CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUSES
ISOLATED FROM COMMERCIAL CHICKENS

by

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ABSTRACT

Infectious bursal disease virus (IBDV) field isolates recovered in 2007 from Delmarva Peninsula broiler farms with a history of poor performance were characterized. The isolates originated from 3- and 4-week-old broilers having only passive breeder vaccinations. A 743 base pair fragment of the VP2 coding region of each of the field isolates was amplified by reverse transcriptase-polymerase chain reaction. VP2 sequencing and phylogenetic analysis showed that all of the isolates distributed into six different clades representing serotype 1 Delaware variant viruses.

Two active vaccination-challenge experiments were performed to test the antigenic and immunogenic properties of the IBDV field isolates and examine their ability to break through immunity to a Delaware variant vaccine 89/03 and a classic vaccine PBG98. Five field isolates representative of each molecular clade were used as challenge viruses in three-week-old specific pathogen free (SPF) White Leghorn chickens vaccinated with the Delaware variant and classic vaccines. The five field isolates induced variant-like gross lesions in all non-vaccinated SPF chicks. However, a single subcutaneous injection of either the 89/03 or PBG98 live, attenuated vaccine was sufficient to protect chickens against challenge with the five field isolates.

Virus neutralization (VN) using antiserum produced against the five field isolates showed no difference in neutralization of the 89/03 and PBG98 strains. This similarity in
neutralization indicated that the two cell-culture-adapted strains may not differ enough on the basis of antigenicity to reveal a difference between the five IBDV field isolates.

The relatedness of the field isolates to other known variant IBDV strains was evaluated using VP2 sequence analysis and two monoclonal antibody (MAb) methodologies, transfection and immunofluorescence and whole virus, with an antigen capture enzyme linked immunosorbent assay (AC-ELISA). Isolate 4813 was determined to be most related to Delaware variant E (Del E) based on a 99.5% identity at the amino acid level and its reactivity with monoclonal antibodies (MAbs) 63 and 67. Isolates 4947 and 4955 were determined to be most related to variant vaccine strain, RS593, based on a 98.1% identity at the amino acid level and similar MAb reactivity patterns. Isolate 5041 was found to be most antigenically similar to the GLS variant by the whole virus/ELISA MAb test, but only 96.8% related at the amino acid level. Isolate 5038 failed to react with MAbs used in either AC-ELISA experiment, which indicated that it may differ antigenically from the IBDV reference strains tested.

Genomic characterization and comparison of the variable region of VP2 of the five field isolates revealed amino acid substitutions in the four hydrophilic peaks previously identified as being important for antigenic variation and the binding of neutralizing antibodies. However, most of the VP2 sequence changes observed for the five field isolates closely resembled those of other characterized variant strains.

A passive immunity progeny challenge was performed to assess passive IBDV immunity in leghorn chicken progeny with maternal antibodies to IBDV. Ten-day-old chicks challenged with isolate 5038 were less protected than chicks challenged with Del
E. This finding indicated that 5038 was able to break through maternally-derived immunity earlier than Del E.

The findings of this study indicated that field isolates 4813, 4947, 4955, and 5041 shared sequence and antigenic similarities with Delaware variant strains of IBDV. The isolation of these viruses from commercial Delmarva broilers at 3-4 weeks of age is not surprising as maternal antibodies to the virus normally are sufficiently low to permit infection. On the other hand, solate 5038 appeared to be antigenically different than IBDV reference strains Del E, STC, GLS, RS593, and AL-2 based on MAb testing and antigenically different from Del E based on progeny challenge findings and warrants further investigation.
Infectious Bursal Disease (IBD) is caused by an acute, highly contagious

*Birnavirus* that results in mortality and immunosuppression of young chickens (1). Since its original isolation in Gumboro, Delaware, the disease has inflicted profound economic losses on the poultry industry worldwide (2). Infectious bursal disease virus (IBDV) and other *Birnaviruses* are single-shelled, non-enveloped viruses that contain a bi-segmented, double-stranded RNA genome (3, 4, 5, 6, 7, 8). The VP2 protein is the major protective antigen of IBDV that contains specific epitopes responsible for inducing neutralizing antibody responses (9, 10, 11). Amino acid changes that occur within the variable region of VP2 can lead to variations in antibody recognition, as well as, antigenicity, immunogenicity, virulence, and tissue tropism of IBDV strains (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21). Two serotypes of IBDV have been identified; however, only serotype I viruses are naturally pathogenic to chickens (22, 23, 24). Serotype I strains are classified as classic, variant or very virulent IBDV strains and differ in their virulent, antigenic, and pathogenic properties (22, 25, 26, 27).
The cloacal bursa is the target organ of IBDV infections; however, IBD viral replication also occurs in other lymphoid structures including the spleen, thymus, Harderian gland, and cecal tonsils (28, 29, 30, 31, 32). The virus preferentially affects actively proliferating and differentiating B lymphocytes, which leads to an age-dependent immunosuppression (33). Chicks infected less than one-week of age suffer severe and permanent B-cell immunosuppression (31, 34, 35, 36, 37). The immunosuppressive effects of IBDV infections not only enhance the chicken’s susceptibility to secondary opportunistic infections such as gangrenous dermatitis, chicken anemia agent, inclusion body hepatitis, respiratory diseases, and \textit{E. coli} infections among others, but frequently interfere with effective immune responses to vaccination (38, 39, 40, 41, 42, 43, 44).

During 2008, the value of broiler chickens produced in the Delmarva poultry industry, alone, was over $2.1 billion (45). Therefore, minimizing immunosuppression and its significant health and economic impacts is an ongoing challenge for broiler companies. The primary method used for controlling IBD in the poultry industry is vaccination (46, 47). The most common strategy is for hyperimmunized breeder flocks to confer high titers of maternal antibodies to progeny chicks (37). Priming vaccinations in the form of live-attenuated vaccines given at two to four weeks of age are followed by an inactivated oil-emulsion vaccination, eight to fourteen weeks later. This boosts immunity in breeder flocks and conveys a longer-lasting immunity to offspring. As a result, chicks are protected from early immunosuppressive IBDV infections (46, 48, 49, 50, 51, 52, 53). Because of differences in the effectiveness of breeder vaccination programs, broilers may be vaccinated in an effort to gain active immunity against IBDV. However, proper timing
is critical to avoid possible neutralization of the vaccine virus by persistent maternal antibodies (46, 49, 54, 55).

Common epitopes between IBDV pathotypes were demonstrated with the production of IBDV variant-derived vaccines. Classic strain-derived vaccines offered limited protection against variant IBDVs, whereas variant vaccines protected against classic strains, as well as, homologous and heterologous variant strains (56, 57, 58). The ability of IBDVs to cross-protect has played an important role in the development of improved vaccines. Both classic and variant strains are often incorporated into commercially available live and killed vaccines, so as to broaden the range of antigenic subtypes and elicit a heightened immune response. However, the dose and strains of both the vaccine and challenge viruses modulate the degree of protection afforded by these vaccines (17, 58, 59). Differences in maternal antibody titers and vaccination route, as well as, coexisting infections and the high mutation rate of RNA viruses, can strongly influence when and if novel variant strains arise within the poultry industry (39, 47, 59).

In late 2007, thirty-three field isolates were obtained from Delmarva Peninsula broiler farms that had a history of poor performance. Poor performing farms typically exhibit clinical or subclinical disease shown by high mortality, morbidity, or condemnations at processing (i.e. air sacculitis, septicemia/toxemia, Leucosis). Below-normal body weight, poor feed conversion, and immunosuppression are also characteristics of these problem farms. Disease monitoring of these farms has been used to track the incidence of Runting and Stunting Syndrome and immunosuppressive diseases in a company with the “Delmarva syndrome,” which is expressed as a
combination of Gangrenous Dermatitis, Respiratory complex, Inclusion Body Hepatitis, and/or RSS. This 28-day health monitoring study involved a weekly sampling of broilers (1-4 weeks of age) from 12 different farms within the Delmarva area. Isolates taken from grossly affected birds were chosen for this study based upon preliminary histopathology, virus isolation, serology, and bursa to body weight ratios.

This research project was designed to characterize strains of recently isolated IBD viruses. Fifteen out of the thirty-three isolates recovered from 3- and 4-week-old broilers were found positive through virus isolation, histopathology, and RT-PCR and designated for further investigation (Table 3.2). The variable region of the VP2 protein of the chosen isolates was compared with that of existing classic and variant strains of IBDV. An evolutionary phylogenetic tree demonstrated that none of the field isolates were related to the classic strain of IBD, as all of the field isolates clustered with the Delaware variant E strain (Figure 4.1). The chosen isolates were distributed into six individual clades (Figure 4.1 and Table 3.3). From each clade, a representative isolate was selected for virus propagation and seed stock production of bursa of Fabricius origin using SPF white leghorn chickens (Table 3.3).

The specific objectives of this research are to sequence the encoding region of VP2 protein from IBDV field isolates, perform a comparative sequence analysis to assess genetic characteristics, and select viruses for further characterization; to perform monoclonal antibody testing on chosen isolates to determine their reactivity pattern compared to IBDV classic and variant reference strains; to determine virus-neutralizing antibody titers for IBDV field isolates against IBDV strains 8903 and PBG98; to evaluate
the cross protection elicited by commercial IBDV vaccines against challenge with each of
the new field isolates; to determine the protection afforded by maternal antibodies against
challenge with a field isolate and reference strain of IBDV.
Chapter 2

REVIEW OF THE LITERATURE

2.1 History of Infectious Bursal Disease Virus

Infectious bursal disease (IBD) was first recognized as a distinct clinical entity in 1957 (2). A. S. Cosgrove initially described the malady as “avian nephrosis” on account of the tubular degenerative lesions found in the kidneys of infected broiler chickens. The syndrome adopted the name “Gumboro disease” since the first outbreaks occurred in and around the area of Gumboro, Delaware, USA. Predominant signs of illness included trembling, ruffled feathers, watery diarrhea, anorexia, depression, severe prostration, and death. In addition, hemorrhages in the thigh and leg muscles, increased mucus in the intestine, liver lobe infarction, renal damage, and enlargement of the bursa of Fabricius were lesions commonly observed at necropsy (2). Early studies suggested that the causative agent was a nephropathogenic strain of infectious bronchitis virus due to similar gross changes observed in the kidney by Winterfield and Hitchner (60). Subsequent studies (42, 61), however, revealed that IBV immunized birds could still be infected with the “infectious bursal agent” (IBA) and develop changes in their cloacal bursas specific for the disease. Following successful isolation of IBA in embryonated
chicken eggs (62), Hitchner (63) proposed that the disease be termed “infectious bursal disease” due to its pathognomonic bursa lesions.

The immunosuppressive effects of infectious bursal disease virus (IBDV) infections were first disclosed by Allen et al. (34). In 1980, a second serotype was reported (22). These factors, along with the high tendency for IBD infections to recur in successive flocks, emphasized the need for stringent measures of prevention and control.

Prior to 1984, spread of both the clinical and subclinical forms of the disease was satisfactorily controlled by vaccination programs. However, in 1984 and 1985, a significant increase in mortality, condemnations, and vaccine failures were reported in the Delmarva Peninsula broiler growing area (64, 65). These newly emergent viruses were capable of breaking through maternal immunity against classic strains of IBDV (25, 56, 57, 65). In vivo reciprocal cross-challenge tests showed that unlike classic or standard strains of IBDV, the field isolates caused rapid atrophy and minimal inflammation of the cloacal bursa when inoculated into susceptible SPF leghorns (56). Studies suggested that a major antigenic shift in serotype I viruses had occurred in the field (26, 66). The IBDV field isolates were characterized as antigenic “variants” of serotype 1 IBDV, while the older serotype 1 viruses discovered prior to these newly emergent viruses were called classic strains of IBDV (65). Currently in the United States, clinical cases are rarely reported and these variant strains are the predominant viruses circulating in the field (47).

Outbreaks of very virulent IBDV (vvIBDV) were first reported in Europe in 1987-1988 (67, 68, 69). Highly virulent IBDV (vvIBDV) infections are characterized by a peracute onset of severe clinical disease and high mortality (67, 68, 70, 71). Although
these new serotype 1 viruses demonstrate increased virulence in their ability to break through the existing level of maternal immunity; they are antigenically similar to the classic strains of IBDV (68, 72, 73). Strains of vvIBDV have rapidly disseminated to every poultry-producing country, except Canada, Mexico, Australia, and New Zealand (69, 74, 75, 76, 77).

2.2 Etiology of IBDV

Infectious bursal disease virus (IBDV) is classified as a member of the
Birnaviridae family (1). The family includes 3 genera: Aquabirnavirus whose type species is infectious pancreatic necrosis virus (IPNV), which infects fish, mollusks, and crustaceans; Avibirnavirus whose type species is infectious bursal disease virus (IBDV), which infects birds; and Entomobirnavirus whose type species is Drosophila X virus (DXV), which infects insects (78). Viruses in this family possess bi-segmented, double-stranded RNA (dsRNA) genomes, which are packaged into single-shelled, non-enveloped virions (3, 79). The capsid shell exhibits icosahedral symmetry composed of 32 capsomeres and a diameter ranging from 55 to 65 nm (6, 8, 80). Its structure is based on a T = 13 lattice composed of trimeric subunits. Cryoelectron microscopy and image processing analysis showed that the outer surface of the viral capsid is made up of 260 trimeric VP2 clusters, while the inner surface is composed of 200 Y-shaped trimeric VP3 structures (81, 82).
2.3 Chemical Composition of IBDV

Infectious bursal disease virus has a sedimentation rate of 460S in sucrose gradients (83, 84). The buoyant density of IBDV in caesium chloride gradients ranges from 1.31-1.34 g/ml for complete viral particles and below 1.33 g/ml for incomplete viral particles (7, 8, 84, 85, 86, 87, 88, 89).

Infectious bursal disease virus is very stable and can persist indefinitely in poultry houses despite thorough routine cleaning and disinfection (90). The virus is resistant to chloroform and ether treatments, and it remains unaffected after a 5 hour incubation at 56°C and pH 2, although, it can be inactivated at pH 12. Exposure to 0.5% phenol and 0.125% thimerosal for 1 hour at 30°C had no effect, while contact with 0.5% formalin for 6 hours markedly reduced virus infectivity (91). Landgraf et al. (92) demonstrated that IBDV is able to withstand 30 minutes at 60°C, but not 70°C and it is killed after 10 minutes in 0.5% chloramine disinfectant. In addition, invert soaps with 0.05% sodium hydroxide can inactivate or strongly inhibit the virus (93). Due to the economics of commercial poultry production, which involve the re-use of litter and short time intervals between flocks, as well as the virus’s resistance toward heat and several physical and chemical agents, IBDV survives in poultry houses for long periods (94).

The genome of IBDV is comprised of two segments of dsRNA as demonstrated by polyacrylamide gel electrophoresis (79, 83). The larger segment, A, is 3261 nucleotides long and contains two open reading frames (ORF). The first ORF, preceding and partially overlapping ORF2, encodes a 17 kDa non-structural protein, known as VP5 (95). The second ORF encodes a 110 kDa precursor polyprotein (NH3-VPX-VP4-VP3-
COOH), which is co-translationally processed to yield the protein capsid precursor VPX or pVP2 (48 kDa), and proteins VP4 (28 kDa) and VP3 (32 kDa) (96). The pVP2 is further processed by serial cleavages near its carboxy-terminus into mature VP2 (41 kDa) and four peptides which remain associated with the virion (9, 97). VP2 and VP3 are the major structural proteins of the virion, whereas VP4 is a virus-encoded protease (9, 98, 99). The pVP2 initially undergoes VP4-mediated processing events to yield shorter pVP2 polypeptides (100). Recently, Irigoyen et al. (100) proposed that VP2 Asp-431 is responsible for catalyzing the last pVP2 to VP2 proteolytic event that occurs during capsid maturation. Genomic segment B, which is 2827 nucleotides long, encodes VP1, a 97-kDa RNA dependent-RNA-polymerase (RdRp) (101). In both genomic segments, short 5’ and 3’ terminal sequences (79 to 111 nucleotides long) flank the coding regions (102). The 3’ untranslated regions of both the A and B segment have the potential to form stem and loop secondary structures that may be essential for RNA replication (103).

2.4 Viral Proteins of IBDV

In virions, VP1 exists as both a covalently bound protein at the 5’ ends of the genomic dsRNA strands, as well as, a free polypeptide (4, 104). It is responsible for viral RNA replication following cellular infection and mRNA synthesis (105). It has also been reported that VP1 may play an important role in IBDV virulence (106). The catalytic motifs of polymerases from birnaviruses are arranged in a permuted order in the sequence (107). As a result, the structure of the birnavirus polymerase VP1 adopts a
unique active site topology that has not been previously found with other RNA and DNA polymerases (107).

