVS-RRV VP3	3	98.44	98.31		97.15	96.63	97.15	97.41	97.15	96.77	97.93	96.76	97.28	96.12	95.74	96.10	95.98
AVS-CN VP3	4	98.06	98.19	97.15		97.02	97.15	97.15	96.50	96.76	97.28	97.28	96.63	96.50	96.25	96.50	96.11
AVS-MD VP3	5	97.67	97.54	96.63	97.02		96.76	97.15	95.98	96.76	96.89	96.37	97.15	95.85	95.86	96.11	95.85
AVS-MT VP3	6	97.80	97.67	97.15	97.15	96.76		97.15	96.50	97.41	97.28	97.28	96.89	96.11	95.86	96.24	95.85
AVS-MB VP3	7	98.44	98.31	97.41	97.15	97.15	97.15		96.89	97.15	96.89	96.89	97.41	96.12	95.61	96.11	95.73
AVS-SCH VP3	8	97.54	97.41	97.15	96.50	95.98	96.50	96.89		96.12	96.50	96.37	96.24	95.47	95.61	96.24	94.82
AVS-AX VP3	9	97.54	97.41	96.77	96.76	96.76	97.41	97.15	96.12		96.76	96.50	96.64	96.24	96.37	96.37	95.85
STC VP3	10	97.93	97.80	97.93	97.28	96.89	97.28	96.89	96.50	96.76		96.89	96.76	95.98	95.99	96.37	96.37
AVS-DX VP3	11	97.67	97.54	96.76	97.28	96.37	97.28	96.89	96.37	96.50	96.89		95.98	95.73	96.12	96.37	95.59
AVS-TV VP3	12	97.80	97.67	97.28	96.63	97.15	96.89	97.41	96.24	96.64	96.76	95.98		95.86	95.22	95.73	95.60
AVS-BA VP3	13	96.64	96.51	96.12	96.50	95.85	96.11	96.12	95.47	96.24	95.98	95.73	95.86		94.95	95.08	96.76
AVS-RO VP3	14	96.64	96.64	95.74	96.25	95.86	95.86	95.61	95.61	96.37	95.99	96.12	95.22	94.95		98.19	94.95
AVS-BM VP3	15	97.15	97.15	96.11	96.50	96.11	96.24	96.11	96.24	96.37	96.37	96.37	95.73	95.08	98.19		95.59
AVS-LA VP3	16	96.63	96.37	95.98	96.11	95.85	95.85	95.73	94.82	95.85	96.37	95.59	95.60	96.76	94.95	95.59	

**Figure 3.11** Pairwise comparison of IBDV structural, scaffolding protein VP3 nucleotide sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AVS-AX VP4	1		99.63	99.26	99.26	99.26	99.63	99.26	99.63	99.63	99.26	98.51	98.14	98.14	98.51	98.14	98.51
AVS-CN VP4	2	99.63		99.26	99.26	99.63	100.00	99.63	100.00	100.00	99.63	98.51	98.51	98.51	98.88	98.51	98.51
AVS-LM VP4	3	99.26	99.26	99.63		98.88	99.26	99.63	99.26	99.26	98.88	99.26	98.51	98.51	98.88	98.51	98.88
AVS-MT VP4	4	99.26	99.26	98.88	98.88		99.26	99.63	99.26	99.26	98.88	98.88	98.51	98.51	98.88	98.51	99.26
AVS-MD VP4	5	99.26	99.63	98.88	98.88		99.63	99.26	99.63	99.63	99.26	98.14	98.14	98.14	98.51	98.14	98.14
AL-2 VP4	6	99.63	100.00	99.26	99.26	99.63		99.63	100.00	100.00	99.63	98.51	98.51	98.51	98.88	98.51	98.51
Var E VP4	7	99.26	99.63	99.63	99.63	99.26	99.63		99.63	99.63	99.26	98.88	98.88	98.88	99.26	98.88	98.88
AVS-MB VP4	8	99.63	100.00	99.26	99.26	99.63	100.00	99.63		100.00	99.63	98.51	98.51	98.51	98.88	98.51	98.51
AVS-DX VP4	9	99.63	100.00	99.26	99.26	99.63	100.00	99.63	100.00		99.63	98.51	98.51	98.51	98.88	98.51	98.51
AVS-TV VP4	10	99.26	99.63	98.88	98.88	99.26	99.63	99.26	99.63	99.63		98.88	98.14	98.14	98.88	98.51	98.14
AVS-BA VP4	11	98.51	98.51	99.26	98.88	98.14	98.51	98.88	98.51	98.51	98.88		97.77	97.77	98.51	98.14	98.14
STC VP4	12	98.14	98.51	98.51	98.51	98.14	98.51	98.88	98.51	98.51	98.14	97.77		99.26	98.14	97.77	97.77
AVS-RRV VP4	13	98.14	98.51	98.51	98.51	98.14	98.51	98.88	98.51	98.51	98.14	97.77	99.26		98.14	97.77	97.77
AVS- BM VP4	14	98.51	98.88	98.88	98.88	98.51	98.88	99.26	98.88	98.88	98.88	98.51	98.14	98.14		99.63	98.14
AVS-RO VP4	15	98.14	98.51	98.51	98.51	98.14	98.51	98.88	98.51	98.51	98.51	98.14	97.77	97.77	99.63		97.77
AVS- SCH VP4	16	98.51	98.51	98.88	99.26	98.14	98.51	98.88	98.51	98.51	98.14	98.14	97.77	97.77	98.14	97.77	

