

DEVELOPMENT OF ADVANCED MOLECULAR AND BIOLOGICAL METHODS FOR
ANTIGENIC CHARACTERIZATION OF EMERGING VARIANT
INFECTIOUS BURSAL DISEASE VIRUSES

by

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ABSTRACT

Infectious bursal disease (IBD) is an immunosuppressive disease in chickens which causes economic losses in the poultry industry worldwide. IBD is caused by infectious bursal disease virus (IBDV), a member of the family *Birnaviridae*. IBDV is a non-enveloped, double-stranded RNA virus targeting proliferating B-lymphocytes causing humoral immunosuppression. Vaccination programs and presence of field viruses probably lead to emergence of antigenically or pathogenically different IBDV due to changes in the viral genome caused by a intrinsic missing proof-reading of the viral replicase. The determination of the antigenicity of IBDV field isolates plays a critical role and is necessary for successful vaccination. To this end, the reverse genetics system (RGS) of IBDV was modified and utilized as a diagnostic tool. In this method, RNA was isolated from bursal samples and amplified with specific primers encompassing the VP2 region responsible for the antigenicity of IBDV. Amplified cDNA was cloned into the segment A of the RGS and nucleotide and amino acid sequences were analyzed. The *in vitro* transcribed cRNA was transfected into cells, followed by determination of the reactivity of the expressed viral protein with a panel of monoclonal antibodies (MAb) to determine the

antigenicity. Using this approach IBDV strains circulating in U.S poultry flocks were identified and resembled a new antigenic subtype of IBDV. Based on this finding, the approach was refined to analyze the antigenicity of IBDV on a global scale. To this end, the RGS was combined with a method where nucleic acids can be transported across borders without importing infectious virus. Using this approach, viruses of the new antigenic subtypes were identified in poultry flocks on different continents. Finally, the relevance of the new variant strains of IBDV were investigated by developing an *in vivo* experimental model. Using this model for one virus of the new antigenic subtype it was shown that it was indeed different from known IBDV. Taken together, a system was established which enables the identification and antigenic characterization of different IBDVs without transporting infectious virus. This complex system will allow the antigenic analysis of IBDV on a global scale.

INDEX WORDS: Infectious bursal disease (IBD), infectious bursal disease virus (IBDV), bursa of Fabricius, bursal lesion score, antigenicity, reverse genetics, FTA cards, *in vivo* experiment, virus neutralization assay

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DEDICATION

I would like to dedicate this work to my parents who encouraged me and provided their
incredible support throughout my career.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction:

Infectious bursal disease (IBD) is an acute contagious disease affecting young chickens. IBD is caused by infectious bursal disease virus (IBDV) which belongs to the family *Birnaviridae* and genus *Avibirnaviridae* (46). IBDV targets proliferating B-lymphocytes (98, 118, 136) but not peripheral B-lymphocytes (118) and thus mainly affects the humoral immune system of chickens. IBDV induced immunosuppression (2, 64, 76, 225, 229) increases susceptibility to respiratory diseases like infectious bronchitis (210, 226), Newcastle disease (226), and infectious laryngotracheitis (226). IBDV infection can also increase the risk of chickens being infected by other non-respiratory viral diseases like chicken anemia (301), Marek's disease (37, 75), inclusion body hepatitis (59), and diseases caused by reoviruses (181). Apart from viral diseases, IBDV infection also increases the incidence of bacterial diseases caused by *Salmonella typhimurium* and *E. coli* (292), *Staphylococcus aureus* (233) as well as parasitic diseases caused by *Eimeria spp.* (4, 206). Another important aspect of IBDV infection is that affected chickens can exhibit a poor immune response to poultry vaccines (99). A common strategy to control IBDV is repeated immunization of breeder hens with a combination of live and inactivated vaccines (288, 293). These breeder hens usually develop high levels of IBDV specific antibodies which are transferred to the offspring via the egg yolk (251, 288, 293). There are two main reasons for evolution of different IBDV. The first reason is that IBDV is an RNA virus. Genetic diversification is more common among RNA viruses because of higher mutation rates due to the lack of proof reading activity of the viral RNA dependent RNA

polymerase. These mutations can lead to the emergence of new viruses. The second reason is basically caused by the intrinsic property of RNA viruses. Due to the presence of neutralizing antibodies at different levels in chicken flocks under production conditions a positive selection of virus mutants occurs. Viral mutants which are able to escape neutralizing antibodies and have a sufficient fitness might eventually become selected and become established in the field. This can lead to variations in antigenicity as well as pathogenicity of those viruses which evolve (15, 268). There are many reasons for the emergence of new IBDV strains, which may lead to the following: (1) variability in the virulence between different IBDV strains (5, 42, 97, 176, 219, 241); (2) susceptibility of the host related to age (155, 157); (3) level of virus challenge (115); (4) level of maternal antibody titer value (78, 166, 192, 282, 291); (5) genetic background of chickens (5); (6) improper vaccination techniques (29); (7) IBDV vaccine strains might be antigenically different from circulating field strains. Any one of these or combination of these factors may lead to vaccination failures. Phylogenetic analysis along with epidemiological surveys could aid in understanding the evolution of emerging new genotypes of IBDV (297) based on genetic relatedness. Thus, surveillance of circulating field strains in poultry flocks plays an essential role in choosing effective vaccines or selecting candidates for new vaccines (51). This research project is primarily aimed at the characterization of the antigenicity of IBDV in different poultry settings across the globe, with the focus on US poultry companies, using reverse genetics of IBDV as a new diagnostic tool.

Literature review:

Infectious bursal disease virus (IBDV) causes a highly contagious, immunosuppressive disease in young chickens called infectious bursal disease (IBD). IBD was first described by Cosgrove in 1962. Initially, IBD was first described as avian nephrosis (40) but later was described as infectious bursal disease due to observed changes in the bursa of Fabricius (95, 102). To date two serotypes of IBDV, serotype 1 and 2, have been described (131, 178). Serotype 1 IBDV causes infectious bursal disease in chickens whereas serotype 2 IBDV (131, 178) are apathogenic to chickens (116) and turkeys (132). IBD outbreaks were observed in chickens between 2-15 weeks of age (155), 16-20 weeks of age (155, 204) with most common incidences occurring 3-6 weeks age (40, 101, 157). In the field, clinical signs of IBDV do not occur before three weeks of age due to the persistence of maternal antibodies derived from vaccinated broiler breeders (104, 287). The half life of maternal derived antibodies is about 4 days (287). IBD causes economic losses in the poultry industry (137, 230) by affecting the efficiency of production in broilers and layers. This virus belongs to the family *Birnaviridae* and genus *Avibirnavirus* (46).

Genomic organization:

Viral particles of IBDV range from 55 to 65 nm in diameter (99, 195) and show an icosahedral symmetry. IBDV virus particles harbor an RNA genome with two segments namely segment A and segment B (41, 47, 137, 171, 186). Segment A is with 3261 basepairs (bp) larger in size compared to segment B which contains 2827 bp (188). Segment A encodes for the polyprotein of 1,012 amino acids with a theoretical molecular weight of 110 kDa (110, 254). This polyprotein is proteolytically cleaved into three viral proteins (VPs), the premature pVP2 (VPX) (48 kDa), VP3 (33kDa) and VP4 (29 kDa) (110), by the viral protease VP4 (18). pVP2 is

further cleaved at its C-terminus to release the mature VP2 (41 to 38 kDa) (20, 41, 72, 184), and four small peptides (43) . These peptides have been shown to associate with the viral capsid, but the function of these interactions is unknown (43). A second open reading frame, partially overlapping with the polyprotein gene (12, 254), encodes 145 amino acids for the nonstructural protein VP5 (188). Segment B encodes VP1 (97 kDa) for which the function as a viral RNA-dependent RNA polymerase (RdRp) was initially proposed (255) and later experimentally shown (274). One of the special features of this virus is that it can discriminate between cellular and viral RNA due to the presence of cis-acting packaging signals in its viral RNA segments (171).