VP2 (441 amino acids [aa]) is the main capsid protein, constituting 51% of the viral proteins in serotype 1 viruses (1). It is the host-protective antigen, as it contains serotype- and strain-specific epitopes responsible for inducing neutralizing antibodies (9, 10, 11, 108). It has also been reported to be an apoptotic inducer in mammalian cells, but not in chicken embryo cells (109). VP2 is highly hydrophobic and folded into three distinct domains termed the base (B), shell (S), and projection (P) (20, 110, 111). The B and S domains are comprised of the conserved N- and C-terminal sequences of VP2, while the P domain contains the conformation-dependent central variable region of VP2 (aa 206 to 350) (13, 73, 112). The significance of protein conformation for VP2 interaction with monoclonal antibodies has been affirmed by several studies where denaturing conditions using Western immunoblotting prevented reactivity (21, 66, 73, 108). Within the central variable region are four stretches of hydrophilic amino acids that are more prone to antigenically significant amino acid changes (12, 13, 15). These areas, known as major hydrophilic peaks A (aa 212-224) and B (aa 314-324) and minor hydrophilic peaks 1 (aa 249-254) and 2 (aa 279-290) (9, 15, 112, 113, 114, 115) reside in the most exposed parts of the P domain (20).

The VP3 protein contains both conformational-independent, group-specific epitopes (common in both serotypes) and serotype-specific epitopes (116, 117) that elicit non-neutralizing and non-protective antibodies (11, 108). This multifunctional protein
interacts with VP1, VP2, and with genomic double-stranded RNA and plays a pivotal role in virus assembly and morphogenesis (100, 118, 119, 120, 121).

VP4 is described as a minor, non-structural viral protease. Using a catalytic serine-lysine (Ser-652 and Lys-692) dyad conserved among bacterial Lon proteases, VP4 is responsible for the proteolytic processing of the precursor polyprotein (122, 123, 124). Sanchez and Rodriguez (124) identified two cleavage sites, $^{511}$LAA$^{513}$ and $^{754}$MAA$^{756}$, that are important for the processing of the pVP2–VP4 and VP4–VP3 precursors, respectfully. In addition, the self-assembling VP4 protease gives rise to specific microtubules (type II tubules), which accumulate within infected cells; however, they are not components of the mature virion (125).

VP5 is a highly basic, cysteine-rich protein that is conserved among all serotype 1 IBDV strains (126). Lombardo et al. (127) identified it as a class II transmembrane protein with a cytoplasmic N-terminal domain and an extracellular C-terminal domain. The VP5 protein is believed to play a role in viral egress, as well as, possess anti-apoptotic function at the early stages of IBDV infection (127, 128, 129). Although it is not essential for viral replication in cell culture (129), it does function in *in vitro* pathogenesis and dissemination (126, 127, 128).

### 2.5 Pathogenesis and Virus Replication of IBDV

Two known serotypes of IBDV, designated serotypes I and II, have been described (22, 89). The natural hosts of IBDV are the chicken and the turkey. Serotype I viruses affect every breed of chicken, but the most severe clinical signs and lesions and
the highest mortality rate have been observed in white leghorns (47). Turkeys, ducks, and ostriches can be naturally and experimentally infected with IBDV serotypes I and II, as evidenced by serological response and isolation; however, the infections are apathogenic (22, 89, 94, 130, 131, 132, 133, 134, 135, 136). Several other avian species including rooks, wild pheasants, crows, gulls, and falcons, were reported to be susceptible to infection or to possess antibodies against IBDV (137, 138). Yet, only serotype I viruses replicate in lymphoid cells and are pathogenic to chickens (23, 24, 89, 139). Pathotypes of serotype I strains are classified in increasing order of virulence as mild, intermediate (serotype I vaccines), variant virulent, classical virulent, and very virulent or hyper-virulent (75). The exhibition of clinical signs and gross and microscopic lesions is contingent upon the virus pathotype and chicken immune status (31).

While viral replication initially occurs in the intestine, the main target organ for pathogenic serotype I IBDV is the bursa of Fabricius, as it is the source for B lymphocytes in avian species (28, 140). This was demonstrated by experiments in which bursectomized chickens failed to develop clinical IBD, despite having IBDV infections lethal for non-bursectomized chickens (141). In vivo and in vitro studies have demonstrated that actively dividing, surface immunoglobulin M-bearing (IgM+) B lymphocytes (B-cells) are the target cell for IBDV cytolytic infection (31, 141, 142, 143). Moreover, the virus seems to affect immature B-cells to a greater degree than mature B cells (33, 144). Throughout the juvenile life of the bursa, IgM+ bearing cells continuously migrate to the bursa where they acquire the characteristics of mature, immunocompetent B cells. Postbursal cells are seeded out to peripheral lymphoid tissues until involution of
the bursa occurs with the onset of oviposition (145, 146). The period of greatest susceptibility to clinical IBDV infections is between 3 and 6 weeks after hatching (37, 147). The exact cellular receptor for IBDV attachment has not been identified; however, it has been suggested that IBDV serotypes I and II may use multiple receptors, serotype-specific or common to both serotypes, on different cell types (47, 140). The mechanism of viral RNA synthesis has not been clearly determined (47, 148). Genome-linked proteins have been described, indicating IBDV replicates its nucleic acid by a semi-conservative mechanism (105, 148, 149, 150).

Following host entry via oral infection or inhalation, IBDV initiates replication in lymphocytes and macrophages of the gut-associated lymphoid tissues (GALT) such as the cecum, duodenum, and jejunum. This stage of viral replication marks the primary viremia. Within 5 hours post-infection, viral antigen reaches the liver, where it is trapped and phagocytized by resident macrophages (Kupffer cells). Virus then enters the bloodstream where it is distributed to other tissues including the bursa of Fabricius. By 13 hours post-infection, most bursal follicles are positive for virus and extensive necrosis of immature B-cells in the medullary and cortical regions of the bursal follicles ensues (28, 151, 152). By 16 hours post-infection, a second massive viremia occurs with secondary replication in other B-lymphocyte-containing tissues including the spleen, thymus, Harderian gland, and cecal tonsils (29, 30, 31, 32). Clinical signs and death may result from the acute phase (7-10 days) of IBD. Factors such as pathogenicity and virulence of a strain, as well as, the chicken’s age, breed, and immune status can influence the outcome and severity of infection (37, 153).
Virus replication during the acute lytic phase results in a dramatic reduction in circulating IgM+ cells and a prolonged suppression of the primary antibody response (152, 153, 154, 155, 156). Secondary antibody responses are unaffected (157). In chickens that survive the acute disease, virus replication subsides, and almost all bursal follicles become repopulated with IgM+ B cells. The primary antibody response is gradually restored to near normal levels (152). The destruction of Ig-producing B cells by IBDV may be one of the main inhibitors of humoral immunity; however, the involvement of other mechanisms such as altered antigen-presenting and helper T cell functions has been proposed (153).

Depletion of B-cells in the bursa is accompanied by an influx of activated CD4+ and CD8+ T-lymphocytes (T-cells) (151, 153, 158, 159). By the seventh day of infection, CD8+ cells are the predominant infiltrating T-cell. T-cells are resistant to infection and replication of IBDV. However, IBDV infection can severely decrease the in vitro proliferative response of T cells to mitogens, indicating that cellular immune responses are also compromised (153). Evidence suggests that T cells may modulate IBDV immunopathogenesis by restricting IBDV replication in the bursa in the early stage of the disease (5 days post-infection). Through their release of cytokines and cytotoxic effects, T-cells may enhance bursal tissue destruction, suppress immunity, and delay recovery of bursa follicles. At the same time, T-cells may promote clearance of IBDV (153, 160, 161).

Along with its direct cytopathic effect on IgM+ cells, IBDV can infect cells of the monocyte-macrophage lineage (141, 152, 162, 163, 164, 165, 166, 167, 168, 169, 170).
IBDV-infected macrophages may play a role in virus dissemination from the gut to the bursa and other peripheral organs (166). In addition, activated macrophages may secrete chemotactic, proinflammatory, and other immunoregulatory cytokines that recruit heterophils, T cells, and macrophages to the site of infection. Elevated levels of these inflammatory mediators and chemokines during the acute phase can enhance local inflammation and tissue destruction (141, 152, 162, 163, 164, 166, 167, 168, 169, 170, 171).

The lack of inflammation observed with variant IBDV infections supports the theory that apoptosis, in addition to necrosis, is responsible for IBDV-induced cellular destruction and immunosuppression (172, 173, 174, 175). Apoptosis, or programmed cell death, occurs in both physiological and pathological conditions and is characterized by several morphological changes including cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation with no inflammation. IBDV induces apoptosis in chicken peripheral bursal lymphocytes, chicken embryos, chicken embryo fibroblasts, and Vero cells (172, 176, 177). Moreover, apoptosis has been reported in lymphoid cells and tissues of young chickens and embryos experimentally infected with pathogenic serotype I IBDV strains (173, 174, 175, 178, 179, 180, 181). Two viral proteins of IBDV, VP2 and VP5, are suspected to play a role in the induction of apoptosis (109, 126, 182). Apoptosis has been detected in antigen-negative cells in the vicinity of productively infected cells, suggesting that indirect mechanisms may also be involved in this process (174, 175, 183).
2.6 Transmission of IBDV

IBDV is highly contagious and ubiquitous. The disease can be spread by direct and indirect contact between infected and susceptible flocks. Once infected with IBDV, chickens are capable of shedding the virus in the feces for as long as 16 days (184). There is no evidence to suggest that IBDV is spread via transovarial transmission (47). Benton et al. (91) reported that poultry houses which previously harbored infected flocks remained infective for at least 122 days and that fomites (water, feed, droppings) contaminated with IBDV contribute to viral dissemination (91). No specific vectors or reservoirs of IBDV have been established, but the virus has been isolated from mosquitos (*Aedes vexans*), rats, and lesser mealworms (*Alphitobius diaperinus*) (47, 185, 186, 187, 188).

2.7 Incubation Period, Clinical Signs, and Mortality

IBDV has a short incubation period of 2 to 3 days and the infection generally lasts 5 to 7 days. One of the earliest signs of IBDV infection is the tendency for birds to engage in vent picking. Clinical signs are described as acute onset of depression, trembling, white or watery diarrhea, anorexia, prostration, ruffled feathers, vent feathers soiled with urates, and hemorrhages in pectoral and thigh muscles. In severe cases, birds become dehydrated, and in the terminal stages subnormal temperatures and death ensue (2, 31). Naive chickens between 3 and 6 weeks of age are most susceptible to the clinical form of IBD, which causes impaired growth, immunosuppression, and mortality (43, 90, 189). Clinical signs are mainly characteristic of IBDV serotype I classic strains (65). In
fully susceptible flocks, mortality associated with classic strain infections may range from 1-60%, with high morbidity of up to 100% (47, 113, 147). Variant IBDV strains do not produce overt clinical signs, but cause immunosuppression, and may cause mortality due to secondary opportunistic infections in immune compromised birds (37, 47, 190). In contrast, vvIBDV strains cause mortality of 50-60% in laying hens, 25-30% in broilers, and 90-100% in susceptible SPF leghorns (37, 67, 68, 70).

Susceptible chickens younger than three weeks of age may not exhibit clinical signs, but develop subclinical infections. This results in a decreased humoral antibody response due to B lymphocyte depletion in the cloacal bursa (184), and a severe and prolonged immunosuppression (34). The most significant economic losses result from subclinical infections. This form of IBD infection greatly enhances the chicken’s susceptibility to sequelae such as gangrenous dermatitis, chicken anemia virus, inclusion body hepatitis, respiratory diseases, and bacterial infections (38, 39, 40, 41, 43, 191, 192). Moreover, it frequently interferes with effective immune responses to vaccination against Newcastle disease, Marek’s disease, and infectious bronchitis (34, 35, 42, 193).

2.8 Gross Lesions

Gross lesions observed in birds that succumb to IBDV infection include dehydration of the breast and leg musculature, darkened discoloration of the pectoral muscles, occasional hemorrhages in the leg, thigh, and pectoral muscles, increased mucus in the intestine, and renal changes. The gross appearance of the kidneys may appear normal in birds that are necropsied during the course of infection. In birds that die or are
in advanced stages of the disease, kidneys frequently show swelling and pallor with heavy accumulation of urates in the tubules and ureters (47).

The bursa of Fabricius is the predominant lymphoid organ affected by IBDV. Infections with classic strains of IBDV cause inflammation and hypertrophy of the bursa as early as day 3 post-infection. By day 4, the bursa is double its original size and weight due to edema and hyperemia. By day 5, the bursa returns to its normal weight, but continues to atrophy until reaching one-third or less of its original weight following day 8 post-infection (47). In contrast, variant strains of IBDV typically cause a rapid atrophy, mucosal edema, and firmness of the bursa in the absence of inflammation (31, 65, 194). Only one variant isolate (IN) has been reported to cause bursal inflammation (195). By day 2 or 3 post-infection, a gelatinous yellowish transudate covers the serosal surface of the bursa and longitudinal striations become visible. The bursa’s normal white color shifts to cream and then, in some cases, gray during and following the period of atrophy. In addition, necrotic foci and petechial or ecchymotic hemorrhages on the mucosal surface may be observed in infected bursas.

Moderate to severe splenomegaly with small gray foci uniformly distributed on the surface has been reported (47, 196). Occasionally, petechial hemorrhages will occur in the mucosa at the juncture of the proventriculus and gizzard (2, 47). Compared to moderately pathogenic IBDV strains, vvIBDV strains induce similar bursal lesions, but cause more severe damages to the cecal tonsils, thymus, spleen, and bone marrow (14, 47, 165).
2.9 Microscopic Lesions

IBDV infections produce microscopic lesions primarily in the lymphoid tissues (i.e. cloacal bursa, spleen, thymus, cecal tonsils, and Hardarian gland). Pathologic observations of experimental cases were reported by Helmboldt and Garner (29) and Cheville (196). Degeneration and necrosis of B lymphocytes in the medullary region of the bursal follicles is apparent within one day of exposure. Depleted lymphocytes are quickly replaced by heterophils, pyknotic debris, and hyperplastic reticuloendothelial (RE) cells. By day 3 or 4 post-infection, IBDV-associated lesions are visible within all bursal follicles. At this time, infections with classic IBDV strains have caused an inflammatory response marked by severe edema, heterophil infiltration, and hyperemia in the bursa. Inflammation diminishes by day 4 post-infection (PI), and as necrotic debris is cleared by phagocytosis, cystic cavities develop in the medullary areas of the lymphoid follicles. Necrosis and phagocytosis of heterophils and plasma cells occur within the follicle, as well as, in the interfollicular connective tissue. In addition, a fibroplasia in the interfollicular connective tissue may appear and the surface epithelium of the bursa becomes involuted and abnormal (36, 196). Proliferation of the bursal epithelial layer generates a glandular structure of columnar epithelial cells that contains globules of mucin. During this stage of the infection, scattered foci of repopulating lymphocytes were observed; however, these did not develop into healthy follicles (29, 197). Microscopic lesions caused by variant strains are characterized by extensive follicular lymphoid depletion and rapid plical atrophy of the cloacal bursa in the absence of an inflammatory response (31, 197).
In the early stages of infection, the spleen exhibits hyperplasia of reticuloendothelial cells surrounding the adenoid sheath arteries. By day 3 PI, diffuse lymphoid necrosis occurs in the germinal centers and around the periarterniolar and periellipsoid lymphatic sheaths. Cell populations in the spleen rapidly recover and the germinal follicles sustain no permanent damage (29, 197).

During the acute phase of the infection, the thymus undergoes a marked atrophy and widespread apoptosis of cortical lymphocytes. However, within a few days of infection, the lesions are overcome and the thymus is restored to its normal state (181). These lesions have not been associated with virus replication in thymic cells (31, 147).

IBDV-induced damage to the cecal tonsils may involve acute heterophilic inflammation and lymphocyte depletion with regeneration on day 5 PI (29). Tanimura and Sharma (151) reported that antigen-positive cells mainly localized to the germinal centers of the cecal tonsils.

Infection with IBDV causes severe plasma cell depletion in the Harderian gland and prevents the normal infiltration of plasma cells into the gland. This reduction is short-lived and plasma cell counts are restored in approximately 14 days PI (47, 198, 199). Lesions characterized by large casts of homogeneous material infiltrated with heterophils have been reported in the kidneys; however, they are minimal and believed to be non-specific (29, 47, 200). Slight perivascular infiltration of monocytes may be observed in the liver (200). There is evidence that IBDV replication also occurs in the bone marrow (165, 197).
2.10 Antigenic Variation of IBDV Strains

Historically, mutations in the IBDV genome have impacted antibody recognition and led to variations in antigenicity, immunogenicity, virulence, and tropism of circulating infectious bursal disease virus (IBDV) strains (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21). Therefore, continuous surveillance, along with rapid identification and characterization of new IBDV isolates and comparison with previously described viruses is of vital importance (30, 75, 201). The molecular basis for these emerging antigenic differences was traced to antigenic domains of the VP2 protein of IBDV (11). The viral capsid protein, VP2, is the major host protective immunogen, as it is the only viral protein responsible for the induction of neutralizing antibodies and for serotype specificity (9, 73, 75, 108, 202). Neutralizing monoclonal antibodies have been shown to bind within a central variable region between amino acids (aa) 206 and 350 (AccI-SpeI fragment) of the VP2 protein. This region is composed of hydrophobic aa, flanked by two short hydrophilic peaks, A and B (aa residues 212-224 and 314-324, respectively) (9, 12, 112, 115). Subsequently, two smaller hydrophilic peaks, 1 and 2 (aa 249-254 and aa 279-290, respectively) were also identified (113, 115). Amino acid changes correlated with antigenic variation are typically found within these peaks (12, 13, 15, 17, 112, 203, 204, 205).