**Figure 3.12** Pairwise comparison of IBDV viral protease VP4 amino acid sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AVS-MB VP4	1		97.81	98.22	97.53	97.12	96.30	96.17	96.44	96.31	96.02	96.30	96.58	95.89	95.89	95.62	95.75
AL-2 VP4	2	97.81		98.22	96.98	96.58	96.03	95.90	95.89	96.31	96.30	96.85	96.16	96.16	96.16	95.49	95.75
Var E VP4	3	98.22	98.22		97.94	97.26	97.26	97.40	97.40	96.72	97.26	96.85	96.58	97.12	97.12	96.85	96.84
AVS-TV VP4	4	97.53	96.98	97.94		97.26	95.48	95.90	96.30	96.03	95.61	96.44	96.03	95.75	96.30	96.03	95.88
AVS-MD VP4	5	97.12	96.58	97.26	97.26		95.34	95.48	96.02	95.89	94.93	96.43	95.75	96.02	95.47	95.75	94.93
AVS-BM VP4	6	96.30	96.03	97.26	95.48	95.34		98.49	96.16	95.48	95.48	95.75	95.06	95.47	95.20	95.34	95.07
AVS-RO VP4	7	96.17	95.90	97.40	95.90	95.48	98.49		96.85	95.75	95.35	95.75	94.93	95.62	95.62	96.30	94.66
AVS-LA VP4	8	96.44	95.89	97.40	96.30	96.02	96.16	96.85		95.48	95.07	95.47	94.92	96.30	95.88	97.26	94.93
AVS-AX VP4	9	96.31	96.31	96.72	96.03	96.89	95.48	95.75	95.48		94.80	96.58	95.75	94.93	94.93	94.93	94.66
AVS-SCH VP4	10	96.02	96.30	97.26	95.61	94.93	95.48	95.35	95.07	94.80		94.66	94.38	96.03	95.34	94.66	95.20
AVS-BA VP4	11	96.30	96.85	96.85	96.44	96.43	95.75	95.75	95.47	96.58	94.66		95.47	96.43	95.47	94.93	95.21
STC VP4	12	96.58	96.16	96.58	96.03	95.75	95.06	95.93	94.92	95.75	94.38	95.47		94.92	94.79	94.66	95.07
AVS-RRV VP4	13	95.89	96.16	97.12	95.75	96.02	95.47	95.62	96.30	95.93	96.03	96.43	94.92		95.88	95.48	95.62
AVS- BM VP4	14	95.89	96.16	97.12	96.30	95.47	95.20	95.62	95.88	94.93	95.34	95.47	94.79	95.88		95.07	96.85
AVS-RO VP4	15	95.62	95.49	96.85	96.03	95.75	95.34	96.30	97.26	94.93	94.66	94.93	94.66	95.48	95.07		94.66
AVS- SCH VP4	16	95.75	95.75	96.84	95.88	94.93	95.07	94.66	94.93	94.66	95.20	95.21	95.07	95.62	96.85	94.66	