VP1:

VP1 plays a major role in encapsidation of the virus particle (182). VP1 encodes for the motifs I, II, III and IV (81) which are typical for nucleic acid template depended polymerases. These motifs are now termed as motifs A, B, C, and E, respectively (274). In general, regardless of whether a polymerase is RNA-or DNA dependent, the motifs A, B, and C are arranged in the order A-B-C. However, a computer-assisted comparative sequence analysis proposed that a small group of viral RNA-dependent RNA polymerases (RdRp) contain a permuted C-A-B arrangement of theses motifs (82). Moreover, although the motifs A, B and C are highly conserved between nucleic acid template depended polymerases (89, 205, 215), the RdRp of IBDV showed a unique structural diversification of the palm subdomain which was compatible with other RdRP due to structural rearrangements which allowed the formation of the enzymatic active domain (82). Later, von Einem et al., 2004 proved with a recombinant IBDV VP1 that this protein was indeed the viral RdRP. Furthermore, von Einem et al., 2004 also suggested that the *in vitro* polymerase activity of VP1 follows a ‘copy-back’ mechanism which leads to the formation of an RNA hairpin. Later, it was shown using mutated recombinant VP1 that the

permuted motif was functional and also responsible for the enzymatic function of VP1 (153). Other experiments showed that VP1 might contain determinants for cell-specific replication of IBDV in Vero cells (24) and also contain capping enzyme activity (185, 274). In the viral particles, VP1 exists either in a free form or covalently linked to the ends of both viral RNA segments (138). Self guanylation was observed for VP1 so that it serves as a primer during RNA synthesis (48). Self guanylation activity of VP1 has been proven by *in vitro* (48) and *in vivo* methods (174). It has been described that among dsRNA viruses, only birnavirus RdRp synthesized RNA by protein priming activity (209). Certain amino acids play a critical role in determining the self guanylation properties. Xu et al., 2004 demonstrated the absence of this guanylation property of VP1 by mutating amino acid 163 from serine to alanine. Different regions of RdRP play an essential role in the pathogenicity and virulence of IBDV without affecting the viral replication (197).

VP2:

VP2 constitutes 51% of the viral particle and is the only structural, capsid protein of IBDV (41, 47) and considered as major immunogen of IBDV (60). The mature VP2 protein contains 441 amino acids and has some unique components of the capsid (see below) (41, 235). A single VP2 molecule is folded into three distinct domains: base (B), shell (S), and projection (P) (Fig. 1.1, 41). These three domains of VP2 form the building blocks for the trimer structure of the protein (41). Azad et al., (1987) performed deletion mapping and expression studies of VP2 and described that the domain encompassing amino acids 206-350 of the polyprotein represents the region to which neutralizing antibodies bind. This study proposed the presence of two hydrophilic regions from amino acid residues 212 to 224 and 314 to 324 in VP2. This finding was further supported by investigation published by Bayliss et al. (1990) in which they

described the existence of a variable region within VP2 which was flanked by these two hydrophilic peaks. These two hydrophilic peaks are now described as major hydrophilic peaks A and B. Others have described that certain amino acid changes in the variable domain affected the viral antigenicity (125, 154, 236, 267). Exchange of one (236) or more amino acids (94) in the hydrophilic peak A and/or B may lead to antigenic drift resulting in new variant strains (94, 236). Schnitzler et al (1993) showed that replacement of four amino acids in the hydrophilic peak A resulted in loss of hydrophilicity as well as amino acid mutations in hydrophilic peak B could generate new serotypes. Later, van den Berg et al. (1996) described two smaller hydrophilic peaks located from residues 248 to 252 and 279 to 290. These hydrophilic peaks are now referred to as the minor hydrophilic peaks. Amino acids 253 and 284 located in these minor hydrophilic peaks play a predominant role for cell culture infectivity (193) and pathogenicity in chickens (271). So far, all epitopes which have been identified to be responsible for inducing neutralizing antibodies in chickens are conformational dependent (9, 13, 41, 62, 235, 267). Letzel et al. (2007b) described more details regarding the molecular background of the antigenicity of VP2. The binding of monoclonal antibodies MAbs characterizing neutralizing epitopes of VP2 (247, 248, 250, 267) was controlled by few amino acid substitutions in VP2. Reactivity with certain MAbs depended on the presence of certain amino acids in the epitope and was thus very fragile (154). It has been shown that the presence of certain amino acids in the VP2 of IBDV was necessary for the binding of certain MAbs (154): serine/threonine at position 222 and alanine at 321 was essential for the binding of MAb 67; glutamic acid at position 321 was important for the binding of MAb 57; alanine at position 321 was not absolutely needed for the binding of MAb R63 to the epitope; glycine at position 318 and aspartic acid at position 323 was important for the binding of MAb 10. Taken together, the variable regions in VP2 play an

important role for the antigenicity of the virus, especially for the selection of neutralizing antibodies producing hybridomas. Thus, characterization of VP2 stays in the center of interest for diagnostics and vaccine development. Besides its function as capsid protein, it has been shown that that VP2 carries the determinants to infect cell culture (161, 193) and induces apoptosis of infected cells (67).

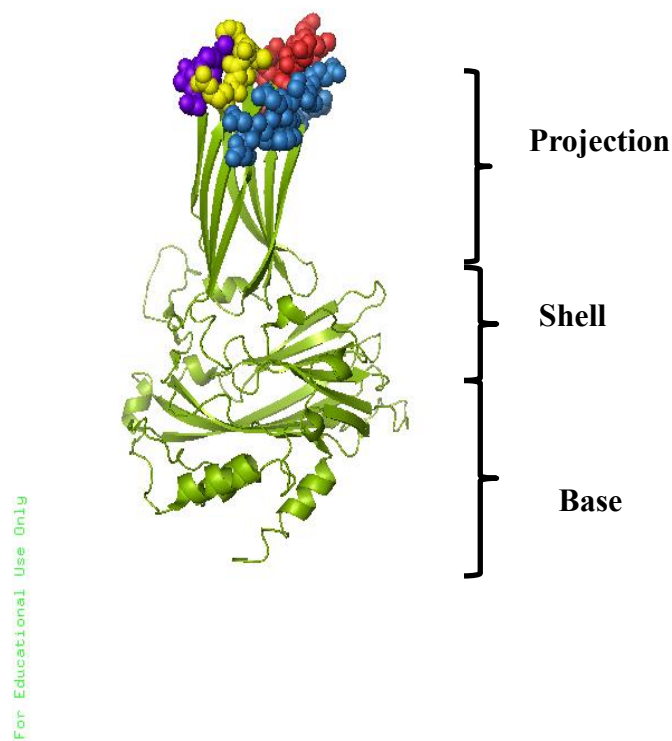


Fig. 1.1: Schematic representation of the VP2 monomer. VP2 has three domains which have been described as projection, shell and base domain. Amino acids as shown in spheres form the major hydrophilic peaks A (red) and B (blue). The amino acids representation the minor hydrophilic peaks are represented in yellow and purple spheres. Figure adapted to Coulibaly et al. (2005) based on the data available in the protein

database (Code 1WCD) using Pymol program (Open-Source PyMOL 1.2r1) available online at <http://www.pymol.org>

VP3:

VP3 constitutes a major structural component of the virus particle representing 40% of the protein content of purified virus particles (47). VP3 builds a dimer structure (Casanas et al., 2008) and is located inside of the viral capsid. Kochan et al. (2003) characterized RNA-binding

activity of VP3. It has been described that VP3 forms group-specific epitopes (61, 110). Initially it was assumed that VP3 was a protein able to induce neutralizing antibodies (8, 61). Later, it was experimentally proven that VP3 was not involved in the induction of neutralizing antibodies. (13, 207, 213). It was proposed that VP3 acts as a scaffolding protein for pVP2 and plays a multifunctional role in the assembly of virus particles, replication, and packing of the viral genome (175, 257). VP3 provides a temporary structure during the assembly of capsid polypeptide (175). Experimental evidence suggested that VP3 acts as an activator of viral transcription (18, 32). Furthermore, it has been shown that VP3 interacts with VP1 forming VP1-VP3 complexes (165, 259). The formation of these complexes may play an important role in IBDV replication (258). VP3 also binds to the genomic dsRNA of segments A and B (257). Besides its function in replication for these complexes it has been proposed that they play an important role in IBDV morphogenesis (15, 24, 72, 165, 259).