Antigenic variation, including the existence of a second serotype, were first introduced by McFerran et al. (22), who reported a lack of in vitro reciprocal virus neutralization between serotype I and serotype II IBDV strains. Serotype I viruses cause immunologic disease in chickens, while serotype II strains only induce sub-acute
infections in turkeys (66). Moreover, serotype II viruses do not protect against challenge with serotype I viruses (23, 58, 206). Heterogeneity in regards to antigenicity, pathogenicity, virulence, and immunosuppressive potential was also demonstrated among serotype I strains in virus neutralization (VN) and in vivo reciprocal cross-challenge tests (22, 25, 26, 58, 64, 65). Jackwood and Saif (26) identified six different subtypes among thirteen serotype I strains in a reciprocal VN study using polyclonal antisera, supporting the existence of antigenic diversity among IBDV commercial vaccine strains and field strains. To help elucidate antigenic variations responsible for vaccination failures, Snyder et al. (207, 208) designed a select panel of monoclonal antibodies (MAbs) (e.g. 8, 179, B69, R63, and 10) for differentiation between classic strains (e.g. D78) and several subtypes of the variant strains. All classic IBDV viruses and vaccines isolated prior to 1985 were efficiently neutralized by MAb R63 and B69 (e.g. STC, D78); whereas, IBDV variants lack either the B69 site (e.g. Delaware variant E (DelE)) or both the B69 and R63 sites (e.g. GLS) (208, 209). Due to the fact that these Mab-defined neutralization sites previously found on all classic strains were absent from new field isolates, it was predicted that a major antigenic shift in serotype I field viruses had occurred in the U.S (30, 66, 208, 209). Neutralizing MAbs including MAbs 8, 179, R63, B69, 57, 10 and 67 and non-neutralizing MAbs such as MAbs B29 and BK9 are commonly used for antigenic analyses of IBDV field isolates by antigen capture enzyme-linked immunosorbent assay (AC-ELISA) (21, 15, 66, 72, 202, 205, 207, 208, 209, 210, 211, 212). For example, the RS593 variant is defined by MAbs 8, 67, and 179, while the AL2 variant is defined by MAbs 8, 57, 67, and 179 (213). Australian variants showing little
similarity to U.S. variants at both the antigenic and genetic level were similarly identified (211). Currently, serotype I IBDV viruses are antigenically grouped as classic (also known as standard) and variant strains based on virus neutralization (47, 212).

In contrast, the vvIBDV strains are considered to be the most antigenically similar to classic IBDV strains, despite their enhanced pathogenic properties and ability to circumvent maternal immunity induced by classical, mild IBDV vaccines (51, 55, 67, 68, 69, 72, 214). Previous findings have suggested that vvIBDVs may be evolving; however, further investigation is needed to determine the biological significance of these antigenic differences (205, 215, 216).

2.11 Prevention and Control of IBDV

Infectious bursal disease virus is both highly contagious and very resistant to inactivation, which accounts for its persistent survival on poultry farms, despite disinfection (37, 47, 91). Therefore, even with strict biosecurity programs (e.g. ‘down time’ between broods, all-in/all-out production, cleaning and disinfection of the premises and equipment), vaccination is especially important to reduce the incidence and impact of IBD in the poultry industry (37, 47). Traditionally, breeder flocks are hyperimmunized with live and killed vaccines in order to confer high titers of maternal antibodies to their progeny (37). This passive immunity protects chicks against early immunosuppressive infections for 1 to 3 weeks; however, protection may be extended to 4 or 5 weeks by boosting the immunity in breeders with oil-adjuvanted vaccines (47, 48, 49, 50, 51, 52, 53). In addition, young broiler chicks are sometimes actively immunized before the total
waning of maternal antibodies. Maternal antibody titers may vary considerably due to
differences in breeder vaccination programs, the age of the breeder flocks supplying
progeny, and normal variation between hen titers in the same flock. This makes the
timing of broiler vaccination in relation to waning maternal antibodies critical, to prevent
persistent maternal antibodies from potentially neutralizing the vaccine virus (46, 49, 54,
55). Skeeles et al. (217) reported that maternal antibody titers must fall below 1:64 before
broiler chicks can be effectively vaccinated with a live-attenuated IBDV strain.
Serological monitoring of antibody levels in a breeder flock or its progeny is usually
necessary to determine the proper time to vaccinate (51).

Prior to 1985, both clinical and subclinical forms of IBD were controlled by
vaccination programs utilizing classic strains. However, in 1984 and 1985 vaccination
failures were reported from the Delmarva poultry-producing area (64, 65). The IBDV
field isolates, characterized as “variant” strains were capable of producing bursal lesions
in vaccinated birds, despite the presence of maternal antibodies to IBDV (25, 56, 57, 65,
66). Common epitopes between IBDV pathotypes were reported with subsequent
production of IBDV variant-derived vaccines. Live and killed vaccines made from classic
strains offered limited protection against variant IBDVs, whereas those made from
variant strains protected against classic strains, as well as, homologous and heterologous
variant strains (56, 57, 58). The ability of IBDVs to cross-protect has played an important
role in the development of improved vaccines. Commercially available live and killed
vaccines have since been reformulated to include both classic and variant virus strains, so
as to broaden the range of antigenic subtypes and elicit a heightened immune response
(17, 58, 59). However, vaccine efficacy highly depends on the dose and strains of the vaccine and challenge viruses, as well as, the route of administration, the appropriate vaccination time, and the levels of maternal antibodies (58, 218). Newly emerging IBDV strains capable of circumventing maternally-derived and active immunity induced by commercial IBDV vaccines have been reported (17).

Classical live attenuated vaccines may induce broad, lifelong protection, but they also carry residual pathogenicity and the potential to revert to virulence (37, 75, 219, 220). Several live-attenuated virus vaccines that differ according to their virulence and antigenic characteristics are available commercially. With regard to virulence, vaccine strains are characterized as mild, mild intermediate, intermediate, intermediate plus, or “hot,” depending on the degree of attenuation (47). Mild strains are predominantly used for breeder vaccinations, but due to their extreme sensitivity to homologous maternal antibody interference, they are normally administered between four and eight weeks of age when maternal antibodies have waned (37). Intermediate vaccines are frequently administered to broilers and pullets (221). In addition, intermediate vaccines may be administered by nebulisation to day-old broiler chicks who possess low levels of maternal antibodies at hatch (37). Less attenuated (hot) strains are known to cause histological lesions in SPF chickens and may provoke immunosuppression (37). Live-attenuated vaccines are administered via drinking water application or nebulisation between the ages of 7 days and 2 or 3 weeks (37, 47).

Killed-virus vaccines in an oil adjuvant are often used to boost levels of maternal antibodies and confer longer-lasting immunity in breeder hens. The duration and
uniformity of this immunity may be influenced by the concentration and antigenic specificity of the vaccine strain (37). These vaccines are not ideal for stimulating a primary antibody response; therefore, they tend to be most effective in chicks that have been “primed” with a live virus vaccine or naturally infected through field exposure to IBDV (47, 52, 53, 220, 222, 223). Currently, many oil-adjuvant vaccines contain both classic and variant IBDV strains (47). These vaccines do not protect against vvIBDV strains (51, 223). Killed-virus vaccines are administered by subcutaneous or intramuscular injection at sixteen to twenty weeks of age (37).

A more recent development in IBD control is the use of in ovo vaccination, which involves inoculating 18-day-old chicken embryos with live IBD vaccine, either alone or complexed in vitro with anti-IBDV antibodies (224, 225). This method helps to avoid interference by maternal antibodies, while effectively initiating a primary antibody response, and is efficacious against IBDV for the duration of the growing period (224, 226, 227).

Expression of IBDV proteins in yeast (228, 229) and the baculovirus system (230, 231, 232, 233, 234, 235) have been studied for use as subunit vaccines. VP2 protein of IBDV expressed in a baculovirus system was tested in commercial broiler breeders and immunity was conferred to their progeny (235). A Pichia pastoris yeast-expressed recombinant VP2 has been used as an oil-adjuvanted vaccine in broilers in Israel (236). Moreover, live recombinant viral vaccines expressing the VP2 protein such as fowlpox virus, herpes virus of turkey (HVT), and Newcastle disease virus have been reported (237, 238, 239, 240, 241); however, only one recombinant vaccine derived from HVT has
been licensed to date (242). Such technologies could allow discrimination between antibodies induced by vaccines (anti-VP2 only) and those induced by infection (anti-VP2 and VP3), which may enhance field monitoring of IBDV (75).

2.12 Clinical and Differential Diagnosis of IBDV

Diagnosis of acute clinical forms of IBD is made according to disease evolution (rapid onset followed by recovery in 5-7 days) and post-mortem examination of gross or microscopic lesions of the bursa of Fabricius (47). Distinct clinical changes in the size and color of the bursa will help differentiate all acute IBD cases from conditions commonly mistaken for IBD such as avian coccidiosis, chicken infectious anaemia, mycotoxicoses, and certain nephropathogenic forms of infectious bronchitis (37, 47, 60). In contrast, diagnosis of subclinical and immunosuppressive forms of IBD is made retrospectively by histopathology of the atrophied bursa (47). Due to the immunosuppressive nature of IBDV, its existence within a flock may be manifested by an increased susceptibility to conditions such as gangrenous dermatitis, inclusion body hepatitis, Marek’s disease, infectious laryngotracheitis, and salmonellosis and colibacillosis (38, 40, 43, 191, 193, 243). However, the histologic response associated with each of these conditions is typically different from that of IBD (244).

IBDV is most commonly isolated from cloacal bursa tissues as the bursa is the primary target organ for viral infection and contains higher concentrations of virus than other non-bursal lymphoid tissues and organs. Propagation of both virulent and vaccine IBDV can be performed in 9-to-11-day-old fertile specific pathogen free chicken eggs.
The most sensitive route of inoculation is via the chorioallantoic membrane (CAM); however, embryos inoculated via the allantoic sac and yolk sac can also be infected (63). CAM inoculation with classic IBDV strains produces embryo mortality in 3 to 5 days post-inoculation. Gross embryo lesions may include cutaneous congestion, edematous distension of the abdomen, petechial hemorrhages along the feather tracts and occasional hemorrhages in the toe joints and cerebral area, necrosis of the liver, pallor of the heart, congestion and some necrosis of the kidneys, extreme congestion of the lungs, and pallor of the spleen with occasional small necrotic foci. Plaques are seldomly present on the CAM, but small surface hemorrhages have been observed (47, 244, 246). In contrast, variant IBDV strains do not cause embryo mortality and rarely produce congestion or hemorrhages. Embryo lesions characteristic of variant IBDV infection include cerebral and abdominal edema, stunting, off-white or cream-colored skin and feathers, necrosis and bile stasis of the liver, and splenomegaly (25, 47, 244). McFerran et al. (22) reported that three out of seven wild type IBDV isolates recovered from infected bursae failed to replicate in chicken embryo fibroblast (CEF) cells, but propagated in embryonating eggs. Very virulent IBDV strains are also propagated in embryonated chicken eggs, producing severe lesions and high mortality at low virus doses (229, 244).

Many IBDV isolates have been successfully adapted to cell cultures of chicken embryo origin, including chicken embryo bursal cells, kidney cells, and fibroblast cells (47, 133, 244, 247, 248). Moreover, IBDV has been grown in cell lines including turkey and duck embryo cells (133), mammalian cell lines derived from rabbit kidneys (RK-13)
Vero cells (6, 250, 251), derived from African green monkey kidneys; BGM-70 cells (251), derived from givet monkey kidneys; and MA-104 cells (251) from fetal rhesus monkey kidneys. Although the virus may be adapted to cell culture, isolation and propagation of some IBDV field strains may be difficult with this technique (22, 244, 252, 253, 254). Initial isolation of IBDV is best accomplished via embryo inoculation (244). However, studies have demonstrated that IBDV pathogenicity and immunogenicity is reduced following propagation in embryos and cell cultures (190, 255).

Direct and indirect immunofluorescence assays, immunocytochemistry, or direct examination by electron microscopy are useful tools for isolation and identification of IBDV in infected tissues (22, 47, 256). Rapid detection and typing of IBD field cases may be accomplished using nucleic acid probes and antigen-capture enzyme immunoassays (207, 257, 258, 259). Conventional reverse transcription (RT) followed by the polymerase chain reaction (PCR) is also commonly used for detection of IBDV serotypes, and less regularly, for differentiating IBDV subtypes (27, 260, 261, 262, 263). A need for more rapid methods of characterizing the VP2 variable region in different subtypes and strains of IBDV led to recent developments in molecular diagnostic techniques based on RT-PCR technology (212). Initial approaches involved the use of restriction enzymes (RE) (e.g. BstNI, MboI, and SspI) for the restriction fragment length polymorphism (RFLP) analysis of RT-PCR products (263, 264, 265, 266, 267, 268, 269, 270, 271, 272). Designed to detect unique banding patterns associated with antigenic variation within the VP2 encoding region, these methods were beneficial for placing
IBDV strains into molecular groups (13, 212, 244, 263, 266, 267). However, these techniques later proved to be unreliable due to inconsistent correlations between RFLP patterns of RT-PCR products and IBDV antigenic subtypes (212, 244, 273). Recent developments of the RT-PCR technique include the use of multiplex RT-PCR or quantitative real-time RT-PCR methods and DNA sequencing of RT-PCR products (212, 274, 275, 276, 277). Quantitative real-time RT-PCR methods allow for rapid, sensitive, and accurate detection and differentiation of IBDV subtypes (classic, variant, and very virulent) by subtype-specific primers and also provide a quantitative measure of the viral load in test samples from poultry flocks (212, 274, 278, 279, 280).

Nucleotide and amino acid sequencing of the entire VP2 encoding region of many IBDV field isolates is frequently used for comparison with previous strains and phylogenetic analysis (14, 15, 59, 194, 215, 281). These comparative analyses are highly useful for identifying putative amino acids within the central variable region that are responsible for molecular and phenotypic variations among IBDV subtypes and strains (21, 59, 73, 201, 211, 282, 283). Both variant and very virulent IBD viruses have distinctive nucleotide and amino acid substitutions, relative to the classic subtype, that can be rapidly detected by DNA sequencing of RT-PCR products (212). For example, residues at positions 253, 279, and 284 of the VP2 protein sequence have been reported to impact tissue culture infectivity (284, 285), virulence, and pathogenic phenotype (16, 18) of IBDV strains. Amino acids that significantly affect antigenicity are at position 222 and within the hydrophilic peak B region aa 314-324 (205, 286). Moreover, the hydrophilic peak B region is involved in the formation of neutralizing epitopes (12, 59,
A serine-rich heptapeptide, S-W-S-A-S-G-S, (residues 326 to 332) is conserved in various vvIBDV strains isolated in Japan, as well as, antigenic virulent variants isolated in the U.S., and may influence virulence and pathogenicity. Mildly pathogenic and tissue-culture-adapted strains contain fewer serine residues in this heptapeptide (12, 264, 285, 288, 289, 290).

Serological detection methods including the enzyme-linked immunosorbent assay (ELISA) test, the agar gel precipitin (AGP) test, and the virus neutralization (VN) test are often employed for confirmatory diagnosis of IBDV (47, 144, 212, 291). The AGP test can be used for the rapid detection of IBDV group-specific soluble antigens or IBDV antibodies in convalescent birds. While simple to perform, low sensitivity, the inability to detect serotypic differences, and non-quantitative results are serious limitations (47, 212, 244). In contrast, the VN test is a more sensitive antibody detection tool and can be used for both antibody quantitation and differentiation of IBDV serotypes and subtypes (23, 26, 206, 212, 292, 293). The ability of serum to neutralize a reference virus is determined and a neutralization endpoint titer (VN titer) is expressed as the reciprocal of the highest dilution of serum that prevents cytopathic effects (47, 212). VN tests are often employed for IBDV identification in embryos and tissue culture after its adaptation to these host systems and for assessment of antigenic and immunogenic changes (94, 190, 211, 212, 294, 295). VN tests serve to compare virus strains by evaluating the ability of antiserum raised against one IBDV strain to neutralize a heterologous strain (212). However, only IBDV strains that efficiently replicate in embryos or cell culture can be used in the VN
assay. Attempts to adapt the virus to a host may result in antigenic and pathological changes to the virus that may render VN results as suspect

Marquardt et al. (144) first described the indirect ELISA test for IBDV antibodies, but it failed to differentiate between antiserum to IBDV serotypes I and II (24, 206). Moreover, many commercial ELISA kits detect antibodies to both serotypes and vary considerably in their sensitivity and specificity (47, 212, 296, 297). The antigen capture ELISA (AC-ELISA) system using monoclonal antibodies (MAbs) was developed for characterizing the antigenic properties and relatedness of IBDV strains (66, 72, 202, 208, 210). Commonly used to measure IBDV serum antibody titers, this sensitive and rapid method allows for differentiation of classic, variant, and vvIBDV subtypes (66, 113, 202, 205, 211, 298). These titers are useful for evaluating the flock immunity level during an outbreak, as well as, the efficacy of a vaccination program (46, 47). Polyclonal antibodies with AC-ELISA assays are more likely to be used for general screening of tissue samples for IBDV (94, 244). The disadvantages to using such ELISA tests are the extensive preparation of the MAbs, the cultivation of IBDV in CEF or chicken bursae, and the fact that results do not always correlate with VN test findings (212).