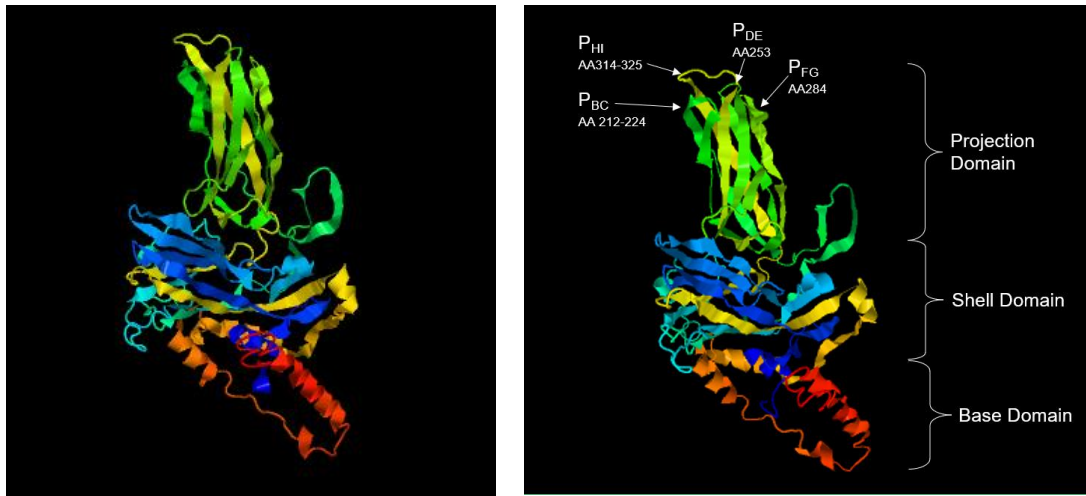
**Figure 3.13** Pairwise comparison of IBDV viral protease VP4 nucleotide sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AVS-BM VP5	1		99.32	98.63	97.95	97.95	97.95	97.95	97.95	96.58	96.58	97.26	96.58	94.52	95.21	94.52	93.84
AVS-RO VP5	2	99.32		97.95	97.26	97.26	97.26	97.26	97.26	95.89	95.89	96.58	95.89	93.84	94.52	93.84	93.15
STC VP5	3	98.63	97.95		99.32	99.32	97.95	99.32	99.32	97.95	96.58	97.26	96.58	95.89	96.58	94.52	95.21
AVS-RRV VP5	4	97.95	97.26	99.32		98.63	97.26	98.63	98.63	97.26	95.89	96.58	95.89	95.21	95.89	93.84	94.52
Var E VP5	5	97.95	97.26	99.32	98.63		98.63	100.00	100.00	98.63	97.26	97.95	97.26	95.21	97.26	93.84	95.89
AVS-MB VP5	6	97.95	97.26	97.95	97.26	98.63		98.63	98.63	97.26	97.26	97.95	97.26	93.84	95.89	93.84	94.52
AVS-DX VP5	7	97.95	97.26	99.32	98.63	100.00	98.63		100.00	98.63	97.26	97.95	97.26	95.21	97.26	93.84	95.89
AL-2 VP5	8	97.95	97.26	99.32	98.63	100.00	98.63	100.00		98.63	97.26	97.95	97.26	95.21	97.26	93.84	95.89
AVS-MT VP5	9	96.58	95.89	95.89	97.95	97.26	98.63	97.26	98.63		95.89	96.58	97.26	95.21	96.58	93.84	94.52
AVS-AX VP5	10	96.58	95.89	96.58	95.89	97.26	97.26	97.26	97.26	95.89		96.58	96.58	92.47	94.52	92.47	93.15
AVS-MD VP5	11	97.26	96.58	97.26	96.58	97.95	97.95	97.95	97.95	96.58	96.58		97.95	93.15	95.21	93.15	93.84
AVS-TV VP5	12	96.58	95.89	96.58	95.89	97.26	97.26	97.26	97.26	97.26	96.58	97.95		92.47	95.21	92.47	93.15
AVS-BA VP5	13	94.52	93.84	95.89	95.21	95.21	93.84	95.21	95.21	95.21	92.47	93.15	92.47		92.47	93.84	94.52
AVS-SCH VP5	14	95.21	94.52	96.58	95.89	97.26	95.89	97.26	97.26	96.58	94.52	95.21	95.21	92.47		91.78	93.15
AVS-CN VP5	15	94.52	93.84	94.52	93.84	93.84	93.84	93.84	93.84	93.84	92.47	93.15	92.47	93.84	91.78		90.41
AVS-LA VP5	16	93.84	93.15	95.21	94.52	95.89	94.52	95.89	95.89	94.52	93.15	93.84	93.15	94.52	93.15	90.41	