VP4:

VP4 is a non-structural polypeptide and its coding region represents the most conserved region in segment A (12). VP4 is responsible for the processing of the precursor polyprotein to generate pVP2, VP3 and VP4 (18). Only after this, during virion maturation pVP2 is cleaved into the mature viral capsid forming VP2 and four small peptides (43). VP4 can be detected in virions (137, 173). VP4 has a unique active center for its protease activity which is similar to the bacterial Lon proteases (18). VP4 is a viral protease with a serine-lysine dyade in its active center (18) which is responsible for self processing (151, 232). Feldman et al. (2006) published the crystal structure of a birnaviral protease from the blotched snakehead virus, which is another member of the family *Birnaviridae*.

VP5:

VP5 is encoded by a second open reading frame on segment A of IBDV (254) preceding and partially overlapping the polyprotein gene. VP5 is a nonstructural viral protein (188) and was not detected in virions (190) but could be detected in IBDV infected bursal tissues (188). VP5 is basic in nature with cysteine rich amino acids (256, 300). VP5 is not essential for *in vitro* (190) or *in vivo* viral replication (300). VP5 of serotype 1 IBDV strains are highly conserved with above 95% identity between different IBDV serotype 1 strains. In contrast, VP5 sequences showed only 73% sequence identity among serotype 2 strains (300). It has been described that VP5 plays an important role during pathogenesis of IBDV (300) and is involved in virulence (299). Additional evidence allowed the assumption that VP5 is involved in viral egress from infected cells (164, 286). VP5 interacts with the voltage-dependent anion channel 2 (VDAC2) in the mitochondria of IBDV infected host cells (160) and it was also shown that VP5 induces apoptosis (299, 300). Lombardo et al. 2000 provided evidence that the expression of VP5 causes changes in the morphological characteristics of the cell, rupture of the plasma membrane, and a significant reduction in the livability of the cells. Tacken et al., (2003) identified an interaction domain in the central region of VP5 which may assemble along with the host cell plasma membrane resulting in forming a pore structure.

Transmission and epidemiology:

IBDV infects chickens by the oral route (14, 136, 183), ocular route (14, 95, 284) or intranasal route (14). Apart from chickens (40, 178) and turkeys (10, 131, 178, 179), IBD infections have been reported in ducks (178), quail (65), quail- chicken hybrid (85), ostriches (83), penguins (71, 128) and other wild birds (201).

Pathogenesis and pathophysiology:

The severity of the disease caused by IBDV depends on the type of virus (42, 97, 176, 219, 241), age of chickens (155, 157), immune status of chickens (1), type of chickens (such as meat type or layer type) (282), the genetic background of the chicken (27, 58, 91) and the level of maternally derived antibodies in young chickens (1, 177, 192, 282). In general, it has been suggested that white leghorn chickens are more susceptible to IBDV infection (282). When chickens are infected with IBDV by the oral route, virus enters the oral cavity and passes through the intestine. During intestinal passage, viral antigen can be detected in the intestinal lymphoid cells and macrophages (136, 186). In case of very virulent IBDV (vvIBDV), the virus severely affects the villus height which causes decreased intraepithelial lymphocyte and mast cell populations (276). It also causes decreased sIgA- producing cells and intestinal alkaline phosphatase accompanied with increased goblet cells in jejunum and ileum leading to impairment of intestinal mucosal immunity (276). Chickens infected with IBDV showed lower levels of IgA when compared to uninfected chickens (76). Virus antigen was detected in the liver and enters the bloodstream to reach various organs (136, 186). Ley et al., 1979b found gamma globulins present in the glomeruli of IBDV infected chickens. This finding might explain the reason for describing this disease as “avian nephrosis” due the presense of immune complexes in the kidney (Ley 1979b). Although the virus is present in several organs, the virus only replicates to detectable levels in the bursa of Fabricius (BF). Approximately eleven hours after infection virus was detected in the BF (136). BF is the primary organ for the development of mature B lymphocytes and thus is involved in synthesis of immunoglobulins (80, 261). IBDV targets the medullary region of bursal follicles (28, 187, 196). The virus infects immature precursors of antibody-producing B lymphocytes located in the BF (24, 98, 109, 187) and particularly targets

IgM bearing B lymphocytes (98, 118, 187, 194). It was proposed that the host range of IBDV is controlled by the presence of viral receptors composed of N-glycosylated proteins which are mostly present on sIgM cells (202). Further studies revealed that λ chain of sIgM interacted with IBDV independent of the virulence of the virus (170). The number of susceptible cells in BF plays an important role in the development and severity of the disease (114). Following IBDV infection in the BF, upregulation of perforin, granzyme-A, high mobility protein groups, poly (ADP-ribose) polymerase (PARP), DNA repair and apoptotic proteins was observed whereas the Nk lysine was downregulated (217). The replication of IBDV of either serotypes were neither restricted by non coding regions of segment A (237, 238) nor VP5 and the N-terminus of VP2 (238).

IBDV serotype 1 infection of chicken results in the depletion of premature B lymphocytes in BF and, to a lesser degree, a lymphoid depletion in caecal tonsils and spleen (219). It has been described that IBDV causes lytic infection of B cells, thymic cells, and peripheral blood lymphocytes (146) and is also able to induce apoptosis in cells located in the BF (203), spleen (147) and chicken lymphocytes (272). In the spleen, IBDV damaged the extracellular matrix resulting in tissue impairment and leading to permanent immunosuppression (19). As a consecutive effect of IBDV infection, lytic infection of B lymphocytes by IBDV leads to immunosuppression in chickens (28, 187, 242, 272). IBDV may also stimulate the suppressor cells which can further worsen the immune status of infected chickens (242). Apart from causing general immunosuppression, IBDV also affects the local immune system such as the harderian gland (49, 50, 210) by lowering the number of plasma cells (49, 50). Although T cells are resistant to acute phase of IBDV infection, minimal changes were described in thymus dependent

tissues (77) with thymic atrophy due to a higher number of death cell caused by apoptosis of thymocytes (241).

Infectious bursal disease virus subtypes:

Initially, there was only one known IBDV subtype which was first described by Cosgrove (1962). Later, IBDV of a second serotype was described. Serotype 2 strains were isolated from turkeys (131, 178, 179), fowl and duck species (178) but were apathogenic to chickens (116). Both serotypes were differentiated by cross neutralization assays (131, 178) and, later by ELISA using a combination of monoclonal antibodies (207). There was no cross protection between two serotypes of IBDV observed (35) but there was cross protection observed between the viruses that share common neutralizing epitopes within a serotype (6). Besides their different antigenic makeup, both serotypes can also be distinguished by their potential to infect chickens and cause disease. In general, and as already mentioned, serotype 1 IBDV strains are pathogenic due to lytic infection of premature B lymphocytes and thus causing humoral immunosuppression in chickens (109, 282). In contrast, serotype 2 IBDV are non pathogenic in chickens (116), turkeys (132), ducks and quail (131). Several distinct pathotypes have been described within serotype 1 IBDV. These pathotypes vary from mild to very virulent. When different pathotypes infected the same host viral interference was observed which competed for host receptor sites (6). The classical pathotype of serotype I IBDV can cause severe clinical signs as well as mortality (40, 104, 168). It has also been described that mortality up to 100% was observed in naïve chickens using the classical IBDV strain Cu1 (136). Acute mortality in chickens may be associated with the formation of immune complexes (118) and a severe decrease in the levels of complement (245, 246). Later, serotype 1 IBDV had been described in the United States which was able to induce immunosuppression but did induce neither morbidity nor mortality (228, 231). These

IBDV were referred to as variant strains. Conventional vaccination with classical serotype 1 IBDV did not induce sufficient protection during challenge studies with these variant isolates (241) which indicated that these viruses were of a different antigenic type. Concurrently, serotype 1 IBDV were described which were able to cause high mortality even in the presence of IBDV neutralizing antibodies. These viruses were consequently designated as very virulent IBDV (vvIBDV) (16, 17, 23, 36). All serotype 1 strains harbour one common property. These strains induce immunosuppression and opportunistic pathogens are able to evade the inefficient humoral immune response system which can lead to multisystemic diseases (37, 59, 181, 210, 226, 301).