In vivo cross-protection studies, vaccination-challenge studies, and progeny challenge studies are frequently performed for assessment of IBDV vaccine efficacy and to determine the pathogenicity and antigenic phenotypes of IBDV strains (26, 58, 194). More recently, an IBDV reverse genetics system was implemented to introduce selected amino acid changes into the VP2 encoding region of the classic IBDV strain D78 in order to assess antigenic determinants of IBDV (21, 201, 299). This process combined with
nucleotide and amino acid sequencing and MAb reactivity patterns may provide a more comprehensive analysis of IBDV strains for better diagnosis and vaccination program design (300).
Chapter 3

MATERIALS AND METHODS

3.1 Chickens

Fertile specific pathogen free (SPF) white leghorn chicken eggs were obtained from SPAFAS, Charles River Laboratories (Norwich, Connecticut). Eggs came from a single breeder flock and were incubated at 37°C for 3 weeks prior to hatching. At one-day of age, the chicks were placed into negative-pressure, gloveport Horsfall isolator units with diptanks in the Charles C. Allen Laboratory at the University of Delaware, Newark, DE. Feed and water were provided ad libitum.

3.2 Chicken embryos

Fertile SPF chicken eggs were purchased from Sunrise Farms (Catskill, NY). The eggs were incubated at 37°C for 9 to 11 days prior to inoculation with infectious bursal disease virus (IBDV) for virus isolation and virus titration.

3.3 Viruses

IBDV reference strains STC and Delaware variant E (DelE) are maintained as seed stocks at the University of Delaware. Thirty-three IBDV field isolates (Table 3.1) were obtained from three- and four-week old commercial broiler chickens raised on the
Delmarva Peninsula. Five cloacal bursas from each commercial broiler submission found to be IBD positive by histopathology and virus isolation were thawed at room temperature and suspended in 5-7mL of tryptose phosphate broth (TPB) (Thermo Fischer Scientific, Lenexa, KS) supplemented with antibiotics (Penicillin G 10,000 IU/mL, Streptomycin Sulfate 10,000 μg/mL, and 25 μg/mL of Amphotercin) (MP Biomedicals, LLC, Solon, OH). Bursas were homogenized using Tenbroeck tissue grinders and 1mL aliquots were stored at -80°C. Subsequent RT-PCR was performed using original virus stocks. Fifteen of the thirty-three field isolates found to be positive by histopathology, virus isolation, and RT-PCR were designated for VP2 sequencing. IBDV variant vaccine 89/03 (Intervet-Schering Plough Animal Health, Millsboro, DE) and the classic PBG98 strain (Lohmann Animal Health, Harrisonburg, VA) of IBDV were propagated in chicken embryo fibroblast (CEF) cells. At 48 hours post-inoculation (PI), cells and supernatant fluids were harvested and aliquoted into 2.0 ml cryogenic vials to serve as viral stocks.

3.4 Virus Isolation

Frozen bursal samples were thawed and prepared as 20% weight to volume (w/v) suspensions in TPB supplemented with antibiotics (Penicillin G 10,000 IU/mL, Streptomycin Sulfate 10,000 μg/mL, and 25 μg/mL of Amphotercin) (MP Biomedicals, LLC, Solon, OH) (244). Using Tenbroeck tissue grinders, the samples were homogenized and frozen and thawed three times. Homogenates were centrifuged at 1000 rpm for 10 minutes and the supernatant fluid was collected. Ten-day-old embryonated SPAFAS eggs were inoculated with 0.1mL of the supernatant fluid via the dropped chorioallantoic
membrane (CAM) route and incubated at 37°C. Eggs were candled daily for one week and mortality was recorded. At 7 days post-inoculation (PI), the eggs were chilled at 4°C for 24 hours. Embryos were examined for gross IBD lesions. Characteristic embryo lesions of classic IBDV include early mortality (3-5 days PI), liver necrosis, hemorrhage, a pale heart, edematous extension of the abdomen, and a pale spleen (25). Characteristic lesions induced by variant IBDV strains include cream-colored, edematous embryos with bile stasis of the liver, liver necrosis, splenomegaly and limited mortality (25). Any mortality within the first 24 hours post-inoculation was considered non-specific and the eggs were discarded.

3.5 Tissues and Histopathology

Tissue samples collected from commercial broiler flocks and from experimental SPF leghorn chickens inoculated with control and field isolates were fixed in 10% volume to volume (v/v) neutral buffered formalin (Richard-Allan Scientific, Kalamazoo, MI). Tissues were embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin (190, 255). Tissues were microscopically evaluated on the basis of the extent of necrosis and degeneration of follicular lymphocytes, the presence of plical, follicle, and muscle wall edema, hyperemia, and heterophilic inflammation and infiltration (226). Cloacal bursa lesions were scored based on the following system: 1 = normal, no IBDV related lesions; 2 = mild, scattered IBDV related cell depletion in a few follicles; 3 = moderate, 1/3 to 1/2 of the follicles with IBDV related atrophy or depletion of follicular lymphocytes; and 4 = severe atrophy of all the follicles, inflammation, and
acute necrosis (301). Bursal scores of 1 and 2 were considered protected and scores of 3 and 4 were considered unprotected.

Thymus tissues were evaluated based on the degree of cortical lymphocyte loss, thymocyte apoptosis, and atrophy of thymic cells. Spleens were assessed for increased germinal center formation, as well as, lymphoid depletion in the germinal centers and around the periellipsoid and periarteriolar lymphoid sheath (197).

### 3.6 Viral RNA Extraction

Field isolates producing microscopic pathology consistent with IBDV infection were designated for molecular testing. Bursal homogenates were treated in equal v/v ratios of phenol/chloroform with isoamyl alcohol (Fischer Scientific, Fair Lawn, NJ). Samples were vortexed and centrifuged at 17,000 rcf for 10 minutes. The supernatant was removed and used in the Qiagen RNeasy Mini kit (QIAGEN Operon, Valencia, CA) to extract total RNA, as per the manufacturer’s instructions.

### 3.7 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

IBDV RT-PCR screening of the bursal homogenates was conducted using a modified version of the GeneAmp RNA PCR kit (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ) protocol. Duplicate reactions were run for each isolate. Denaturation of dsRNA was performed using 4μL of 90% Dimethylsulfoxide (ATCC, Manassas, VA), 2μL of random hexamers, and 4μL of dsRNA extracted from IBDV isolates. Complementary DNA (cDNA) was synthesized from viral RNA in the same
tube by the addition of 32µL of a reverse transcriptase master mix containing 4µL of MgCl, 2µL of 10x PCR buffer, 8µL of DXTP mixture, 1µL of RNase inhibitor, and 1µL of reverse transcriptase. Twenty-one microliters of sample were removed and placed in a 200µL PCR tube to serve as a duplicate PCR reaction. Seventy-eight microliters of a PCR master mix containing 4µL of MgCl, 8µL of 10x PCR buffer, 65.5µL of DEPC treated water, and 0.5µL of Taq polymerase, were added to each tube. Two microliters of the forward primer 5’-GCCAGAGTCTACACC-3’ and reverse primer 5’-CCCAGATTATGCTTT-GA-3’ (59) set were also added to each tube (Eurofins MWG Operon, Huntsville, AL). RT and PCR were conducted using the AB Applied Biosystems 2720 Thermal Cycler and the PE Applied Biosystems GeneAmp PCR System 9700 Version 2.25. Thermal cycling was performed in three sequential phases. The cycle parameters for the denaturation phase of dsRNA consisted of one 94°C hold for 7 minutes. The RT phase involved one 42°C hold for 42 minutes, followed by a 99.9°C hold for 5 minutes. The PCR cycle parameters were as follows: one 94°C hold for 2 minutes; 35 cycles at three temperatures (respectively): 95°C for 40 seconds, 53°C for 40 seconds, and 69°C for 50 seconds; one 72°C hold for 7 minutes, followed by a 4°C hold. PCR products were electrophoresed in 1.8% GenePure LE Agarose (ISC BioExpress, Kaysville, UT) containing ethidium bromide (25ng/mL) (MP Biomedicals, Inc., Solon, OH) and observed under UV light. Bands were excised from the gel and DNA was extracted according to the Illustra™ GFX PCR DNA and Gel Band Purification Kit, as per the manufacturer’s instructions (GE Healthcare UK Limited, Buckinghamshire, UK).
DNA was resuspended in 50µL of elution buffer and quantitated using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

3.8 Genome Sequencing and Analysis

Genome sequencing was performed at the University of Delaware Sequencing and Genotyping Center, Newark, DE. Forward primer 5’-GCCAGAGTCTACACCATTCC-3’ and reverse primer 5’-CCCGGAATTATGTTTTGA-3’ specific for the VP2 encoding region (59) was provided at 2pm/µL (Eurofins MWG Operon, Huntsville, AL). Comparative analysis of IBDV VP2 nucleotide and protein sequences was conducted by creating a CLUSTAL W alignment (MegAlign; Version 8.0.2; DNASTar, Inc., Madison, WI). Amino acid identity values were determined based on amino acid sequence data of fifteen IBDV infected bursal homogenates, IBDV variant strains, DelE (Delmarva Peninsula, University of Delaware), GLS (Delmarva Peninsula, University of Maryland), and AL-2 (Perdue Farms Inc., Vangs Farm, NC), variant vaccine strain, RS593 (Foster Farms Inc., CA), and classic challenge strain, STC (USDA-APHIS, Ames, IA) (Table 4.1). Deduced amino acid sequences from the fifteen RT-PCR positive IBDV samples (Table 3.2) were analyzed by Dr. Daral Jackwood (The Ohio State University). Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (302) and the unweighted pair-group method using arithmetic averages (UPGMA) method were used to conduct a VP2 phylogenetic analysis (303). The phylogenetic tree was evaluated for evolutionary relationships between the 14 field isolates and existing classic and variant strains.
3.9 Cultivation of Seed Stocks

Seed stocks were made for six IBDV field isolates (DMV/4813/07, DMV/4947/07, DMV/4955/07, DMV/5036/07, DMV/5038/07, DMV/5041/07) and two IBDV reference strains (DelE and STC) (Table 3.3). Seventy-one, 1-day-old SPF leghorn chickens were divided into eight groups of seven birds each and one negative control group of fifteen birds. Birds were placed into negative-pressure, Horsfall isolator units in the Allen Laboratory at the University of Delaware, Newark, DE and provided food and water *ad libitum*. Groups were challenged on separate days to avoid cross-contamination. For each isolate, seven chickens were inoculated via ocular and intranasal routes with 250μL of viral inoculum prepared from bursal homogenates as previously described. Chickens were evaluated daily for the development of clinical signs and mortality. Three days post-inoculation (PI), five birds and one negative control bird were euthanized via cervical dislocation. Birds were assessed for gross IBDV-specific lesions and cloacal bursas were removed.

From each harvested bursa, a small section of tissue was cut and placed in 10% volume to volume (v/v) neutral buffered formalin (Richard-Allan Scientific, Kalamazoo, MI) for histopathology. Remaining bursal tissues were pooled according to virus treatment and homogenates were prepared as 20% weight to volume (w/v) suspensions in TPB containing antibiotics (Penicillin G 10,000 IU/mL, Streptomycin Sulfate 10,000 μg/mL, and 25 μg/mL of Amphotericin) (MP Biomedicals, LLC, Solon, OH). Bursas were homogenized using a sterile electric tissue grinder (PRO Scientific Inc.) and subjected to 1 freeze-thaw cycle. Tubes were incubated at room temperature for 1 hour and
centrifuged at 4°C for 30 to 40 minutes at 2500 rpm to pellet cellular debris. Supernatant was aliquoted into sterile 2 mL cryogenic vials (Fisher Scientific, Fair Lawn, NJ) to serve as virus seed stocks and stored at -80°C. Viral material from a single aliquot was streaked onto a nutrient agar plate to screen for bacterial contamination. The following day, plates were examined for bacterial growth and results were recorded.

Viral RNA was extracted from the seed stock preparations, amplified by RT-PCR, and sequenced, as previously described. Nucleotide and amino acid sequence results for each virus strain were compared with the initial sequence data to determine if mutations had occurred. In addition, each viral seed stock was tested for potential contamination with infectious bronchitis virus (IBV) (304, 305), reovirus (306), Newcastle disease virus (NDV) (307) using RT-PCR, and adenovirus (308) using PCR.

3.10 Antiserum Production

During the production of seed stocks, two birds were retained in each treatment group for serum antibody production. At 4 weeks PI, blood was collected via cardiac puncture. Clotted blood samples were incubated at room temperature for 1 to 2 hours and were then placed at 4°C for 24 hours. Samples were centrifuged at 1000 rpm for 15 minutes and the serum was harvested and pooled according to virus treatment. Sera were incubated at 56°C for 30 minutes to inactivate complement. Stocks of antisera were maintained in aliquots in sterile 2 mL cryogenic vials and stored at -20°C.

Serum was tested for the presence of IBDV specific antibodies using the agar gel precipitin (AGP) test (38). Agar was prepared by dissolving 1 gram of 1% Bacto agar
(Becto, Dickinson and Company, Sparks, MD) and 8 grams of NaCl (Fisher Scientific, Fair Lawn, NJ) in 100 mL of distilled water by heating. Ten to twelve mL of melted agar were poured into 60mm x 15mm plates. After solidifying at room temperature for 12 hours, seven 5 mm holes were punched into the gel. For each plate, National Veterinary Services Laboratory (NVSL) IBD Antiserum (APHIS, Ames, IA) was loaded in each of the three alternate outer wells. Antisera from the field isolates were placed in the remaining three alternate outer wells. The center well contained NVSL IBD Antigen (APHIS, Ames, IA). Each of the wells was loaded with 12μL of material four times, allowing for adequate absorption between each loading. Plates were incubated at room temperature and observed for precipitin lines of identity consistent with positive control samples for up to 72 hours. Results were recorded daily.

3.11 Virus Titration

Ten-fold, serial dilutions (10⁻¹ to 10⁻⁷) were prepared in TPB containing antibiotics (Penicillin G 5000 IU/mL, Streptomycin Sulfate 5000 μg/mL, and 12.5 μg/mL of Amphotercin) and incubated for 1 hour at room temperature. Ten to eleven day old embryonated chicken eggs were inoculated via the chorioallantoic membrane route with 0.1 mL of virus dilution per egg. Five embryos were inoculated for each dilution, and candled daily. Any mortality within the first 24 hours was discarded, and subsequent mortality was held at 4°C. At one week PI, living embryos were chilled at 4°C for 24 hours. The following day, embryos were examined for gross IBDV lesions. Virus titers
were calculated for each IBDV seed stock by the Reed and Müenich method (309) and expressed as embryo infectious dose (EID₅₀/mL).

3.12 Monoclonal Antibody Characterization

Bursal-derived seed stocks were analyzed by Dr. Egbert Mundt, at the University of Georgia, using a panel of IBDV-specific monoclonal antibodies (MAbs) 57, 63, 67, 69, and 10 by an antigen capture enzyme-linked immunosorbent assay (AC-ELISA) (72, 207, 208) as described by Icard et al. (201). Polyclonal rabbit VP1 antiserum served as a control for transfection and monoclonal antibody (MAb) tests were performed in duplicate (21, 122). With this system, the VP2 encoding region of the IBDV field isolate is cloned into an IBDV backbone (D78) and transfected into cell culture along with other viral elements. Successful cotransfection of cRNA, as indicated by VP1 fluorescence, results in IBDV infection of the cells. Cells are reacted with the panel of IBDV-specific MAbs and processed for immunofluorescence. VP2 epitopes of the IBDV field isolates are determined by a positive or negative reactivity pattern and compared to those of IBDV reference strains (Table 3.4) (201).