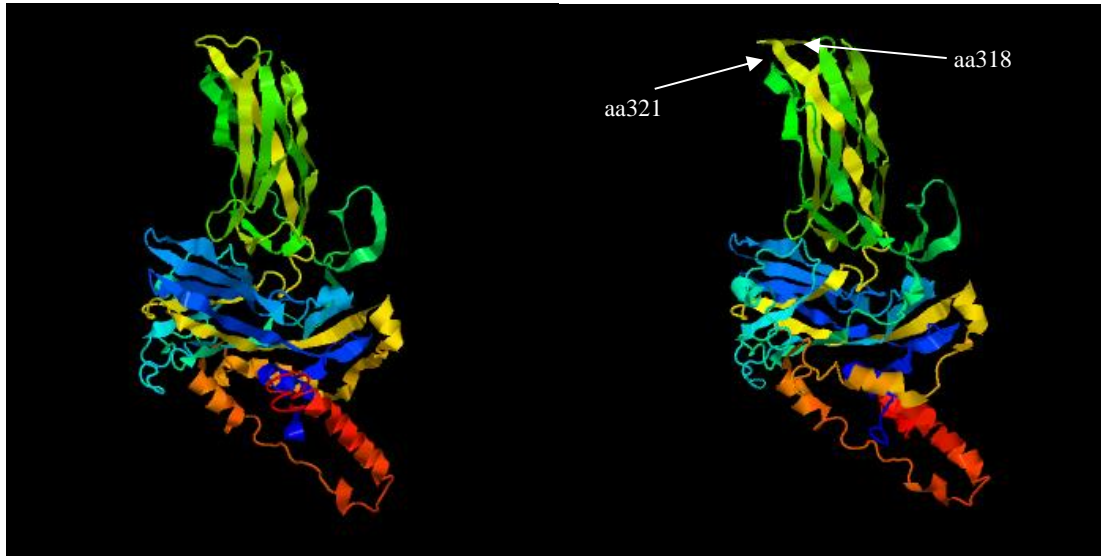
**Figure 3.14** Pairwise comparison of IBDV non-structural protein VP5 amino acid sequence percent identities.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AL-2 VP5	1		100.00	99.54	99.09	99.77	99.32	99.09	99.09	99.32	98.86	98.63	99.09	98.86	98.17	97.95	98.40
Var E VP5	2	100.00		99.54	99.09	99.77	99.32	99.09	99.09	99.32	98.86	98.63	99.09	98.86	98.17	97.95	98.40
STC VP5	3	99.54	99.54		99.54	99.32	98.86	98.63	99.09	99.32	98.40	98.17	98.63	98.40	98.17	97.95	97.95
AVS-RRV VP5	4	99.09	99.09	99.54		98.86	98.40	98.17	98.63	98.86	97.95	97.72	98.17	97.95	97.72	97.49	97.49
AVS-DX VP5	5	99.77	99.77	99.32	98.86		99.09	98.86	98.86	99.09	98.63	98.40	98.86	98.63	97.95	97.72	98.17
AVS-MD VP5	6	99.32	99.32	98.86	98.40	99.09		98.86	98.86	99.09	98.63	98.86	98.40	98.17	97.49	97.72	97.72
AVS-MB VP5	7	99.09	99.09	98.63	98.17	98.86	98.86		98.63	98.86	98.40	98.17	98.17	97.95	97.26	97.49	97.49
AVS-RO VP5	8	99.09	99.09	99.09	98.63	98.86	98.86	98.63		99.77	98.40	98.17	98.17	97.95	97.72	97.95	97.49
AVS-BM VP5	9	99.32	99.32	99.32	98.86	99.09	99.09	98.86	99.77		98.63	98.40	98.40	98.17	97.95	98.17	97.72
AVS-AX VP5	10	98.86	98.86	98.40	97.95	98.63	98.63	98.40	98.40	98.63		98.63	98.40	97.72	97.03	97.26	97.26
AVS-TV VP5	11	98.63	98.63	98.17	97.72	98.40	98.86	98.17	98.17	98.40	98.63		99.09	97.72	96.80	97.03	97.03
AVS-MT VP5	12	99.09	99.09	98.63	98.17	98.86	98.40	98.17	98.17	98.40	98.40	99.09		98.17	97.72	97.49	97.49
AVS-SCH VP5	13	98.86	98.86	98.40	97.95	98.63	98.17	97.95	97.95	98.17	97.72	97.72	98.17		97.03	96.80	97.26
AVS-BA VP5	14	98.17	98.17	98.17	97.72	97.95	97.49	97.26	97.72	97.95	97.03	96.80	97.72	97.03		97.49	97.72
AVS-CN VP5	15	97.95	97.95	97.95	97.49	97.72	97.72	97.49	97.95	98.17	97.26	97.03	97.49	96.80	97.49		96.58
AVS-LA VP5	16	98.40	98.40	97.95	97.49	98.17	97.72	97.49	97.49	97.72	97.26	97.03	97.49	97.26	97.72	96.58	