Classical IBDV:

Serotype 1 IBDV belonging to the classical subtype can cause clinical signs in infected chickens, although with different levels of virulence. During acute infection of susceptible chickens whitish or watery diarrhea, soiled vent, ruffled feathers, trembling, anorexia, depression, severe prostration and death was observed (40). Clinical signs appear within 2-3 days after exposure (168). Winterfield and Hitchner (1964) described vent pecking as one of the initial symptoms after IBDV infection. Classical strains cause edematous and hypertrophied BF (95) which is accompanied by heterophil infiltration (241). Bursal hypertrophy as a result of inflammatory processes due to elevated IL-6 and iNOS expression (218) was observed from 24 hours after infection and the BF reaches its largest size at 48 hours after infection (38). This was associated with B cell depletion due to lytic infection of B lymphocytes (168). Some examples of classical IBDV strains are STC (139, 226), Faragher 52/70 (30, 36), PBG 98 (11), Cu1-wt (191, 195), Faragher 52/70 (2, 64), Edgar (38), Lukert (167), 2512 (169), Irwin Moulthrop (IM) (24), BVM (169), 002-73 (110), and NC (199).

Very virulent IBDV:

Very virulent IBDV causes an instant inflammation in follicles of the BF which is characterized by heterophil infiltration accompanied by depletion of B lymphocytes. The hallmark lesions are strong hemorrhages in the BF, the leg and breast muscles. Chickens infected with vvIBDV were found to have elevated levels of mast cells, eosinophilic major basic protein, tryptase activity and eotaxin expression which might explain the reason for severe lesions (275). Also, vvIBDV resulted in upregulation of Th1 and Th2 cytokines (162). Also, the severity of lesions in the lymphoid organs is more intensive in case of vvIBDV infection (260). Very virulent IBDV (vvIBDV) strains are able to cause high mortality in affected birds (23, 36) in the presence of maternal IBDV-specific antibodies following vaccination with classical strains (16). But Jackwood (2011b), reported that although vvIBDV infects maternally immune chickens, the clinical signs and macroscopic lesions were less severe in these chickens. UK661 (25, 271), OKYM (298), HLJ-5 (NCBI accession number -EU042141.1), HLJ-7 (EU042143.1), DV 86 (36), Ehime/91 (264), 94432 (56), D6948 (21), ZJ2000 (301), SH/92 (140), Harbin-1 (294) YS07 (220), HLJ-3, HLJ-4, HLJ-6, HLJ-7, HLJ-8, HLJ-9, HuB-1 (302) are some examples for vvIBDV strains. Sequence analysis revealed that vvIBDV encode for unique amino acid residues in segment A and B. VP2 of vvIBDV contains certain amino acids at certain positions of the polyprotein gene encoded on segment A (25): 222 (A), 256 (I), 294 (I) and 299 (S) (25). It was assumed that VP2 residues in vvIBDV might determine the virulence, cell tropism and pathogenicity of viruses (24, 298). The proof of that hypothesis has been provided by van Loon et al., (2002) where the exchange of two amino acid in the VP2 region (aa 253 and 284) resulted in a complete attenuation of the virus in susceptible chickens. Several amino acids were identified which were only present in the VP1 encoding region on segment B of IBDV showing

the vvIBDV phenotype (163): 146 (D), 147 (N), 242 (E), 390 (M), 393 (D), 511 (S), 562 (P), 687 (P), 695 (R). Etteradossi et al. (1998) described that critical amino acid changes in the hydrophilic peaks A and B of VP2 were related to the antigenicity of vvIBDV. Studies by Boot et al. (2005) revealed that the virulence of vvIBDV is partly determined by segment B. Studies by Yamaguchi et al. (1997) and Hon et al. (2006) suggest that vvIBDV might have evolved due to genetic reassortment and not due to genetic recombination. Jackwood et al., 2011b. reported reassortment of serotype 1 vvIBDV and serotype 2 IBDV. Sometimes, in commercial chicken flocks vvIBDV may be unnoticed because of less severe clinical signs due to the presence of maternal antibodies and competition with variant viruses circulating in the flock (130).

Variant IBDV:

It has been described that variant strains of IBDV were able to affect the broiler chickens (228, 231) as well as chickens of the layer type (117). Emergence of variant strains of IBDV resulted in substantial economic losses in the poultry industry in the US (250). These strains caused subclinical infection which was characterized by absence of clinical signs (224). Variant strains were able to cause rapid bursal atrophy (117, 227) within 72 hours post infection (227) which was associated with the depletion of B lymphocytes (241). Sharma et al., (1989a) showed that vaccination using serotype I vaccines did not induce sufficient immunity against variant strains. Some examples for IBDV variant strains are E/Del (228), GLS (250), AL2 (262), DS 326 (250), IN (variant A) (227) and MD (231).

Diagnosis of IBDV:

Virus isolation:

Initially IBD virus isolation was performed in 9-11 day old embryonated chicken eggs by the chorioallantoic route of inoculation (284), but later it was recognized that inoculation into

the chorioallantoic membrane (CAM) was the most sensitive route for virus isolation (103). Most IBDV field isolates do not have the ability to infect cell culture due to the presence of the particular amino acids at positions 253(Gln) and 284 (Ala) as described before (193), making cell cultures the least sensitive diagnostic system. Although, it was observed that for certain IBDV isolates the presence of threonine in position 284 was necessary and sufficient to grow in tissue culture (154), most of the field isolates do not encode for this amino acid. The isolation of IBDV requires several passages in either cell culture (92), chorioallantoic membranes (103), or in the yolk sac of embryonated eggs (298) to adapt an IBDV to cell culture. Cell culture system and embryonated eggs are more sensitive titrating IBDV (92). But, this method is not reliable, labor intensive, and seldom yields virus isolation.

Detection of IBDV based on serology and viral antigen:

The detection of IBDV specific antibodies is a valuable tool for the evaluation of vaccination programs. The presence of IBDV antibodies are expected in field serum samples due to the omnipresence of IBDV in the field. IBDV antibodies can be detected by agar-gel precipitation test (AGPT) (100, 290), quantitative gel diffusion precipitation test (QGPT) (287), virus neutralization assay (280, 284), and indirect ELISA (106). Each system has its own merits and demerits. AGPT is not IBDV subtype specific, but easy to perform. ELISA results vary from lab to lab and from assay to assay (144, 145). Virus neutralization assay can be used as a specific tool to detect antibodies against a particular IBDV strain that is used as antigen for vaccination. The virus neutralization assay led to the discovery of serotype 2 by McFerran et al., (1980) in Europe and Jackwood et al., (1982) in the United States. This assay fails to provide the immune status of the flock vaccinated with other IBDV subtypes (119). In addition, the assay is laborious, expensive, and time consuming. The isolates described before 1984 were considered

as classical or standard viruses as they produced clinical signs. New isolates of IBDV were discovered in the United States in the early 1980's. These isolates were categorized as variant strains (228, 231) as their antigenicity and pathogenicity varied from previously described classical viruses (224). In the mid 1980s, kinetic-based ELISA (kELISA) was used to measure the presence of IBDV-specific maternal antibodies in the offspring (252, 253). The assay can be used for the determination of the optimal time point for vaccination of the offspring (253). As a field diagnostic tool the one-strip test was used to identify IBDV antigen in the bursal tissues (305). Protein chips were used to detect the antibodies against IBDV (277). Immunochromatographic gold based test strips were applied for rapid detection of antibodies against IBDV in 2 minutes (198). Also an one-step reverse-transcription loop-mediated isothermal amplification was described to detect IBDV (150, 279). Later, optical surface plasmon resonance (SPR) biosensor was developed to detect IBDV antigen. In this method, the antigen was detected in less than 30 min and the method was label-free (111).