MAb testing using a whole virus and ELISA assay was performed by the Poultry Service Laboratory of Intervet/Schering-Plough Animal Health as described by Snyder et al. (66) and Intervet-Schering Plough Animal Health. In this system, the whole viral genome of the IBDV field isolate is reacted directly with the panel of monoclonal antibodies. Included in this panel were IBDV-specific MAbs 10, 57, 63, 67, and 69. MAbs B29, 8, and BK9 were used as positive controls. MAb testing was performed on
the original bursal homogenates from field cases, as well as, the bursal-derived seed stocks (Intervet-Schering Plough Animal Health).

To determine the binding ability of each field virus to the specific monoclonal antibodies, absorbance data was used to calculate a MAbX/MAb B29 binding index, where MAbX is the specific MAb that was tested and MAb B29 reacts with an epitope found in all IBDV (positive control). The MAb index for each IBDV field virus was calculated based on the following formula: Absorbance of virus antigen against MAb X – Absorbance of MAb X normal bursa / Absorbance of the virus antigen against MAb B29 – Absorbance of MAb B29 normal bursa. A value that was greater or equal to 0.7 was expressed as positive (+). A value that was between 0.3 and 0.69 demonstrated moderate to low binding activity. A value that was equal to or less than 0.29 was expressed as negative (-) (Table 3.5) (310).

As a quality control and for comparison, six control samples were run simultaneously. These included the previously characterized variant viruses, DelE (56, 57) GLS (66), and AL-2 (310), one variant vaccine strain, RS593 (Intervet, Inc., Millsboro, DE), one classic strain, STC (APHIS, Ames, IA), and normal bursa as a negative control (Table 3.5).

3.13 Vaccines

Two commercially available live vaccine strains, 89/03 (Intervet-Schering Plough Animal Health, Millsboro, DE) and PBG98 (Lohmann Animal Health, Harrisonburg, VA) were obtained. Vaccine 89/03 contains an attenuated IBDV serotype 1 variant strain
(Delaware variant E) that elicits cross-protection against Delaware variant E, STC, GLS, and RS593 strains of IBDV (311). PBG98 contains an attenuated IBDV serotype 1 classic strain (312).

### 3.14 Cell Cultures

Chicken embryo fibroblast (CEF) cells were prepared from 9- to 11-day-old fertile SPF chicken eggs (Sunrise Farms, Catskill, NY) as previously described (313). A 10ml volume of 2x trypsin was added to tissue fragments prior to trypsinization for 7 minutes in a 37°C shaker cabinet. Cells were collected in a mesh filtered flask containing 5% fetal bovine serum (FBS) (Hyclone Laboratories, Inc, Logan, UT). Remaining tissue fragments were re-trypsinized for 5 minutes in 10ml of 2x trypsin. Collected cells were centrifuged for 10 minutes at 1000 rpm and trypsin solution was discarded. Pelleted cells were resuspended in medium 199 (M199) and F10 medium (Hyclone Laboratories, Inc, Logan, UT) supplemented with antibiotics (Penicillin G 100 IU/mL, Streptomycin Sulfate 100 μg/mL, and 0.25 μg/mL of Amphotericin) (MP Biomedicals, LLC, Solon, OH) and 5% FBS at a 1:500 dilution.

### 3.15 Virus-neutralization

Virus-neutralization (VN) antibody titers were determined for IBDV field isolates 4813, 4947, 4955, 5038, 5041 against IBDV strains 89/03 and PBG98. Beta-VN assays (constant-virus diluted-serum) were performed using viral stocks diluted in cell-culture medium to contain 100 TCID$_{50}$. To confirm that 100 TCID$_{50}$ per 0.1ml was used, back-
titrations of the viruses were performed with each virus neutralization test. Antiserum collected from birds inoculated with IBDV field isolates was diluted in cell culture media. After an initial 1:100 dilution, serial two-fold dilutions (1:100 to 1:12800) were prepared in 96-well flat-bottomed microplates (Costar, Cambridge, MA) in duplicate. Each well contained 25µL of diluted serum, to which was added 25µL of virus suspension containing 100 TCID$_{50}$ per 0.1ml. Back titrations of the viruses using known negative control serum from SPF leghorn chickens (SPAFA$\text{S}$ Charles River Laboratories, Norwich, CT) were performed to verify that the test virus dose contained 100 TCID$_{50}$. Plates were incubated for 1 hour at 37°C before 100µl of CEF cells diluted 1:500 was added to each well. Cell cultures were incubated at 37°C in 5% CO$_2$ atmosphere and examined at 48 hours PI. Cell culture media was discarded and 100 ul of 10% volume to volume (v/v) neutral buffered formalin (Richard-Allan Scientific, Kalamazoo, MI) were added to the wells of the microtiter dishes. Cells were fixed for 5 minutes and formalin was discarded. Cells were stained with 250 ul of 0.05% Crystal Violet (CV) (Fischer Scientific, Fair Lawn, NJ) in distilled water for 30 minutes and CV was discarded. Microtiter dishes were washed 2 times with tap water and drained for 2-3 minutes. Cell cultures were scored as positive or negative for cytopathogenic effect (CPE). Virus-neutralizing antibody titers were determined based on the reciprocal of the highest dilution of serum that neutralized 100 TCID$_{50}$ of IBDV (244). A four-fold difference in neutralization of the viruses was considered to be significant (J. Gelb, Jr., personal communication, March 11, 2010).
3.16 Challenge of Immunity

IBDV vaccine 89/03 was diluted in TPB containing antibiotics (Penicillin G 5000 IU/mL, Streptomycin Sulfate 5000 μg/mL, and 12.5 μg/mL of Amphotercin) according to the manufacturer’s instructions. Seven groups of fifteen SPF leghorn chickens per group were vaccinated at one day of age. Each chicken was subcutaneously injected in the back of the neck with 0.2 ml of the vaccine. Seven groups of five birds per group served as unvaccinated challenge controls, and they were subcutaneously injected in the back of the neck with 0.2 ml of TPB. Unvaccinated challenge controls and IBDV vaccinated chickens were raised separately until challenge. At 21 days of age, unvaccinated challenge controls and IBDV vaccinated chickens were re-housed in the same isolation unit according to the specific challenge virus to be administered (Table 3.6). Blood samples were collected from 10 birds per group and serum was harvested, as previously described. Each treatment group was challenged via eye drop with $10^{2.0}$ TCID$_{50}$/0.1 ml of the following IBDV field isolates: 4813p1; 4947p1; 4955p1; 5038p1; and 5041p1. Reference strains, STC and Delaware variant E (Del E), served as positive controls. One group of fifteen birds was not challenged and served as non-vaccinated, non-challenged controls. At 28 days of age (7 days post-challenge), all birds were bled, euthanized, and weighed. Tissues (cloacal bursa, thymus, spleen) were removed from each bird, and cloacal bursas were weighed and assessed for IBDV gross lesions. Sections of each tissue were placed in 10% volume to volume (v/v) neutral buffered formalin solution (Richard-Allan Scientific, Kalamazoo, MI) for histological evaluation. Birds with gross or microscopic bursa lesions and bursa to body weight indices of less than two standard
deviations below the mean of the negative control group were considered unprotected against challenge. IBDV-specific antibody levels in serum were evaluated prior to challenge at 21 days of age and at 7 days post-challenge by the IBDV Antibody Test Kit (IDEXX Laboratories Inc., Westbrook, ME), as per the manufacturers’ instructions. A second vaccine challenge study was performed with PBG98 (Table 3.7).

3.17 Progeny Challenge

A progeny challenge study was performed in collaboration with Intervet/Schering-Plough Animal Health, Millsboro, DE. Three groups of 18-week-old SPF leghorn breeders were vaccinated with a licensed inactivated IBDV vaccine containing classic and variant strains, as well as, a strain of reovirus (Intervet/Schering-Plough Animal Health) (Table 3.8). Each leghorn breeder was given a single intramuscular (IM) injection of vaccine (0.5mL per bird) in the breast muscle. No live priming vaccination was administered. Progeny chicks were hatched and housed in filtered-air negative-pressure isolation units. Two groups of SPF (IBDV antibody negative) leghorn chickens served as non-vaccinated challenge controls, while a third served as non-vaccinated, non-challenged controls. Progeny and SPF controls were challenged via the intraocular route with $10^{2.0}$ EID$_{50}$ per bird at 10 and 17 days of age with IBDV field isolate 5038 and IBDV reference strain Del E. Isolate 5038 was selected for the progeny challenge trial based on its lack of binding with neutralizing monoclonal antibodies (Tables 3.4 and 3.5). At 7 days post-challenge, all birds were euthanized, and bursa and body weights were measured. Tissue sections of each bursa were placed in
10% volume to volume (v/v) neutral buffered formalin solution (Richard-Allan Scientific, Kalamazoo, MI) for histological evaluation. Birds with microscopic bursa lesions and bursa to body weight indexes of less than two standard deviations below the mean of the negative control group were considered unprotected against challenge.

### 3.18 Bursa to Body Weight Calculations and Statistical Analysis

A bursa of Fabricius/body weight ratio for each bird was calculated according to the following formula: bursa weight in grams X 1000/body weight in grams (31). The geometric mean and standard deviation of bursa/body weight ratios was determined for each treatment group and the negative control group. A percent protection score was determined based on the percentage of birds within a treatment group that were protected (314). The significance of differences in percentages was analyzed by two-tailed chi-square tests (P < 0.05).
Table 3.1. Accession numbers and broiler flock information for IBDV isolation attempts.

<table>
<thead>
<tr>
<th>Accession #</th>
<th>Grower Name</th>
<th>Submitted</th>
<th>Histology</th>
<th>Virus Isolation (VI)</th>
<th>Age (weeks)</th>
</tr>
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<tbody>
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<td>07-4810</td>
<td>Atlanta</td>
<td>8/7/2007</td>
<td>Negative</td>
<td>N/A(^A)</td>
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<td>07-4811</td>
<td>Ball Farm</td>
<td>8/7/2007</td>
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<td>N/A</td>
<td>3</td>
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<td>Schmick</td>
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<td>IBDV</td>
<td>3</td>
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<td>N/A</td>
<td>3</td>
</tr>
<tr>
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<td>8/7/2007</td>
<td>Negative</td>
<td>N/A</td>
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<td>07-4816</td>
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<td>IBDV</td>
<td>3</td>
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<td>N/A</td>
<td>3</td>
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<td>8/9/2007</td>
<td>Negative</td>
<td>N/A</td>
<td>3</td>
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</tr>
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<td>07-5038</td>
<td>Dennis Shockley</td>
<td>8/14/2007</td>
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<td>IBDV</td>
<td>4</td>
</tr>
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<td>IBDV</td>
<td>4</td>
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<tr>
<td>07-5041</td>
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<td>8/14/2007</td>
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<td>IBDV</td>
<td>4</td>
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<td>07-5042</td>
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<td>Positive</td>
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<td>07-5043</td>
<td>Oak Hall</td>
<td>8/14/2007</td>
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<td>IBDV</td>
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<tr>
<td>07-4810</td>
<td>Atlanta</td>
<td>8/7/2007</td>
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</table>

\(^A\)VI not performed.
Table 3.2. IBDV RT-PCR results from screening of the bursal homogenates from 3- and 4-week-old commercial broiler chickens raised on 12 farms in the Delmarva Peninsula region.

<table>
<thead>
<tr>
<th>Accession #</th>
<th>Bursal Histology</th>
<th>Virus Isolation</th>
<th>Age (weeks)</th>
<th>RT-PCR Result</th>
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<td>3</td>
<td>Positive</td>
</tr>
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<td>IBDV</td>
<td>3</td>
<td>Positive</td>
</tr>
<tr>
<td>07-4947</td>
<td>Positive</td>
<td>IBDV</td>
<td>3</td>
<td>Positive</td>
</tr>
<tr>
<td>07-4952</td>
<td>Positive</td>
<td>IBDV</td>
<td>4</td>
<td>Positive</td>
</tr>
<tr>
<td>07-4955</td>
<td>Positive</td>
<td>IBDV</td>
<td>4</td>
<td>Positive</td>
</tr>
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</tr>
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<td>Positive</td>
</tr>
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<td>07-5034</td>
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<td>Positive</td>
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<td>Negative</td>
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Table 3.3. Propagation of Selected Field Viruses for Seed Stocks\textsuperscript{A}.

<table>
<thead>
<tr>
<th>Virus Clade</th>
<th>IBDV Strain</th>
<th>Isolator Group</th>
<th>Viable Virus Reisolated\textsuperscript{B}</th>
</tr>
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<tbody>
<tr>
<td>N/A</td>
<td>Neg. Control</td>
<td>Negative Control</td>
<td>N/A</td>
</tr>
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<td>Clade 1</td>
<td>4813</td>
<td>1</td>
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</tr>
<tr>
<td>Clade 2</td>
<td>5038</td>
<td>2</td>
<td>Yes</td>
</tr>
<tr>
<td>Clade 3</td>
<td>4947</td>
<td>3</td>
<td>Yes</td>
</tr>
<tr>
<td>Clade 4</td>
<td>4955</td>
<td>4</td>
<td>Yes</td>
</tr>
<tr>
<td>Clade 5</td>
<td>5036</td>
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<td>Clade 6</td>
<td>5041</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td>Classic Reference strain</td>
<td>STC</td>
<td>7</td>
<td>Yes</td>
</tr>
<tr>
<td>Variant Reference strain</td>
<td>DelE</td>
<td>8</td>
<td>Yes</td>
</tr>
</tbody>
</table>

\textsuperscript{A} One IBDV field isolate from each of the six clades identified in the phylogenetic tree was randomly selected for propagation in SPF leghorn chickens. Classic challenge virus, STC, and Delaware variant E (DelE) served as reference strains.

\textsuperscript{B} Confirmed by histopathological examination.
Table 3.4. Monoclonal antibody (MAb) reactivity patterns of infectious bursal disease virus field isolates using VP2 transfection and immunofluorescence assays<sup>A</sup>.

<table>
<thead>
<tr>
<th>Isolate&lt;sup&gt;BC&lt;/sup&gt;</th>
<th>MAb and dilution tested</th>
<th>10&lt;sup&gt;D&lt;/sup&gt;</th>
<th>57&lt;sup&gt;D&lt;/sup&gt;</th>
<th>63&lt;sup&gt;D&lt;/sup&gt;</th>
<th>67&lt;sup&gt;D&lt;/sup&gt;</th>
<th>69&lt;sup&gt;D&lt;/sup&gt;</th>
<th>VP1 his&lt;sup&gt;E&lt;/sup&gt;</th>
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<tbody>
<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<tr>
<td>4813p2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>-</td>
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</tr>
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<td>+</td>
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<td>4955p2</td>
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<td>5038p1</td>
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<td>5041p1</td>
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<td>+</td>
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<tr>
<td>5041p2</td>
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<td>-</td>
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<td>+</td>
</tr>
</tbody>
</table>

<sup>A</sup>Performed by Egbert Mundt, University of Georgia.

<sup>B</sup>Bursal-derived seed stocks of each field isolate were used in the AC-ELISA.

<sup>C</sup>Two plasmids were constructed for each field isolate; p1 and p2.

<sup>D</sup>MAbs diluted in phosphate buffered saline.