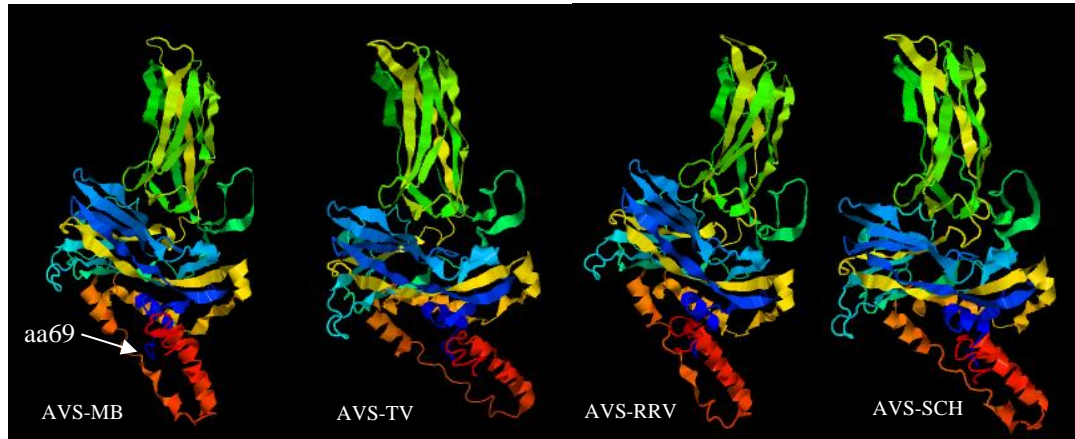
**Figure 3.15** Pairwise comparison of IBDV non-structural protein VP5 nucleotide sequence percent identities.



**Figure 3.16** Comparison of STC and Delaware Variant E 3D proteins models. STC is on the left and Delaware Variant E is on the right. The structure of the projection domain of STC and Delaware Variant E is different, while the base and shell domains are more conserved.



**Figure 3.17** Comparison of 3D proteins models of Delaware Variant E sequence with and without mutations D318N and A321E. These amino acids are located within loop P<sub>HI</sub>, which has previously been associated with antigenicity. The model on the left does not contain mutations, while the model on the right does. The position of amino acids 318 and 321 are labelled. The projection region of the sequence with mutations at 318 and 321 appears different from the model without mutations.



**Figure 3.18** Three-dimensional protein models of AVS-MB, AVS-TV, AVS-SCH and AVS-RRV. There are visible differences in the projection domain. AVS-MB and AVS-TV have an amino acid change at position 69, which is located in the base domain.

## **Chapter 4**

### **SUMMARY AND CONCLUSIONS**

The purpose of the work described in this thesis was to serologically and genomically characterize several IBDVs isolated from commercial broiler flocks affected with bursal lesions at less than 24 days of age. Preliminary virus neutralization assays were performed to test field IBDVs against prevalent vaccine virus Delaware Variant E antiserum. Results showed poor neutralization indices, demonstrating the necessity for a new IBDV vaccine candidate which elicits highly cross-neutralizing antibodies.

Serological evaluation was utilized to assess antigenicity of field isolates compared to each other and to contemporary IBDVs to evaluate cross-neutralization potential of field IBDVs and identify potential vaccine candidates. The whole genomes of contemporary IBDV strains and field isolates with varying levels of cross-neutralization potential were sequenced with the objective of finding correlations between genotypes and serotypes or pathotypes.

Serological evaluation was performed using Virus stocks and antiserum that were created in specific-pathogen-free leghorns inoculated with bursal homogenate from field or contemporary IBDVs. Virus neutralization assays were performed to evaluate cross-neutralizing potential of isolates. Field IBDVs were shown to neutralize each other to varying extents. Two highly cross-protective isolates, AVS-MB and AVS-TV were identified, which should be further evaluated as potential vaccine candidates. AVS-MB and AVS-TV antiserum neutralized 10/10 and 9/10 evaluated IBDVs respectively. Two other field IBDVs, AVS-SCH and AVS-RRV did not

effectively neutralize any of the evaluated IBDVs. Cross-neutralizing potential of other field isolates and contemporary strains fell between these ranges.