Antigenic characterization of IBDV:

Several attempts were made to determine the antigenicity of IBDV in the middle of the 1980's in an effort to differentiate the field isolates based on their antigenic makeup. With the establishment of panels of neutralizing IBDV monoclonal antibodies (MAb), attempts were made to determine the antigenicity of IBDV (57, 63, 247, 248, 249, 250, 267). The neutralizing monoclonal antibodies used for determining the differentiating antigenicity of IBDV were 10, 57, R63 (248), 67 (267), and B69 (248, 250). In an antigen capture ELISA these MAb's were used to characterize the antigenicity of IBDV (270). E/Del variant strain reacts with MAbs R63 (154, 248, 267) and 67 (154, 267). Variant strains GLS and DS326 reacts with MAbs 10 and 57

(154, 267). Classical strains like D78, STC, PBG98, 52/70 reacts with MAbs 10, 63 and 69 (267).

Genetic characterization of IBDV:

In the middle of the 1990's, reverse transcription-polymerase chain reaction (RT-PCR) followed by restriction fragment length polymorphism (RFLP) was used in detecting and genotyping IBDV (123, 304). This method employs the amplification of a 394-bp fragment of VP2 by RT-PCR followed by restriction enzyme (RE) digestion using specific restriction enzymes (120, 121, 122, 135). This method was used to determine the genotype based on the presence of these RE sites (122, 123, 124) resulting in a certain cleavage pattern of the RT-PCR fragment. RT-PCR coupled with RFLP was used to predict similarities and differences between IBDV strains but it lacked the ability to determine the antigenic relatedness between IBDV strains (127). This method mainly relies on the presence of the restriction enzyme site. Zierenberg et al., 2001 applied RT-PCR along with restriction enzyme analysis to differentiate classical IBDV from very virulent IBDV. Ashraf et al. (2007) developed differential RT-PCR assays to differentiate vvIBDV from classical IBDV strains as well as to differentiate serotype 1 and 2 viruses. The other technique used to diagnose and differentiate IBDV strains is real-time RT-PCR (39, 55, 126, 127, 180). SYBR green I was used to quantitate the yield of real-time RT-PCR products (285). Hairul Aini et al, 2008 reported that SYBR green I based real-time RT-PCR is at least ten times more sensitive than conventional PCR methods to detect IBDV. Kong et al., 2009 applied this technique for detecting and differentiating between classical and very virulent strains. Multiplex RT-PCR amplification of partial fragments in segment A and segment B followed by restriction enzyme analysis allowed to differentiate between low and high pathogenic strains of IBDV (96). Another approach involving real time RT-PCR and high

resolution melt curve analysis was used to detect and differentiate IBDV strains (74). RT-PCR amplification of segment B followed by restriction enzyme analysis was described to differentiate classical and vvIBDV strains (113).

Genetic and antigenic characterization:

Antigenic differences related to protective immune response were found to be present in the VP2 protein of IBDV (8). Bayliss et al (1990) determined that a certain portion of the VP2 protein was different than other regions on the IBDV genome and coined the term variable region. Also, Lana et al (1992) reported that minor changes in the VP2 region resulted in emergence of variant IBDV. The next step was the combination of the antigenic and genetic characterization of IBDV. This was performed by combining the reverse genetics system (189) and neutralizing IBDV VP2-specific MAb's (247, 248, 250, 267) into one system (112). Using this method, the VP2 encoding region of IBDV (nt) 121-1181, including the variable region of VP2, was amplified by RT-PCR. The obtained RT-PCR fragment was cleaved with the restriction enzymes Rsr II and Spe I. The cleaved fragment was ligated into an appropriately cleaved cDNA copy of segment A of the vaccine strain D78 which had been previously ligated into a plasmid backbone (pD78APD, 112). By transferring the region coding for the neutralizing epitopes (154) the antigenic region was transferred into a carrier backbone without being limited to cell culture infectivity. Using a panel of monoclonal antibodies, the antigenicity of the IBDV field isolate was determined. This method relies on the monoclonal antibody panel pattern reaction in correlation with nucleotide and amino acid sequence. It was described that this might be an efficient method to characterize the antigenicity of IBDV (52, 112).

Control of IBDV:

Intense vaccination programs accompanied with strict biosecurity measures are considered to be effective to control IBD in the field (168). IBDV remains infectious in contaminated poultry houses for an extended period of time. Benton et al. (1967) described that IBDV was infectious in contaminated poultry house for a period of 122 days even after the removal of IBDV infected birds (14). IBDV is highly resistant to commonly used disinfectants such as phenol and merthiolate (14). When exposed to metam-sodium at the same concentration levels influenza A virus was inactivated within one hour but IBDV was still infectious for 48 hours after treatment (73). Even after proper cleaning and disinfection, IBDV can persist in poultry flocks due to its hardy nature (168). Timing of vaccination (143, 149), choice of vaccine (79, 143, 149), and the body weight of chickens (273) play an important role in determining successful rates of IBDV vaccination. In contrast, Mundt et al., (2003) has shown that the time point of vaccination has no role in vaccine intake, whereas level of maternally derived antibody titer value plays a critical role in the level of vaccine take.

It is impossible to adopt a global vaccination program due to factors such as maternal antibody titer value in young chickens (78, 192), variability in virulence of IBDV strains (42, 97, 176, 219, 241), and different management structures (168). IBDV vaccines are broadly classified into mild, intermediate and intermediate plus, or hot vaccines based on their virulence (200) in susceptible, antibody free chicken. Although, mild strains were safe in specific-pathogenic-free chickens, the efficacy was poor in the presence of virulent and classical IBDV. The effectiveness of the same vaccines was poor when the birds were challenged with vvIBDV (265). Winterfield et al., (1978) proved that the use of mild strains of IBDV as a vaccine did not induce sufficient protection in chickens when a significant challenge infection was performed. Whereas by using

more virulent viruses as vaccine strains offered a better protection (282). This resulted into the use of intermediate and hot IBDV vaccines in the field. Intermediate and hot IBDV vaccine strains induced a better protection but caused moderate to severe bursal lesions (26, 282). Administration of intermediate vaccines by intramuscular route induced high titers against the vaccine strain along with overexpression of proinflammatory cytokines such as IL-6, IL-15 and gIFN γ (31). This property may cause immunosuppression. The severity of the immunosuppression varies from strain to strain (176). These circumstances require a qualified vaccination program, where the presence of maternal derived antibodies needs to be assessed (265, 282). The choice of the right vaccine depends on the type of circulating IBDV strains in that particular area. Also, propagation of IBDV in different host systems such as cell culture, chicken embryos and BF affected the antigenicity, immunogenicity and pathogenicity of these viruses (90, 221, 222, 223). Since, IBDV have various subtypes in virulence and antigenicity, it cannot be expected that a single IBDV vaccine offers protection against all IBDV subtypes. In such cases, two or more IBDV vaccines representing several subtypes need to be combined to induce sufficient protection (79). A broader protection can be achieved by generating a broad spectrum vaccine as it has been described by the application of the reverse genetics system (192).

Vaccination of breeder flocks with serotype 1 IBDV protects their offspring by transfer of maternal derived antibodies (MDA) (192, 293). This protection persists for the first few weeks after hatch depending on the MDA titer levels (3). Breeder flocks vaccinated at 12 weeks of age with vaccines based on infectious virus followed by the application of an inactivated IBDV conjugate vaccine at 20 weeks of age offers better protection than breeders vaccinated with the live virus only at 12 weeks of age (53). Vaccines based on inactivated virus along with an oil adjuvant can also be administered in breeder flocks during 16-18 weeks of age (168).