<sup>E</sup>Rabbit VP1 antiserum (122) used as a control for transfection.
Table 3.5. Monoclonal antibody (MAb) reactivity patterns of infectious bursal disease virus field isolates and IBDV reference strains using whole virus/ELISA assays.A

<table>
<thead>
<tr>
<th>Isolate</th>
<th>10</th>
<th>57</th>
<th>63</th>
<th>67</th>
<th>69</th>
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<td>+</td>
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<td>-</td>
</tr>
</tbody>
</table>

APerformed by Intervet/Schering-Plough Animal Health, Millsboro, DE.
BOriginal and p1 (single passage seed stocks in SPF chickens) bursal homogenates of each field isolate were used in the AC-ELISA.
CClassic virus STC (USDA-APHIS, Ames, IA).
DVariant viruses Delaware Variant E (Delmarva Peninsula, University of DE), GLS (Delmarva Peninsula, University of MD), RS593 (ISPAH, Millsboro, DE) and AL-2 (Perdue Farms Inc., Vangs Farm, NC).
Table 3.6. Vaccination-challenge of SPF leghorns using Delaware variant vaccine 89/03.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Vaccination Age</th>
<th>No. Birds</th>
<th>Challenge Virus</th>
<th>Challenge Titer&lt;sup&gt;C&lt;/sup&gt; (EID&lt;sub&gt;50/bird&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>89/03&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1 day&lt;sup&gt;B&lt;/sup&gt;</td>
<td>15</td>
<td>4813p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
<tr>
<td>89/03</td>
<td>1 day</td>
<td>15</td>
<td>4947p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
<tr>
<td>89/03</td>
<td>1 day</td>
<td>15</td>
<td>4955p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
<tr>
<td>89/03</td>
<td>1 day</td>
<td>15</td>
<td>5038p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
<tr>
<td>89/03</td>
<td>1 day</td>
<td>15</td>
<td>5041p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
<tr>
<td>89/03</td>
<td>1 day</td>
<td>15</td>
<td>STC</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
<tr>
<td>89/03</td>
<td>1 day</td>
<td>15</td>
<td>Del E</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
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<td>n/a</td>
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<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
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<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
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<td>n/a</td>
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<td>4955p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
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<td>None</td>
<td>n/a</td>
<td>5</td>
<td>5038p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
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<td>n/a</td>
<td>5</td>
<td>5041p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
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<td>STC</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
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<td>Del E</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
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<td>n/a</td>
<td>15</td>
<td>None</td>
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</tbody>
</table>

<sup>A</sup>Intervet/Schering Plough Animal Health, Millsboro, DE
<sup>B</sup>0.2 mL per bird via subcutaneous injection.
<sup>C</sup>0.1 mL per bird via the intracocular route at 21 days of age.
Table 3.7. Vaccination-challenge of SPF leghorns using classic vaccine PBG98.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Vaccination Age</th>
<th>No. Birds</th>
<th>Challenge Virus</th>
<th>Challenge Titer (^{\text{C}}) (EID(_{50})/bird)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBG98(^{\text{A}})</td>
<td>1 day(^{\text{B}})</td>
<td>15</td>
<td>4813p1</td>
<td>(10^{2.0})</td>
</tr>
<tr>
<td>PBG98</td>
<td>1 day</td>
<td>15</td>
<td>4947p1</td>
<td>(10^{2.0})</td>
</tr>
<tr>
<td>PBG98</td>
<td>1 day</td>
<td>15</td>
<td>4955p1</td>
<td>(10^{2.0})</td>
</tr>
<tr>
<td>PBG98</td>
<td>1 day</td>
<td>15</td>
<td>5038p1</td>
<td>(10^{2.0})</td>
</tr>
<tr>
<td>PBG98</td>
<td>1 day</td>
<td>15</td>
<td>5041p1</td>
<td>(10^{2.0})</td>
</tr>
<tr>
<td>PBG98</td>
<td>1 day</td>
<td>15</td>
<td>STC</td>
<td>(10^{2.0})</td>
</tr>
<tr>
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<td>(10^{2.0})</td>
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<td>(10^{2.0})</td>
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<tr>
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<td>5</td>
<td>4955p1</td>
<td>(10^{2.0})</td>
</tr>
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<td>5</td>
<td>5038p1</td>
<td>(10^{2.0})</td>
</tr>
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<td>(10^{2.0})</td>
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<tr>
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<td>5</td>
<td>STC</td>
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<td>5</td>
<td>Del E</td>
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<td>n/a</td>
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<td>None</td>
<td>n/a</td>
</tr>
</tbody>
</table>

\(^{\text{A}}\) Lohmann Animal Health, Harrisonburg, VA

\(^{\text{B}}\) 0.2 mL per bird via subcutaneous injection.

\(^{\text{C}}\) 0.1 mL per bird via intraocular route at 21 days of age.
Table 3.8. Progeny challenge using progeny of 18-week-old SPF leghorn breeder chickens vaccinated with a licensed inactivated IBDV vaccine containing classic and variant strains, as well as, a strain of reovirus and challenged with IBDV field isolate 5038 and reference strain Delaware variant E.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Age of Hens at Vaccination&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Age of Hens at Lay</th>
<th>No. Birds</th>
<th>Challenge Virus</th>
<th>Challenge Titer&lt;sup&gt;C&lt;/sup&gt; (EID&lt;sub&gt;50&lt;/sub&gt;/bird)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sup&gt;A&lt;/sup&gt;</td>
<td>18 weeks</td>
<td>34 weeks</td>
<td>12</td>
<td>5038p1</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>18 weeks</td>
<td>34 weeks</td>
<td>12</td>
<td>Del E</td>
<td>10&lt;sup&gt;2.0&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>18 weeks</td>
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<sup>A</sup>Intervet/Schering Plough Animal Health, Millsboro, DE

<sup>B</sup>0.5 mL per bird by intramuscular injection.

<sup>C</sup>0.1 mL per bird via the intraocular route.
Chapter 4

RESULTS

4.1 VP2 Sequence Analysis

To assess genetic characteristics of IBDV field isolates and identify specific viruses for further characterization, VP2 sequencing and comparative analysis was performed. A primer pair that amplifies a 743 base pair fragment of the VP2 encoding region was used to sequence the variable regions of IBDV field isolates and five IBDV reference strains. Fifteen deduced amino acid sequences of the major structural VP2 protein were obtained from RT-PCR positive samples (Table 3.2). Percent amino acid sequence identity values among the field isolates, reference strains, STC (USDA-APHIS, Ames, IA), Delaware Variant E (Delmarva Peninsula, University of DE), GLS (Delmarva Peninsula, University of MD), AL-2 (Perdue Farms Inc., Vangs Farm, NC) and variant vaccine strain, RS593 (Intervet/Schering-Plough Animal Health, Millsboro, DE) are shown in Table 4.1. Field isolates showed less than a 6% divergence from the classic STC strain and less than a 3% divergence from the Delaware variant E strain. The isolates showed less than a 5% divergence from GLS and less than a 4% divergence from AL-2 and RS593 (Table 4.1). A VP2 phylogenetic tree was constructed by comparing deduced amino acid sequences of the field isolates with known VP2 sequences contained
in an IBD database (Figure 4.1). All of the field isolates clustered under the Delaware variant E strain and were distributed into six distinct clades (Figure 4.1 and Table 3.3). Analysis of the VP2 sequences of IBDV field isolates and reference strains showed that the majority of the amino acid substitutions were observed in major hydrophilic peaks A (aa 210-225) and B (aa 312-325) and minor hydroophilic peaks 1 (aa 248-252) and 2 (aa 279-289) (Figure 4.2) (9, 15, 112, 113, 114, 115)

4.2 Propagation and Pathology of Selected Viruses for Seed Stocks

Five field isolates (4813, 4947, 4955, 5038, and 5041) representative of each of the six clades determined in the phylogenetic analysis were selected for propagation in SPF leghorn chickens. In addition, two IBDV reference strains, STC and Delaware variant E (Del E) were used for the cultivation of seed stocks (Table 3.3). As a result of STC infection, clinical signs included ruffled feathers, reddish, almond-shaped eyes, watery diarrhea, and depression. Upon harvest at three days post-inoculation, cloacal bursas appeared slightly enlarged, round or oval-shaped, cream-colored, and contained longitudinal striations and a gelatinous transudate around the serosal surface. Spleens were normal in size and color. Birds infected with Del E and IBDV field isolates showed minimal clinical signs; however, ruffled feathers and depression were observed with some birds. Cloacal bursas were normal to small in size, firm, and displayed slight mucosal edema. Slight splenomegaly was observed with some birds. Histological data demonstrated that the two IBDV reference strains and five of the six propagated IBDV field isolates were viable, and they were subsequently used for further analysis.
4.3 Monoclonal Antibody Testing

To characterize the antigenic properties of the IBDV field isolates 4813, 4947, 4955, 5038, and 5041 and to determine their relatedness to classical and variant IBDV strains, two antigen capture enzyme-linked immunosorbent assays (AC-ELISA) were performed using IBDV-specific monoclonal antibodies (MAbs) (15, 21, 66, 114, 207). MAb reactivity pattern results from the MAb test using a transfection and immunofluorescence assay system (E. Mundt, University of Georgia) are presented in Table 3.4. All of the isolates reacted with the polyclonal anti-VP1 rabbit serum, indicating that cotransfection of cRNA was successful and the tests were valid (E. Mundt, unpubl. data; 21). Epitope B69 was absent from the five field isolates, thereby differentiating them from the classic strains of IBDV. Isolate 4813 reacted with MAb 63 and MAb 67, and isolate 4955 showed a positive reactivity to MAb 67. Isolates 4947, 5038, and 5041 displayed no reactivity with MAbs 10, 57, 63, 67, and 69.

MAb reactivity pattern results from the MAb test using a whole virus/ELISA assay system (P. Lynch, Intervet/Schering-Plough Animal Health) are presented in Table 3.5. Original bursal samples obtained from commercial broilers of 4947, 4955, and 5041 isolates did not react with MAbs B29, 8, or BK9; however, bursal samples taken from one passage (p1) of 4955 and 5041 in SPF chickens did react with B29 and 8 MAbs. The p1 sample of 4947 only reacted with MAb B29. Epitope B69 was absent from the five field isolates, again, differentiating them from the classic strains of IBDV. Isolate 4813 shared a similar reaction pattern with Delaware variant E (Del E) in its binding of MAbs R63 and 67. Isolate 4947 showed a similar reaction pattern to RS593 in its binding of
MAbs B29 and 67. Isolate 4955 reacted with MAb 67 in both assays. In addition, MAbs B29 and 8 reacted with isolate 4955, which together demonstrated its similarity to the IBDV variant vaccine strain RS593. Isolate 5041 shared a similar reaction pattern to the GLS variant in its binding of MAbs 57 and 10. Aside from a reaction with B29, 8, BK9, and VP1 MAbs, IBDV field isolate 5038 displayed no reactivity with the other MAbs used in the panel.

4.4 Monoclonal Antibody Epitope Mapping

Correlations between predicted amino acid substitutions and monoclonal antibody (MAb) reactivity patterns were previously described by Vakharia et al. (15) and Sellers et al. (114) (Tables 4.2 – 4.6). A glutamine at position 249 has been reported to be essential for the binding of MAb 69 (Table 4.2). All five field isolates had a glutamine to lysine substitution at this position and escaped the binding of neutralizing MAb 69. Amino acids likely to be important in the binding of MAb 10 are threonine at position 286, glycine at position 318, and aspartic acid at position 323 (Table 4.3). This is supported by the positive reactivity with reference strains STC and GLS. Isolate 5038 and reference strain RS593 differ by only one amino acid (i.e. isolate 5038 showed an aspartic acid to glutamic acid substitution at position 323, and RS593 showed a threonine to isoleucine substitution at position 286) and escaped the binding of MAb 10.

The reactivity of the MAb 57 epitope has been previously shown to depend on a glutamic acid at position 321, which is supported by the positive reactivity with isolate 5041, and reference strains GLS and AL-2 (Table 4.4). Interestingly, isolate 5038
contained a glutamic acid residue at position 321, but did not react with MAb 57. This suggests that recognition of the MAb 57 epitope may involve other critical amino acid residues. A glutamic acid to alanine substitution at position 321 appears to inhibit the binding of MAb 57 and allows for the binding of MAb 63, as previously reported by Letzel et al. (21). This is supported by the positive reactivity with isolate 4813 and reference strains Del E and STC. Isolates 4947 and 4955, and reference strain RS593 contained an alanine at position 321 and did not react with MAb 63 (Table 4.5). This suggests that substitutions at other positions along the sequence may play a role in the binding of MAb 63.

Three amino acids that appear to be essential for the binding of MAb 67 include asparagine at position 213, isoleucine at position 286, and aspartic acid at position 318 (Table 4.6) (15). Isolate 4947 and reference strains RS593, and AL-2 reacted with Mab 67, despite variation in their residues at position 318. This suggests that substitutions at position 318 may be less critical for the binding of MAb 67. Previous reports identified a serine/threonine residue at position 222 as important for the binding of MAb 67 (Table 4.6) (21). However, isolates 5038 and 5041 and reference strain GLS contained serine/threonine residues at position 222 and did not bind MAb 67.

4.5 Virus Neutralization in Cell Culture

The virus neutralizing (VN) antibody titers in serum from SPF leghorn chickens infected with IBDV field isolates and reference strains against IBDV viruses 89/03 and PBG98 are summarized in Table 4.7. All of the sera cross-neutralized the variant 89/03
strain and the classic PBG98 strain. A four-fold difference was considered to be significant; however, with the exception of isolate 4813 and the two IBDV reference strains (Del E and STC), no significant difference in neutralization of the two viruses was observed.

4.6 Active Immunity – Challenge with 89/03 Vaccine

SPF leghorn chickens vaccinated with IBDV strain 89/03 and challenged with IBDV field isolates 4813, 4947, 4955, 5038, and 5041 and reference strains STC and Delaware variant E exhibited no clinical signs or mortality throughout the duration of the study. At 2 days post-challenge, clinical signs characteristic of classic IBDV including dehydration, depression, and ruffled feathers were observed in one non-vaccinated SPF leghorn chicken infected with field isolate 4947. All other non-vaccinated, challenged controls and non-vaccinated, non-challenged control birds were normal.

Mean bursa to body weight ratios were calculated and percent protection values were determined based on a minimum protection level of two standard deviations less than the mean of the negative control group (Table 4.8). All chickens vaccinated with 89/03 were completely protected against challenge with the five field isolates and reference strains. Disease incidence observed in non-vaccinated, chickens challenged with field isolates 4947, 5038, STC and Delaware variant E ranged from 20 to 40%. Non-vaccinated chicks challenged with isolates 4955 and 5041 showed 60% disease incidence, while non-vaccinated chicks infected with isolate 4813 displayed 80% incidence. Bursal
atrophy was observed in all non-vaccinated, challenged chicks, whereas all bursas removed from 89/03-vaccinated chicks were normal in size.

Figure 4.3 presents the geometric mean antibody titers (GMTs) for antisera collected prior to challenge at 21 days of age as measured by ELISA. Non-vaccinated, non-challenged control birds and non-vaccinated, challenged control birds had low antibody titers (GMTs of 2 and 4, respectively), whereas, all birds vaccinated with 89/03 showed high antibody titers (GMTs ranging from 1485 to 2294). Antibody GMTs for antisera collected 7 days post-challenge showed an increase in anti-IBDV ELISA titers in both 89/03-vaccinated (GMTs ranging from 2156 to 3206) and non-vaccinated (GMTs ranging from 2154 to 3178) chickens. Non-vaccinated, non-challenged control birds tested seronegative for IBDV antibodies (Figure 4.3).

Histopathological examination of the bursa of Fabricius of non-vaccinated SPF leghorn chickens challenged with IBDV field isolates and reference strains revealed lesions typical of IBDV (Table 4.9). All non-vaccinated challenge controls showed no protection based on histopathology. All of the non-vaccinated, non-challenged controls showed no disease incidence. SPF leghorn chickens vaccinated with 89/03 and challenged with STC showed 94% protection. No other 89/03-vaccinated birds displayed histological IBDV lesions in the bursa.

Microscopic evaluation of the thymus revealed significant lesions in one 89/03-vaccinated SPF leghorn chicken challenged with isolate 4947, where there was diffuse, moderate to severe cortical lymphocyte loss. Two non-vaccinated SPF leghorns infected with STC also showed significant thymic lesions, with minimal to moderate cortical
lymphocyte loss with and without associated heterophilic infiltration. Microscopic changes in the thymus were not observed in non-vaccinated, non-challenged control birds or the remainder of 89/03-vaccinated and non-vaccinated, challenged birds. An increase in the number of germinal centers in the spleen was observed in several 89/03-vaccinated and non-vaccinated chicks; however, this finding was not considered a significant microscopic lesion of IBDV infection.

4.7 Active Immunity – Challenge with PBG98

SPF leghorn chickens vaccinated with IBDV strain PBG98 and challenged with IBDV field isolates 4813, 4947, 4955, 5038, and 5041 and reference strains STC and Delaware variant E exhibited no clinical signs or mortality after virus challenge. Similarly, non-vaccinated challenged controls and non-vaccinated, non-challenged control birds displayed no clinical signs or mortality during the study. Percent protection of SPF leghorn chickens vaccinated with PBG98 as determined by mean bursa to body weight indexes ranged from 80 to 100% (Table 4.10). Non-vaccinated, challenged SPF leghorns showed 100% disease incidence (Table 4.10). Bursal atrophy was observed in all non-vaccinated, challenged chicks, whereas all bursas from PBG98-vaccinated chicks were normal in size.

Figure 4.4 presents the geometric mean antibody titers (GMTs) for antisera collected prior to challenge at 21 days of age as measured by ELISA. Non-vaccinated, non-challenged control birds and non-vaccinated, challenged control birds had antibody titers below the baseline of 18, whereas, all birds vaccinated with PBG98 showed
antibody titers ranging from 1934 to 4362. Antibody GMTs for antisera collected 7 days post-challenge showed an increase in anti-IBDV ELISA titers in both PBG98-vaccinated (GMTs ranging from 5610 to 9097) and non-vaccinated, challenged (GMTs ranging from 1335 to 3122) chickens. Non-vaccinated, non-challenged control birds tested seronegative for IBDV antibodies (Figure 4.4).

Histopathological examination of the bursa of Fabricius of non-vaccinated SPF leghorn chickens challenged with IBDV field isolates and reference strains revealed lesions typical of IBDV (Table 4.11). Based on histopathology, all non-vaccinated, challenged controls were unprotected. All of the non-vaccinated, non-challenged controls showed 100% protection. Birds vaccinated with PBG98 displayed 60% protection against challenge with 4813, 87% protection against challenge with 4947, 40% protection against challenge with 4955, 67% protection against challenge with 5038, 73% protection against challenge with 5041, 93% protection against challenge with STC, and 93% protection against challenge with Del E.