In addition to serological evaluation, genomic characterization was performed. The whole genomes of sixteen IBDVs with varying levels of cross-neutralization potential were sequenced using Illumina high-throughput technology with the objective of finding correlations between genotype and serotype or pathotype. Pairwise comparisons of amino acid sequences of the five viral proteins encoded by IBDV showed that overall, the IBDV genome was highly conserved (>90%). The most conserved proteins were VP1 (98.3% to 100.0%) and VP4 (98.8% to 100.0%) were most conserved, followed by capsid proteins VP2 (96.0% to 100%) and VP3 (97.9% to 100.0%), while VP5 had the lowest percent identity (90.4% to 100.0%).

Amino acid sequence alignments were created for all viral proteins, and specific amino acid mutations in capsid protein VP2, which has previously been associated with antigenicity, were identified. Two three-dimensional predictive protein models were created for Delaware Variant E sequences with and without insertion of a pair of amino acid changes, D318N and A321E. This mutation resulted in a conformational change in an antigenically significant region of VP2.

Three-dimensional predictive protein models were also created for highly neutralizing IBDVs AVS-MB, AVS-TV and poorly neutralizing IBDVs AVS-SCH and AVS-RRV. The projection domains of AVS-SCH and AVS-RRV appeared different from AVS-SCH, AVS-TV, STC, and DE Variant E models. This change was expected, because changes in known antigenic epitopes of VP2 may cause IBDVs with these changes to elicit poorly cross-neutralizing antibodies.

Research conducted here demonstrated that contemporary field IBDVs neutralized each other to varying extents, and two of the evaluated isolates, AVS-MB and AVS-TV elicited highly cross-neutralizing antibodies. These IBDV should be tested further as potential vaccine candidates. Genome sequencing showed several amino acid changes in field isolates. At least one of the amino acid changes located in VP2 resulted in a conformational change of an antigenically significant location when evaluated in a 3D protein model.

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**Appendix A**  
**PCR PRIMERS**

<b>Test Virus</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
Astrovirus	<i>CAstV-pol-F</i> 5'-KCA TGG CTY CAC CGY AAD CA-3'	<i>CAstV-pol-R</i> 5'-CGG TCC ATC CCT CTA CCA GAT TT-3'
Reovirus	<i>ARV-L1-F</i> 5'- TCTCTCTCAGCGCCCGTCCC- 3'	<i>ARV-L1-R</i> 5'- TGGACGACGCACACCGTTCG- 3'
Adenovirus	<i>FAdV-F</i> 5'- CAARTTCAGRCAGACGGT-3'	<i>FAdV-R</i> 5'- TAGTGATGMC GSGACATCAT- 3'

**Appendix B**

**IACUC PROTOCOL APPROVAL**

UNIVERSITY OF DELAWARE  
COLLEGE OF AGRICULTURAL & NATURAL RESOURCES INSTITUTIONAL  
ANIMAL CARE AND USE COMMITTEE

Animal numbers used  
for Use of Agricultural Animals in Research and Teaching

IACUC Protocol Approval Number: 56R-2019-O

Please check appropriate box: Teaching/Outreach

Research

Proposal Title: Characterization of IBDV/Reovirus field isolates seed stock and polyclonal antibody preparation

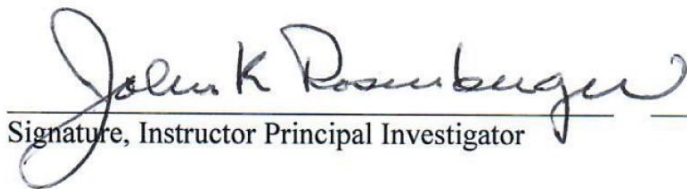
Instructor/Principal Investigator or Supervisor: Dr. John K. Rosenberger (AviServe LLC)

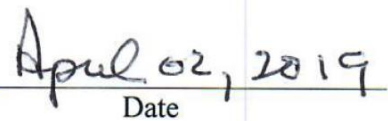
Co-investigator(s) if applicable: Drs. Sandra C. Rosenberger and Milos Markis  
Description of Animals: Chickens Common Name: Leghorns

Estimated Number: 100

Breed: White leghorns

Source: Charles River SPAFAS

  
Signature, Instructor Principal Investigator

  
Date