Combinations of vaccines containing infectious virus with vaccines based on inactivated virus enhanced the quality and quantity of MDA transferred to the young chickens (168, 289). Offspring obtained from breeder flock which were vaccinated with inactivated vaccine showed higher and uniform IBDV specific antibody titers when compared to the offspring derived from a breeder flock which was vaccinated with live IBDV vaccine only (53).

In ovo vaccination was described as a practice to control IBDV (134, 234, 240). By this method IBD vaccines based on infectious, attenuated virus were inoculated into 18-day-old embryonated chicken eggs. The vaccine inoculation did not interfere with either hatchability or livability of the hatched chickens. Chickens vaccinated by this method developed neutralizing antibodies which were present even at four weeks of age. These antibodies persisted in the chickens for at least ten weeks of age (239, 240). The automation of this method also reduces the labor involved in the vaccination procedure of large chicken flocks.

Immune complex vaccines were another approach used to generate an effective immune response in the offspring of either IBDV- vaccinated or nonvaccinated breeder flocks. Immune complex vaccines were generated by mixing infectious IBDV with IBDV-specific antibodies, usually derived from polyclonal serum obtained from chickens repeatedly vaccinated following a schedule as described above (86, 281). When this immune complex vaccine was subcutaneously administered, the antibodies present in the immune complex vaccine prevented the immediate release of the vaccine virus for the first seven days after vaccination. A slow release of the virus infected B lymphocytes in the BF and stimulated the onset of protective immunity (281). The efficacy of this vaccine has been studied in SPF birds (281) and broiler chickens (86). *In ovo* vaccination of an immune complex vaccine in 18-day-old embryonated chicken eggs also

induced protection to the offspring, although the precise mechanism is not well understood (133).

Another and more recent approach in the poultry industry is the use of vector vaccines. In general, recombinant vaccines are based on a vector system which encodes for the protective antigen. Within this system are two types of recombinant vaccines. One group is based on an antigen-producing system where the IBDV antigen is produced and used similar to inactivated vaccines. This includes recombinant vaccines developed in insect cells using a recombinant baculovirus (214, 266, 296) or recombinant yeast (172). In both cases, the VP2 protein was used as the IBDV-specific antigen. The other vaccine group comprises of recombinant vaccines where the protective antigen has been cloned into a replication competent viral vector system. The vector is used as an infectious self-replicating vaccine virus. This group includes recombinant vaccines encoding the VP2 of IBDV in herpesvirus of turkeys (HVT) (45, 152, 211, 263), fowl pox virus (93) and fowl adenovirus (68, 84, 212, 243). HVT-based virus vaccines and baculovirus-based vaccines have been licenced and are used in the poultry industry to induce protection in the offspring.

DNA vaccination as a method to induce a protective immune response in chickens has been described (33, 69, 107, 108). The initial experiment showed that recombinant DNA could be used for transient expression in body tissues of the recipient animals (44). In general, DNA vaccines encode for either the complete virus antigen (88), the protective antigen (158), or a peptide which forms a protective epitope (159). The coding sequence of the target antigen is usually under the control of a DNA-dependent RNA polymerase II promoter and will be transiently expressed after transferring into the cell. This induces an effective immune response even in the presence of maternal antibody and offers protection to young chickens (107).

Priming with DNA vaccine at day 1 of age, followed by boosting with an inactivated vaccine at 1 or 2 weeks of age induced 80-100% protection against an IBD challenge infection (108). DNA vaccines induce an effective response of the CD4⁺ and CD8⁺ population of T cell- mediated immunity (216), even in the presence of high levels of maternal antibody (54, 107, 244). DNA vaccines offer protection by delayed appearance as well as aiding in the rapid clearance of viruses in the affected tissues (34). The disadvantages of such vaccines in the poultry industry are the high costs associated with the vaccine itself and the costs of labor associated with the necessary repeated application of the vaccine.

Another approach was to develop tailor-made vaccines against IBDV using reverse genetics. Mundt et al. (2003) generated an IBDV chimera containing classical and variant IBDV sequences encoding for VP2 using reverse genetics. Administration of this rescued chimeric IBDV resulted in high neutralizing antibody titers against both classical and variant IBDV. Gao et al. (2011) developed a vaccine against vvIBDV using reverse genetics approach.

Recently, Wang. et al.(2012) used a multi-mimotope vaccine approach to induce immunity against IBDV by using E.coli expressed polypeptide (5EPIS) which had five mimotopes of VP2 protein. They constructed a chimeric human hepatitis B virus (HBc) VLP displaying this multi-mimotopes and immunized the chickens. The immunized chickens were protected, when challenged with virulent IBDV. Although the HBc-based VLP vaccine strategy offers protection, the drawback of this method is that the immunogenicity developed is weak due to the small size of 5EPIS (~9 kDa).

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CHAPTER-2

INVESTIGATION ON THE ANTIGENIC EVOLUTION OF FIELD ISOLATES USING THE REVERSE GENETICS SYSTEM OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) *

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Abstract:

The antigenic profile of over 300 infectious bursal disease virus (IBDV) isolates has been analyzed using a panel of monoclonal antibodies in a reverse genetics approach. In addition, the sequences of a large portion of the neutralizing antibodies inducing VP2 of IBDV have been determined. Phylogenetic analysis of nucleotide and amino acid sequences in combination with the antigenic profile obtained using the monoclonal antibody panel pattern, revealed a lack of correlation between antigenicity and isolate's location within the phylogenetic tree. In depth analysis of amino acid exchanges revealed that changes within a certain region of the VP2 molecule resulted in differences in the antigenicity of the virus. This comprehensive analysis of VP2 sequences indicated a high selective pressure in the field likely due to vaccinations programs which forces the evolution of the virus.

Index words: IBDV, antigenicity, phylogenetic analysis.

Introduction:

Infectious bursal disease virus (IBDV) was described for the first time almost 40 years ago [10] in the Delmarva region as the causative agent of an unknown disease, which was called avian nephritis due to changes observed in the kidneys [10]. Due to varying morphologic and histologic changes observed in the bursa of Fabricius (BF), the term infectious bursal disease was proposed [18], and the virus was designated as IBDV. The immunosuppressive effect of IBDV is caused by a lytic infection of immature B lymphocytes [41], which develop in the BF. IBDV belongs to the genus *Avibirnavirus* within the family *Birnaviridae*. IBDV is a non-enveloped virus with a capsid containing a genome of two segments (segment A and B) of double-stranded RNA [13]. Segment B encodes, in a single open reading frame (ORF), the viral RNA-dependent RNA polymerase, designated as viral protein (VP) 1 [37, 40, 42, 62]. Segment A contains two ORFs of different lengths. The viral protein VP5 is encoded by the smaller ORF [55], which results in a translated protein of an apparent molecular weight of 21 kDa [44]. The larger ORF encodes a polyprotein, which is autoproteolytically cleaved into the viral proteins VP2, VP3, and VP4 [1]. The proteolytical function of VP4 was described by Azad et al. [2], and the active center of the protease was characterized by Birghan et al. [6] as a non-canonical lon protease. The only known IBDV antigen capable of inducing neutralizing antibodies in chickens is VP2 [5], which has been shown to be the only capsid protein of IBDV [11]. Within the coding region of VP2, an unconserved region was identified in IBDV isolates, and the term “variable region” was coined [4]. Furthermore, this variable region contains two major [3] and two minor hydrophilic regions [59]. Although VP3 was initially thought to be the most important viral antigen for the induction of neutralizing antibodies [2, 15], it later became clear that VP2, in fact, induces neutralizing antibodies and is the basis for IBDV protection [3]. The genetic basis for the

antigenicity of VP2 has been determined [32, 51, 58], and the identification of amino acids responsible for antigenicity has been reported recently [33].