Microscopic evaluation of the thymus revealed significant lesions in one non-vaccinated SPF leghorn chicken challenged with STC, where there was diffuse, moderate cortical lymphocyte loss via apoptosis. In the same bird, diffuse ellipsoid degeneration in the spleen was observed along with many foamy macrophages and plasma cells in the red pump. Microscopic lesions in the thymus and spleen were not observed in non-vaccinated, non-challenged control birds or the remainder of PBG98-vaccinated and non-vaccinated, challenged birds.
4.8 Passive Immunity - Progeny Challenge

Percent protection of white leghorn progeny challenged on day 10 and day 17 as determined by bursa to body weight indexes is displayed in Table 4.12 and Table 4.13, respectively. Progeny challenged on day 10 showed greater protection against challenge with Del E (64%) than those challenged with 5038 (33%). Day 10 SPF leghorn control birds showed 100% disease incidence following challenge with both Del E and 5038. Progeny challenged on day 17 displayed equal protection (42%) against challenge with isolate 5038 and Del E. Day 17 SPF leghorn controls exhibited 92% disease incidence following challenge with both Del E and 5038. Bursal atrophy was observed in all SPF leghorn non-vaccinated, IBDV-challenged chicks following the day 10 and 17 challenge. Isolate 5038 and Del E induced minimal to moderate (33-64%) bursal atrophy in progeny challenged on day 10 and 17.

Results of histopathological examination of the bursa of Fabricius of progeny and SPF leghorn chickens challenged with isolate 5038 and Del E are displayed in Table 4.14. Percent protection for day 10 challenge as determined by microscopic pathology showed 42% protection in progeny challenged with Del E; whereas, no protection was observed in progeny challenged with 5038. SPF leghorn non-vaccinated, IBDV challenge control chickens showed 100% disease incidence following day 10 challenge with both Del E and 5038. Percent protection for day 17 challenge showed minimal protection (8%) in progeny challenged with 5038 and progeny challenged with Del E. SPF leghorn non-vaccinated, IBDV challenge control chickens showed 100% disease incidence following day 17 challenge with both Del E and 5038.
Table 4.1. Percent identity values of protein sequences of the variable region of VP2 protein of IBDV field strains isolated from Delmarva broiler farms and reference strains Delaware variant E, STC, GLS, AL-2, and RS593 as determined by multiple sequence alignment using the CLUSTAL W method.<sup>A</sup>

Protein sequences were generated using an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and analyzed with MegAlign v8.0.2 software (DNASTAR, Inc., Madison, WI).

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<td>0.9</td>
<td>3.2</td>
<td>2.8</td>
<td>95.1</td>
<td>96.9</td>
<td>98.2</td>
<td>97.8</td>
<td>16</td>
</tr>
<tr>
<td>17</td>
<td>5.1</td>
<td>6.1</td>
<td>6.1</td>
<td>5.1</td>
<td>6.1</td>
<td>4.6</td>
<td>4.8</td>
<td>5.1</td>
<td>5.8</td>
<td>6.1</td>
<td>5.1</td>
<td>5.1</td>
<td>5.6</td>
<td>5.6</td>
<td>5.8</td>
<td>5.1</td>
<td>100.0</td>
<td>94.6</td>
<td>95.1</td>
<td>17</td>
</tr>
<tr>
<td>18</td>
<td>3.7</td>
<td>4.6</td>
<td>3.7</td>
<td>4.6</td>
<td>4.2</td>
<td>3.3</td>
<td>3.7</td>
<td>4.3</td>
<td>4.6</td>
<td>1.4</td>
<td>1.4</td>
<td>3.7</td>
<td>3.2</td>
<td>3.3</td>
<td>3.2</td>
<td>4.2</td>
<td>97.3</td>
<td>96.4</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>2.3</td>
<td>3.2</td>
<td>2.7</td>
<td>3.2</td>
<td>3.2</td>
<td>2.7</td>
<td>2.3</td>
<td>3.3</td>
<td>3.2</td>
<td>3.2</td>
<td>2.3</td>
<td>2.7</td>
<td>2.7</td>
<td>2.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

<sup>A</sup>Protein sequences were generated using an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and analyzed with MegAlign v8.0.2 software (DNASTAR, Inc., Madison, WI).
Table 4.2. Correlation between predicted amino acid substitutions and monoclonal antibody (MAb) 69 reactivity patterns for passage 1 seed stock samples of IBDV field isolates and IBDV reference strains\(^A\).

<table>
<thead>
<tr>
<th>Amino Acid Position</th>
<th>MAb 69 Transfection and Immunofluorescence(^B)</th>
<th>Whole Virus/ELISA(^C)</th>
<th>Virus</th>
<th>249</th>
<th>280</th>
<th>326</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>4813</td>
<td>Lys</td>
<td>Asn</td>
<td>Ser</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>4947</td>
<td>Lys</td>
<td>Asn</td>
<td>Ser</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>4955</td>
<td>Lys</td>
<td>Asn</td>
<td>Ser</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>5038</td>
<td>Lys</td>
<td>Asn</td>
<td>Ser</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>5041</td>
<td>Lys</td>
<td>Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>STC</td>
<td>Gln</td>
<td>Asn</td>
<td>Ser</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>DelE</td>
<td>Lys</td>
<td>Asn</td>
<td>Ser</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>GLS</td>
<td>Lys</td>
<td>Asn</td>
<td>Ser</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>RS593</td>
<td>Lys</td>
<td>Asn</td>
<td>Ser</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>AL2</td>
<td>Lys</td>
<td>Asn</td>
<td>Ser</td>
<td></td>
</tr>
</tbody>
</table>

\(^A\) p1 sample is SPF bursal origin stock.

\(^B\) Performed by Egbert Mundt, University of Georgia.

\(^C\) Performed by Intervet/Schering-Plough Animal Health, Millsboro, DE.
Table 4.3. Correlation between predicted amino acid substitutions and monoclonal antibody (MAb) 10 reactivity patterns for passage 1 seed stock samples of IBDV field isolates and IBDV reference strains\textsuperscript{A}.

<table>
<thead>
<tr>
<th>MAb 10 Transfection and Immunofluorescence\textsuperscript{B}</th>
<th>Whole Virus/ELISA\textsuperscript{C}</th>
<th>Amino Acid Position</th>
<th>Virus 286</th>
<th>318</th>
<th>323</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>4813</td>
<td>Ile</td>
<td>Asp</td>
<td>Glu</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>4947</td>
<td>Ile</td>
<td>Asn</td>
<td>Asp</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>4955</td>
<td>Ile</td>
<td>Asp</td>
<td>Asp</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5038</td>
<td>Thr</td>
<td>Gly</td>
<td>Glu</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>5041</td>
<td>Ile</td>
<td>Ser</td>
<td>Asp</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>STC</td>
<td>Thr</td>
<td>Gly</td>
<td>Asp</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>DelE</td>
<td>Ile</td>
<td>Asp</td>
<td>Glu</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>GLS</td>
<td>Thr</td>
<td>Gly</td>
<td>Asp</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>RS593</td>
<td>Ile</td>
<td>Gly</td>
<td>Asp</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>AL2</td>
<td>Ile</td>
<td>Asn</td>
<td>Asp</td>
</tr>
</tbody>
</table>

\textsuperscript{A} p1 sample is SPF bursal origin stock.
\textsuperscript{B}Performed by Egbert Mundt, University of Georgia.
\textsuperscript{C}Performed by Intervet/Schering-Plough Animal Health, Millsboro, DE.
Table 4.4. Correlation between predicted amino acid substitutions and monoclonal antibody (MAb) 57 reactivity patterns for passage 1 seed stock samples of IBDV field isolates and IBDV reference strains

<table>
<thead>
<tr>
<th>MAb 57</th>
<th>Amino Acid Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transfection and Immunofluorescence</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

A1 sample is SPF bursal origin stock.
BPerformed by Egbert Mundt, University of Georgia.
CPerformed by Intervet/Schering-Plough Animal Health, Millsboro, DE.
Table 4.5. Correlation between predicted amino acid substitutions and monoclonal antibody (MAb) 63 reactivity patterns for passage 1 seed stock samples of IBDV field isolates and IBDV reference strains

<table>
<thead>
<tr>
<th>Amino Acid Position</th>
<th>MAb 63 Transfection and Immunofluorescence B</th>
<th>Whole Virus/ ELISA C</th>
<th>Virus 321</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>4813</td>
<td>Ala</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>4947</td>
<td>Ala</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>4955</td>
<td>Ala</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5038</td>
<td>Glu</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5041</td>
<td>Glu</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>STC</td>
<td>Ala</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>DeIE</td>
<td>Ala</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>GLS</td>
<td>Glu</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>RS593</td>
<td>Ala</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>AL2</td>
<td>Glu</td>
</tr>
</tbody>
</table>

A p1 sample is SPF bursal origin stock.
B Performed by Egbert Mundt, University of Georgia.
C Performed by Intervet/Schering-Plough Animal Health, Millsboro, DE.
Table 4.6. Correlation between predicted amino acid substitutions and monoclonal antibody (MAb) 67 reactivity patterns for passage 1 seed stock samples of IBDV field isolates and IBDV reference strains\textsuperscript{A}.

<table>
<thead>
<tr>
<th>MAb 67</th>
<th>Amino Acid Position</th>
<th>213</th>
<th>286</th>
<th>318</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfection and Immunofluorescence\textsuperscript{B}</td>
<td>Whole Virus/ ELISA\textsuperscript{C}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>4813</td>
<td>Asn</td>
<td>Ile</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>4947</td>
<td>Asn</td>
<td>Ile</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>4955</td>
<td>Asn</td>
<td>Ile</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5038</td>
<td>Asp</td>
<td>Thr</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5041</td>
<td>Asp</td>
<td>Ile</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>STC</td>
<td>Asp</td>
<td>Thr</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>DelE</td>
<td>Asn</td>
<td>Ile</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>GLS</td>
<td>Asp</td>
<td>Thr</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>RS593</td>
<td>Asn</td>
<td>Ile</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>AL2</td>
<td>Asn</td>
<td>Ile</td>
</tr>
</tbody>
</table>

\textsuperscript{A}p1 is SPF bursal origin stock.

\textsuperscript{B}Performed by Egbert Mundt, University of Georgia.

\textsuperscript{C}Performed by Intervet/Schering-Plough Animal Health, Millsboro, DE.
Table 4.7. Virus-neutralization (VN) serum antibody titers of SPF leghorn chickens against IBDV strains 89/03 and PBG98 as measured by beta VN assays in chicken embryo fibroblast cells\textsuperscript{A}.

<table>
<thead>
<tr>
<th>Antiserum\textsuperscript{B}</th>
<th>89/03\textsuperscript{C}</th>
<th>PBG98\textsuperscript{D}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>4813</td>
<td>&gt;12800</td>
<td>3200</td>
</tr>
<tr>
<td>4947</td>
<td>1600-3200\textsuperscript{E}</td>
<td>1600-3200\textsuperscript{E}</td>
</tr>
<tr>
<td>4955</td>
<td>3200-6400\textsuperscript{E}</td>
<td>1600</td>
</tr>
<tr>
<td>5038</td>
<td>3200</td>
<td>800</td>
</tr>
<tr>
<td>5041</td>
<td>1600</td>
<td>3200</td>
</tr>
<tr>
<td>STC</td>
<td>3200</td>
<td>&gt;12800</td>
</tr>
<tr>
<td>Delaware Variant E</td>
<td>&gt;12800</td>
<td>6400</td>
</tr>
</tbody>
</table>

\textsuperscript{A}Reciprocal of the highest dilution of serum that neutralized 100 TCID\textsubscript{50} of IBDV.

\textsuperscript{B}Antiserum was harvested at 4 weeks post-inoculation from SPF leghorn chickens infected with IBDV field isolates and reference strains.

\textsuperscript{C}Variant virus

\textsuperscript{D}Classic virus

\textsuperscript{E}Value range due to discrepancy between duplicates.
Table 4.8. Percent protection determined by bursa to body weight ratios of one-day-old SPF leghorn chickens subcutaneously vaccinated with IBDV strain 89/03 and challenged at 21 days post-vaccination via the intraocular route with IBDV field isolates and reference strains STC and Delaware variant E.

<table>
<thead>
<tr>
<th>Challenge Virus</th>
<th>Treatment</th>
<th>Percent Protection(^B)</th>
<th>Mean Bursa to Body Weight Ratio(^A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>n/a</td>
<td></td>
<td>4.98</td>
</tr>
<tr>
<td>4813</td>
<td>Vaccinated</td>
<td>100(^*)</td>
<td>5.31</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>20</td>
<td>1.33</td>
</tr>
<tr>
<td>4947</td>
<td>Vaccinated</td>
<td>100</td>
<td>4.69</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>80</td>
<td>1.89</td>
</tr>
<tr>
<td>4955</td>
<td>Vaccinated</td>
<td>100(^*)</td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>40</td>
<td>1.39</td>
</tr>
<tr>
<td>5038</td>
<td>Vaccinated</td>
<td>100(^*)</td>
<td>5.62</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>60</td>
<td>1.59</td>
</tr>
<tr>
<td>5041</td>
<td>Vaccinated</td>
<td>100(^*)</td>
<td>5.31</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>40</td>
<td>1.62</td>
</tr>
<tr>
<td>STC</td>
<td>Vaccinated</td>
<td>100(^*)</td>
<td>5.08</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>60</td>
<td>1.55</td>
</tr>
<tr>
<td>Delaware variant E</td>
<td>Vaccinated</td>
<td>100(^*)</td>
<td>5.60</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>60</td>
<td>1.69</td>
</tr>
</tbody>
</table>

\(^A\)Minimum protection level (Control Group Mean – 2 Standard Deviations)

\(^B\)Astericks indicate a significant difference by two-tailed chi-square analysis compared with non-vaccinated challenge controls within the same treatment group (P < 0.05).
Table 4.9. Microscopic pathology of the bursa of Fabricius of SPF leghorn chickens vaccinated with IBDV strain 89/03 and challenged with IBDV field isolates 4813, 4947, 4955, 5038, and 5041 and IBDV reference strains STC and Delaware variant EA.

<table>
<thead>
<tr>
<th>Bursal lesion scores</th>
<th>Histopathological bursa of Fabricius lesions</th>
<th>4813</th>
<th>4947</th>
<th>4955</th>
<th>5038</th>
<th>5041</th>
<th>STC</th>
<th>Del E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>16/16</td>
<td>16/16</td>
<td>16/16</td>
<td>16/16</td>
<td>6/6</td>
<td>14/16</td>
<td>16/16</td>
</tr>
<tr>
<td></td>
<td>No IBDV related lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mild scattered IBDV related cell depletion in a few follicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/16</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/16</td>
</tr>
<tr>
<td></td>
<td>1/3 to 1/2 of the follicles with IBDV related atrophy or depletion of cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Severe atrophy of all follicles</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/4</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acute necrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A\textsuperscript{a} Birds were challenged via the intraocular route at 21 days of age and bursas were collected 7 days post-challenge.

B\textsuperscript{b} BV = Vaccinated

NV = Non-vaccinated
Table 4.10. Percent protection determined by bursa to body weight ratios of one-day-old SPF leghorn chickens subcutaneously vaccinated with IBDV strain PBG98 and challenged at 21 days of age via the intraocular route with IBDV field isolates and reference strains STC and Delaware variant E.

<table>
<thead>
<tr>
<th>Challenge Virus</th>
<th>Treatment</th>
<th>Percent Protection(^\text{B})</th>
<th>Mean Bursa to Body Weight Ratio(^\text{A})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>n/a</td>
<td></td>
<td>6.08</td>
</tr>
<tr>
<td>4813</td>
<td>Vaccinated</td>
<td>87(^*)</td>
<td>5.68</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>0</td>
<td>1.14</td>
</tr>
<tr>
<td>4947</td>
<td>Vaccinated</td>
<td>100(^*)</td>
<td>4.69</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>0</td>
<td>1.25</td>
</tr>
<tr>
<td>4955</td>
<td>Vaccinated</td>
<td>93(^*)</td>
<td>5.55</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>0</td>
<td>1.51</td>
</tr>
<tr>
<td>5038</td>
<td>Vaccinated</td>
<td>87(^*)</td>
<td>5.57</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>0</td>
<td>1.57</td>
</tr>
<tr>
<td>5041</td>
<td>Vaccinated</td>
<td>80(^*)</td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>0</td>
<td>1.50</td>
</tr>
<tr>
<td>STC</td>
<td>Vaccinated</td>
<td>100(^*)</td>
<td>5.74</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>0</td>
<td>1.77</td>
</tr>
<tr>
<td>Delaware variant E</td>
<td>Vaccinated</td>
<td>87(^*)</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>0</td>
<td>1.60</td>
</tr>
</tbody>
</table>

\(^{A}\)Minimum protection level (Control Group Mean – 2 Standard Deviations)

\(^{B}\)Asterisks indicate a significant difference by two-tailed chi-square analysis compared with non-vaccinated challenge controls within the same treatment group (\(P < 0.05\)).
Table 4.11. Microscopic pathology of the bursa of Fabricius of SPF leghorn chickens vaccinated with IBDV strain PBG98 and challenged with IBDV field isolates 4813, 4947, 4955, 5038, and 5041 and IBDV reference strains STC and Delaware variant E^A.