The first IBDV isolates, now known as classical IBDV, were clinically characterized by depression, anorexia, ruffled feathers, trembling, whitish or watery diarrhea, prostration, and mortality [36]. A second IBDV serotype was later described in Europe [38] and the United States [23]. Virus isolates belonging to serotype 2 IBDV were not pathogenic in chickens. Since the mid-1980 s, new antigenic subtypes of serotype 1 IBDV (now called variant strains) were isolated in the USA and characterized by the depletion of B lymphocytes [49, 50] in the absence of a clinical disease. These variant strains were later characterized by a panel of Mabs [52–54, 58] and found to have a different antigenic makeup. An antigen capture ELISA was developed using the Mabs and was utilized to characterize the antigenicity of IBDV [60]. At the same time, very virulent (vv) IBDV [7, 8] emerged in Europe. vvIBDV is capable of causing up to 100% mortality in susceptible chickens [27, 31], which can also be observed with some classical serotype 1 IBDV [29]. The new characteristic was that even in the presence of relatively high maternally derived antibody titers, the offspring was not protected from the clinical symptoms and death caused by vvIBDV. This IBDV subtype spread very quickly and was already present in Brazil by 1997 [12]. The first reported outbreak of vvIBDV in the US occurred in California in 2008 [56], leaving only New Zealand and Australia free of this IBDV subtype. Control of IBD can only be accomplished with a solid vaccination program accompanied by a solid biosecurity program. In addition, circulating field isolates should be characterized for their antigenic and virulent properties to provide a scientific basis for vaccine selection. Initially, IBDV diagnosis was accomplished by virus isolation, agar-gel precipitation,

and electron microscopy [36]. These methods are not capable of identifying antigenic or pathogenic characteristics. Due to this fact, and the tremendous increase in poultry production, it is necessary to have rapid and accurate methods for typing IBDV field isolates. For conventional typing using the virus neutralization (VN) assay, propagation of the virus in cell culture is required, but most field isolates are not able to infect cell cultures. In addition, antigenic and pathologic characteristics of the virus may change during the adaptation process [36]. For direct antigenic characterization of bursal material, an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) using Mabs has been used [60]. The detection of viral nucleic acid by reverse transcription (RT)-polymerase chain reaction (PCR) has become an important tool along with restriction fragment length polymorphism (RFLP) for the detection and genotyping of IBDV field isolates [21, 22, 24, 30, 35]. The next generation of detection and characterization of IBDV field isolates by molecular techniques was the use of real-time RT-PCR [9, 39]. Using TaqMan primers and probes designed to target specific epitopes, viruses with nucleotide sequences identical to the TaqMan probes [28] were detected. However, this approach was not applicable for analysis of emerging European IBDVs [20]. Icard et al. [19] reported the use of reverse genetics as a diagnostic tool along with the use of the Mabs and the deduced amino acid sequence of the VP2 protein. Due to the degeneracy of the genetic code, the prediction of antigenic differences between IBDV isolates based on nucleotide sequence is not possible. Using the reverse genetics method, several IBDV isolates that lacked reactivity with any of the Mabs [19] were identified. These data suggest the need to utilizing a combination of three diagnostic components, the Mab reactivity pattern, nucleotide sequence, and amino acid sequence, to help refine the in vitro process of antigenic characterization of IBDVs. The advantage of the reverse genetics system is that viruses can be characterized on the basis of their antigenic makeup

directly from bursal samples without virus propagation in embryonated eggs or inoculation of susceptible chickens to obtain sufficient virus material for the AC-ELISA [60].

In the work described in this paper, the nucleotide and amino acid sequences of 302 virus isolates were analyzed along with their Mab reactivity patterns. The results showed that amino acid exchanges located in hydrophilic peak B resulted in the most dramatic changes in the antigenic phenotype of IBDV. In addition, it was observed that changes located outside of the previously described hydrophilic regions of VP2 [3, 51, 59] also influenced the antigenicity of IBDV, making prediction of changes in IBDV antigenicity based on sequence data alone highly unreliable.

Material and methods:

Cells and virus:

Transfection and determination of monoclonal antibody binding reactivities were performed in a chicken fibroblast cell line DF-1 [17]. The resulting Mab reaction patterns were determined using indirect immunofluorescence. Cells were cultivated in Dulbecco's modified Eagle medium with 4.5 g glucose per liter (DMEM-4.5, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Mediatech, Manassas, VA, USA) in the presence of penicillin (100 IU/ml) and streptomycin (100 µg/ml). Field samples were taken from diagnostic submissions to the Poultry Diagnostic and Research Center (Athens, GA, USA), and these included samples from seven states of the USA (Alabama, California, Delaware, Florida, Georgia, Mississippi, and South Carolina; see also Table 2.1).

Construction of recombinant segment A:

The reverse genetics system used for these studies utilized plasmids containing the full-length cDNA of segments A and B, pD78A-SpeI and pD78BPD, respectively, as described previously [19]. The plasmid pD78APD contains restriction enzyme cleavage sites at position 201 (Rsr II) and 1181 (Spe I), which enables the substitution of a major part of VP2 from aa 23 to aa 350. The nucleotide numbering is based on Mundt and Müller [45]. This part of the polyprotein gene encodes the variable region of VP2 and flanking regions that are highly conserved between different serotype I IBDV isolates [4]. The diagnostic submissions were bursal samples from field surveys or flocks showing poor performance, including higher feed conversion rate, higher condemnation rates at the processing plant, and/or an increased observation of respiratory problems. This triage is considered an indicator of the presence of an immunosuppressive agent in the chicken flock. The bursal samples were either taken directly for RNA isolation using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) or following virus isolation in nine-day-old embryonated SPF eggs (Sunrise Farms, Catskill, NY, USA) inoculated via the chorio-allantoic membrane (CAM). For virus isolation, the bursal samples were minced in serum-free medium at a ratio 1:10 (w/v) and centrifuged at 700 x g for 10 min at 4°C. The supernatant was filtered with a 0.45-µm filter, and 100 µl of the filtrate was incubated with a chicken serum specific for avian reovirus for 60 min at 37°C. One hundred µl of this mixture was inoculated onto the dropped CAM of the SPF embryos and sealed with nontoxic glue. The eggs were incubated for seven days and candled daily. Death occurring within the first 24 h after inoculation was regarded as nonspecific. Dead embryos and embryos surviving seven days post-inoculation were opened and inspected for lesions. CAMs from embryos that showed lesions were harvested. Bursal and CAM samples were treated as described previously [19]. The

extracted RNA was used for reverse transcription-polymerase chain reaction (RT-PCR, see below). Samples that did not induce embryo lesions were passaged two additional times in embryonated eggs. If the third CAM passage was negative for embryo death or lesions, the sample was considered negative for IBDV.

Detection of viral RNA by RT-PCR and generation of chimeric plasmids:

The extracted RNA from either bursa or CAM samples was used for RT-PCR using a single step RT-PCR kit with platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA). To this end, eluted RNA was used for amplification of a cDNA encompassing the viral genome from nucleotide 107 to 1199 using oligonucleotides IBDVFP1 (GAGATCAGACAAACGATCGCAGC) and IBDVRP4-Spe I (CTCTTTCGTAGGCCACTAGTGTGACGGGACGG) following the manufacturer's instructions. The resulting PCR fragment was gel-purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and incubated with the restriction enzyme endonucleases Rsr II and Spe I, gel-purified again and ligated into the appropriately cleaved pD78A-Spe I. This resulted in the chimeric plasmid pD78A-Chim. Using this approach, the VP2-encoding sequence of the field isolate was ligated in frame with the ORF of the polyprotein encoded by segment A. The sequence of the viral cDNA was determined using three oligonucleotides that were highly conserved among known IBDV VP2 sequences. Using this approach, a chimeric IBDV segment A was obtained for subsequent experiments. The general approach is depicted in Fig. 2.1.