<table>
<thead>
<tr>
<th>Bursal lesion scores</th>
<th>Histopathological bursa of Fabricius lesions</th>
<th>4813</th>
<th>4947</th>
<th>4955</th>
<th>5038</th>
<th>5041</th>
<th>STC</th>
<th>Del E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal No IBDV related lesions</td>
<td>9/15</td>
<td>12/15</td>
<td>6/15</td>
<td>10/15</td>
<td>11/15</td>
<td>14/15</td>
<td>14/15</td>
</tr>
<tr>
<td>2</td>
<td>Mild scattered IBDV related cell depletion in a few follicles</td>
<td>3/15</td>
<td>1/15</td>
<td>4/15</td>
<td>2/15</td>
<td>1/15</td>
<td>1/15</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Moderate 1/3 to 1/2 of the follicles with IBDV related atrophy or depletion of cells</td>
<td>1/15</td>
<td>2/15</td>
<td>1/15</td>
<td>2/15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Severe atrophy of all follicles Inflammation Acute necrosis</td>
<td>2/15</td>
<td>5/5</td>
<td>1/15</td>
<td>5/5</td>
<td>3/15</td>
<td>5/5</td>
<td>2/5</td>
</tr>
</tbody>
</table>

^A^Birds were challenged via the intraocular route at 21 days of age and bursas were collected 7 days post-challenge.

^B^V = Vaccinated

NV = Non-vaccinated
Table 4.12. Percent protection determined by bursa to body weight ratios of progeny of 18-week-old SPF leghorn breeder chickens vaccinated with a licensed inactivated IBDV vaccine containing classic and variant strains, as well as, a strain of reovirus and challenged with field isolate 5038 and reference strain Delaware variant E at 10 days of age.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Challenge Virus</th>
<th>Percent Protection^B</th>
<th>Mean Bursa to Body Weight Ratio^A</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5038</td>
<td>33^*</td>
<td>2.53</td>
</tr>
<tr>
<td>A</td>
<td>Del E</td>
<td>64</td>
<td>2.95</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>100</td>
<td>4.22</td>
</tr>
<tr>
<td>Non-vaccinated</td>
<td>5038</td>
<td>0</td>
<td>1.01</td>
</tr>
<tr>
<td>Non-vaccinated</td>
<td>Del E</td>
<td>0</td>
<td>1.22</td>
</tr>
<tr>
<td>Non-vaccinated</td>
<td>None</td>
<td>100</td>
<td>4.00</td>
</tr>
</tbody>
</table>

^A Minimum protection level (Control Group Mean – 2 Standard Deviations).  
^B Asterisks indicate a significant difference by two-tailed chi-square analysis compared with non-vaccinated challenge controls within the same treatment group (P < 0.05).
Table 4.13. Percent protection determined by bursa to body weight ratios of progeny of 18-week-old SPF leghorn breeder chickens vaccinated with a licensed inactivated IBDV vaccine containing classic and variant strains, as well as, a strain of reovirus and challenged with field isolate 5038 and Delaware variant E at 17 days of age.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Challenge Virus</th>
<th>Percent Protection^B</th>
<th>Mean Bursa to Body Weight Ratio^A</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5038</td>
<td>42</td>
<td>2.93</td>
</tr>
<tr>
<td>A</td>
<td>Del E</td>
<td>42</td>
<td>2.36</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>100</td>
<td>4.28</td>
</tr>
<tr>
<td>Non-vaccinated</td>
<td>5038</td>
<td>8</td>
<td>1.33</td>
</tr>
<tr>
<td>Non-vaccinated</td>
<td>Del E</td>
<td>8</td>
<td>1.43</td>
</tr>
<tr>
<td>Non-vaccinated</td>
<td>None</td>
<td>100</td>
<td>3.76</td>
</tr>
</tbody>
</table>

^A Minimum protection level (Control Group Mean – 2 Standard Deviations).

^B Asterisks indicate a significant difference by two-tailed chi-square analysis compared with non-vaccinated challenge controls within the same treatment group (P < 0.05).
Table 4.14. Microscopic pathology of the bursa of Fabricius of progeny from 18-week-old SPF leghorn breeder chickens vaccinated with a licensed inactivated IBDV vaccine containing classic and variant strains, as well as, a strain of reovirus and challenged with field isolate 5038 and Delaware variant E\(^A\).

<table>
<thead>
<tr>
<th>Bursal lesion scores</th>
<th>Histopathological bursa of Fabricius lesions</th>
<th>Day 10 Progeny Group Challenge(^B)</th>
<th>Day 17 Progeny Group Challenge(^B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5038 V NV</td>
<td>Del E V NV</td>
</tr>
<tr>
<td>1</td>
<td>Normal No IBDV related lesions</td>
<td>3/12</td>
<td>11/11</td>
</tr>
<tr>
<td>2</td>
<td>Mild scattered IBDV related cell depletion in a few follicles</td>
<td>2/12</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Moderate 1/3 to 1/2 of the follicles with IBDV related atrophy or depletion of cells</td>
<td>1/12</td>
<td>1/12</td>
</tr>
<tr>
<td>4</td>
<td>Severe atrophy of all follicles Inflammation Acute necrosis</td>
<td>11/12</td>
<td>12/12</td>
</tr>
</tbody>
</table>

\(^A\)Birds were challenged via the intraocular route at 10 and 17 days of age and bursas were collected 7 days post-challenge.

\(^B\)V = Vaccinated

NV = Non-vaccinated
Figure 4.1. VP2 phylogenetic analysis of IBDV field isolates and reference strains constructed using Molecular Evolutionary Genetics Analysis software version 4.0 (302) and the unweighted pair-group method using arithmetic averages method (303).
Figure 4.1 continued.
Figure 4.2. Deduced amino acid sequences of the variable region of VP2 protein of IBDV field isolates and reference strains Delaware variant E, STC, GLS, AL-2, and RS593. Major (9, 112, 315) and minor (113, 115) hydrophilic peaks are indicated.
Figure 4.3. Serum antibody geometric mean titers (GMTs) of 89/03-vaccinated and non-vaccinated SPF leghorn chickens before and after challenge with IBDV field isolates and reference strains as measured by ELISA.
Figure 4.4. Serum antibody geometric mean titers (GMTs) of PBG98-vaccinated and non-vaccinated SPF leghorn chickens before and after challenge with IBDV field isolates and reference strains as measured by ELISA.
Infectious bursal disease virus is among the most important pathogens of poultry, because it replicates and damages tissues of the immune system (94, 316). Permanent immunosuppression results from infection of chickens less than one-week of age (141). The virus is ubiquitous in poultry production systems because of its unusually resistant physicochemical characteristics combined with the infrequent clean-out and disinfection of poultry houses (94, 316). Although IBDV vaccination is regularly practiced in the industry, selection of novel antigenic types has occurred (25, 56, 57, 64, 201). Thus, ongoing surveillance for new IBDV isolates is important so that new isolates may be antigenically characterized and evaluated for the ability to break through immunity provided by current vaccines.

Thirty-three IBDV field isolates were recovered from 3- and 4-week-old broilers, located on 12 different farms on the Delmarva Peninsula. Flocks raised on these farms had a history of poor performance due to recurrent cases of gangrenous dermatitis, inclusion body hepatitis, runting and stunting syndrome, and respiratory complex. Fifteen of the isolates found to be IBDV positive by histopathology, virus isolation, and RT-PCR were used for sequencing of the variable region of VP2. VP2 phylogenetic analysis of the deduced amino acid sequences showed that the isolates distributed into six different
clades, all representing serotype 1 Delaware variant viruses. Five field isolates representative of the six molecular clades were selected for further analysis.

The characterization of the Delmarva isolates showed that one of the five field isolates, 5038, was antigenically different from reference strains STC, Del E, GLS, RS593, and AL-2 based on monoclonal antibody (MAb) reactivity patterns. This was surprising because, in both of the active vaccination-challenge studies, vaccinated leghorn chickens exhibited solid immunity against the 5038 virus. Virus neutralization tests correlated with these findings in that 5038 antiserum neutralized 100 TCID$_{50}$ of both the 89/03 virus and the PBG98 virus. In contrast to the active vaccination-challenge studies, a passive immunity progeny challenge indicated that 5038 was able to break through maternally-derived immunity earlier than Del E due to its antigenic difference.

Use of the amino acid sequence in combination with the antigenic profile as determined by MAb reactivity has been shown to be highly informative in the antigenic characterization of field isolates (201). Genomic characterization and comparison of the variable region of VP2 of 5038 showed that the sequences at amino acid positions 249K and 254S were similar to other variant strains (16, 17, 194, 286, 317, 318). Residues at positions 253, 279, and 284 of the VP2 protein sequence have been reported to impact virulence and pathogenic phenotype (16, 319), while residues at position 222 and within the hydrophilic peak B region (aa 314-324) have been found to affect antigenicity (205, 286). In addition, amino acid positions 318 and 322 are thought to play a role in the pathogenicity of IBDV (17). Interestingly, the VP2 sequence of 5038 at amino acid positions 222T, 253Q, 279N, 284A, and 322G, was also similar to other characterized
variant strains (16, 17, 194, 201, 286, 317, 318). The deduced amino acid sequence showed that 5038 shared one amino acid substitution, 294I, that is unique to vvIBDV strains (14, 215, 318); however, none of the other genetic elements common to vvIBDV were present. Unfortunately, VP2 sequence analysis did not allow the pinpointing of the exact mutations responsible for the lack of MAb binding with 5038. Studies involving selection and comparative nucleotide sequencing of escape mutants may help to determine the genetic basis of this unique reaction pattern with the MAbs.

In this study, we also demonstrated that four of the five field viruses, 4813, 4947, 4955, and 5041, were antigenically similar to several well-characterized IBDV strains (i.e. Delaware variant E, RS593, GLS). Genomic characterization of the variable region of VP2 of the four isolates showed that the sequence at amino acid positions 222T/S, 249K, 253Q, 254S/N, 279N, 284A, and 322G/E was similar to other characterized variant strains (16, 17, 194, 201, 286, 317, 318). Position 318 was the most mutated with four different amino acids, aspartic acid, asparagine, glycine, and serine. Jackwood et al. (17) reported similar findings from a sequence comparison of 25 IBDV strains placed in the same or different molecular groups by RT/PCR-RFLP. It is possible that the 318 mutation played a role in the emergence of these four field isolates. However, active vaccination-challenge studies indicated that a single dose of either classic or variant live, attenuated vaccine was sufficient to protect maternal antibody free chicks challenged at 3 weeks of age.

The fact that isolate 5038 appeared to be antigenically different from STC, Del E, RS593, GLS, and AL-2 by MAb testing and antigenically different from Del E by the
progeny challenge warrants further investigation. In the past, genetic mutation in the variable region of VP2 of the IBDV genome has enabled the virus to evade immunity, as well as, exhibit variations in antigenicity, virulence, cell tropism, and pathogenic phenotype (16, 17, 58, 263, 320). While SPF leghorn chickens serve as an acceptable model for in vivo IBDV challenge studies, variation in susceptibility to IBDV infection has been observed among different chicken breeds (321, 322). Thus, experimentation using broiler chickens would provide a more accurate overview about the situation in the field. The next step will be to examine the ability of 5038 to break through maternal immunity to commercial IBDV classic and variant vaccines in broiler chickens. If this virus once again proves to be antigenically different from the vaccines used in in vivo testing, then the issue of vaccine strain selection may be raised. On the other hand, if little to no vaccine breakthrough is observed, then the MAbs used in the antigen capture ELISA tests may need to be re-evaluated for their usefulness against IBDV strains currently circulating in the field.

A hypothesis to explain the apparent vaccine breakthrough of field isolates 4813, 4947, 4955, and 5041, is that these viruses could be the result of factors such as poor vaccine delivery or the natural waning of maternal antibodies rather than antigenic drift (59, 283). The Delmarva broilers from which these IBDV field isolates originated only received passive breeder vaccinations, which normally afford protection for up to 2-3 weeks of age. Therefore, it is not surprising that the broilers were susceptible to infection at 3 and 4 weeks of age. Active immunization of broiler progeny would extend the life of IBDV protection (323); however, high maternal antibodies can sometimes
neutralize the vaccine virus and delay or prevent stimulation of a protective immune response (51, 324). Variation in the natural waning of maternal antibodies makes it difficult to determine the optimal timing for broiler vaccinations (46, 47, 283, 325, 326, 327). Consequently, active immunization is not always a practical solution to vaccination failures.

As shown by this study, the vaccine response of optimally vaccinated birds is not always a true picture of the situation in the field. Highly industrialized poultry-producing systems such as those located on the Delmarva Peninsula involve high density poultry houses and hatcheries with litter that may be removed perhaps every three years. Such conditions can have a significant effect on stress during rearing, nutrition, and the prevalence and control of major poultry diseases. Moreover, clean-out and disinfection is not always handled properly and, at times, may not be effective at eliminating highly resistant viruses such as IBDV (46, 59, 321, 325). In addition, inconsistencies of vaccine dosage, the indirect targeting of tissues that generate immunity, unequal distribution, and variability in water quality are common problems that accompany vaccination methods used in large scale poultry operations (325).

Although current vaccination programs and strict sanitary procedures help to minimize the occurrence of IBD outbreaks, field cases continue to emerge among broiler flocks (273, 318, 328, 329). The immunosuppressive nature of IBDV makes this virus a major economic concern to the poultry industry as it not only contributes to vaccination failures (26, 318), poor broiler performance, and increased condemnations at processing, but it frequently results in an increase of secondary infections (46, 115, 153, 330, 331). It
is not clear whether IBDV was the underlying cause for the recurring poor performance of the Delmarva poultry flocks involved in this study. However, IBDV-associated immunosuppression may have played a role in the prevalence of gangrenous dermatitis, inclusion body hepatitis, runting and stunting syndrome, and respiratory complex on these problem farms. We observed by molecular characterization and in vivo studies that field isolate 5038 may differ antigenically from vaccine strains currently used in the field. For this reason, continuous surveillance is necessary to identify, isolate, and characterize what may be new IBDV strains circulating within the poultry industry.
CONCLUSION

Fifteen infectious bursal disease virus field isolates were recovered from 3-4 week old commercial broiler chickens raised on the Delmarva Peninsula in 2007. The viruses were identified by histopathology, virus isolation, and RT-PCR. VP2 phylogenetic analysis identified all of the isolates as serotype 1 Delaware variant viruses.

Five of the field isolates distributed into six clades and were selected for further study. The five isolates, 4813, 4947, 4955, 5038, and 5041 produced gross and microscopic pathology of the bursa of Fabricius consistent with Delaware variant infection. Active immunization of specific-pathogen-free leghorn chickens with highly attenuated Delaware variant 89/03 and classic PBG98 live vaccines produced solid immunity upon challenge with each of the field isolates.

Based on the antigenic similarity of field isolates 4813, 4947, 4955, and 5041 to well-characterized strains of IBDV, it is highly probable that the broilers from which these isolates originated became infected due to waned maternal antibodies. Therefore, in order to improve the performance of these problem farms in the Delmarva Peninsula, broiler vaccination programs for IBDV may need to be implemented.
Isolate 5038 was found to be antigenically different from STC, Del E, GLS, RS593, and AL2 based on its inability to react with monoclonal antibodies. *In vivo* testing showed that 5038 was antigenically different from Del E based on its ability to break through maternally-derived immunity earlier than Del E challenge in 10-day-old SPF leghorn chickens.

On the other hand, in both active vaccination-challenge studies, vaccinated birds exhibited solid immunity against 5038, and genomic characterization and comparison of the variable region of VP2 did not indicate specific mutations that may be responsible for the lack of monoclonal antibody binding. It is possible that the MAbs used in the antigen capture ELISA tests are not entirely effective for IBDV strains currently circulating in the field. For a more comprehensive analysis, new MAbs may need to be produced.

VP2 sequencing and phylogenetic analysis may be useful molecular tools for identifying differences among strains, but they do not always predict how the viruses will behave biologically.
APPENDIX A

Epitope profiles of infectious bursal disease viruses recognized by monoclonal antibodies used in antigen capture enzyme-linked immunosorbent assays.

Ruud Hein (Intervet/Schering Plough Animal Health)
APPENDIX B

Standard Amino Acid Abbreviations and Side Chain Properties Table

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>3-Letter</th>
<th>1-Letter</th>
<th>Side chain polarity</th>
<th>Side chain charge (pH 7)</th>
<th>Hydropathy index</th>
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<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
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<td>1.8</td>
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<td>Arg</td>
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<td>Asp</td>
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<td>negative</td>
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</table>

http://en.wikipedia.org/wiki/Amino_acid
REFERENCES