Transfection and analysis of antigenicity:

Transfection of cells with the chimeras was performed as described previously [19, 46]. Briefly, the recombinant plasmids pD78A-Chim and pD78BPD were linearized with Bsr GI and Pst I, respectively. The T7 RNA polymerase transcription of viral cRNA and subsequent co-

transfection were performed as described previously [46] with two modifications. DF-1 cells grown in 48-well tissue culture plates were transfected using a TransIT[®]-mRNA Kit (Mirus Bio, Madison, WI, USA) following the manufacturer's instructions. Sixteen hours after transfection, the supernatants were removed, and the cells were rinsed with phosphate-buffered saline (PBS) and fixed with ice-cold ethanol (96%) for 10 min. The ethanol was removed, and the cells were air-dried. An indirect immunofluorescence assay using the monoclonal antibodies R63, B69 [52], 10, 57 [54] and 67 [58] and rabbit VP1 antiserum [6] was performed as described by Letzel et al. [33]. The monoclonal antibodies were kindly provided by Rudolf Hein (Intervet/Schering Plough, Millsboro, DE, USA). The binding of antibodies was visualized using the appropriate species-specific FITC-conjugated antibodies: goat anti-mouse or goat anti-rabbit FITC (Jackson ImmunoResearch, West Grove, PA, USA). The fluorescence was documented using an inverted Zeiss microscope Axiovert 40 CFL (Zeiss GmbH, Jena, Germany).

Sequence analysis and manipulation of the crystal structure:

The sequences obtained in this study were analyzed using the DNASTAR Lasergene 8 (DNASTAR, Inc., Madison, WI, USA) software. The sequence from nucleotide 201 to 1181 was translated *in silico* using an online translation tool (<http://www.expasy.org/tools/dna.html>). Nucleotide as well as amino acid sequences were aligned using Clustal W (<http://www.ebi.ac.uk/clustalw>). Phylogenetic analysis was performed using MEGA 4.1 following the algorithms described by Tamura et al. [57]. All phylogenetic analyses were performed using two methods: the neighbor-joining method and minimum evolution. The robustness of nodes was evaluated by bootstrapping (1000 replications). Bootstrap values <75 were judged as invalid. The crystal structure of VP2 was manipulated using the Pymol program

(Open-Source PyMOL 1.2r1), which is available online at <http://www.pymol.org>, using the structural data for the VP2 protein [PDB ID Code: 1WCD, [11]].

Results:

Molecular characterization of IBDV field isolates:

Three hundred two IBDV sequences from different IBDV samples were analyzed from nucleotide 200 to 1181 of segment A (NCBI GenBank accession numbers JF748873-JF749174). The GenBank accession number, USA state, year of isolation, and MAb reactivity pattern for each isolate are shown in Table 2.1. The following VP2 sequences were included for the analysis of phylogenetic similarities between IBDV subtypes: E/Del subtype (E/Del, GenBank accession number X54858), GLS subtype (GLS, AY368653), subtype Bel-IBDV [33], the classical subtype (D78, 46), and the very virulent subtype (UK661, 61). To maximize the calculation stringency, the VP2 nucleotide sequence of the serotype 2 strain 23/82 [51] was used (Fig. 2.2). In general, most of the newly generated sequences were not related to sequences from viruses isolated more than 10 years ago (E/Del, GLS, UK661, D78). Phylogenetic analysis revealed only three sequences grouped with the E/Del subtype out of the 302 sequences analyzed (bootstrap value of 83). One sequence each grouped with the GLS subtype and with the classical strain D78, with bootstrap values of 99 and 100, respectively. Interestingly, two sequences grouped with a bootstrap value of 100 with the sequence of the vvIBDV strain UK661, while four sequences were very closely related to the Bel-IBDV isolate (bootstrap value of 100). Fifty-four sequences formed a clade with a bootstrap value of 87. This branch contained only two sequences (JF748970, JF748990, highlighted with an asterisk) with a known antigenic subtype (GLS-subtype, 10 and 57 positive, 58). All other sequences in this clade were phylogenically different from any known subtype sequences, indicating a significant difference. Furthermore, 26

out of the 96 sequences analyzed, representing viruses that did not react with any of the Mabs, formed an clade (bootstrap value of 100). Most of the sequences that showed an E/Del antigenic subtype (63 and 57 positive, 58) grouped in one clade (bootstrap value of 77). In this clade, other antigenic IBDV subtypes were also present, including sequences from viruses that did not react with any of the Mabs (see Fig. 2.2). Within this clade, a subclade was formed (bootstrap value of 86) that consisted mainly of sequences from viruses that showed the E/Del antigenic subtype. Since the bootstrap value of this subclade was calculated to be 86, they were significantly different from the other sequences that showed the same antigenic subtype. Interestingly, two sequences grouped in that subclade but reacted either with Mab 67 (JF749141) or with none of the MABs (JF749012). These findings indicate that due to evolution of IBDV in the field, current IBDV field isolates have drifted away from the viruses isolated in the mid-eighties. Furthermore, it was noticed that the Mab reactivity patterns were different for some IBDV isolates even though they were phylogenically located in the same clade.

Antigenic characterization of field viruses:

To analyze the antigenic profiles of the new field isolates, we analyzed the reactivity pattern using the Mabs 10, 57, R63, 67, and B69 by applying the reverse genetics system described previously [19]. Mab B69 was generated using the classical IBDV strain D78 as antigen, while the hybridoma secreting Mab R63 was obtained after immunizing mice with a mixture of different IBDVs [52]. The immunization of mice with the GLS virus yielded Mabs 10 and 57 [54]. Mab 67 was first described by Vakharia et al. [58] and was obtained after the fusion of spleen cells from mice that had been were immunized with the E/Del virus. It has been shown that all of the Mabs were able to neutralize the virus used for immunization [33, 52, 54, 58] In total, the Mab panel pattern was determined for 302 samples (Table 2.1). The classification of

isolates into the groups of E/Del-like, GLS-like, and classical IBDV-like was based on the Mab reactivity pattern as described previously [58]. Approximately one third of all samples (100 samples) reacted with two of the Mabs (R63 and 67), indicating that they belong to the antigenic E/Del subtype (Table 2.1). Based on their reactivity, eight samples could be grouped with the GLS antigenic subtype (only positive for Mabs 10 and 57), and three samples could be grouped with the classic antigenic subtype (only positive for Mabs 10, R63 and B69). Interestingly, some samples showed a panel pattern that, to date has not been described. The new viruses were only positive for Mab 67 (44 samples), Mab 57 (23 samples), Mab R63 (18 samples), or Mabs 10, 57 and 67 (3 samples). Interestingly, approximately one third of the samples (96 samples) did not react with any of the monoclonal antibodies. To verify their non-reactivity, transfection experiments were repeated with each of the non-reactive samples using a 50% dilution of the Mab previously used in the optimized assays. Again, no reactivity was observed for the field isolates and appropriate reactivity patterns were observed for the controls (pD78A-8903PD for E/Del subtype, pD78A-GLS-05PD for GLS subtype, and pD78A-SpeI for classic subtype; see ref. [19]). These findings indicate that IBDVs are circulating in the field that are antigenically different from the known IBDV subtypes.

Analysis of amino acid exchanges:

The nucleotide sequences were translated *in silico* into the appropriate amino acid sequence using online translation tools. The deduced amino acid sequences were used to evaluate exchanges in VP2 compared to the sequence of the E/Del (Fig. 2.3). In general, it was observed that most amino acid exchanges occurred in the four hydrophilic regions of IBDV. The hydrophilic peaks are located at amino acids 210 to 225 (peak A, 3), amino acids 247-254 (minor peak 1, 59), amino acids 281-292 (minor peak 2, 59), and amino acids 312 to 324 (peak B, 3).

These regions are located at the outer part or projection domain of the viral capsid [11]. This indicates that the selection pressure for the evolution of the virus is directed toward regions of the capsid that are immediately exposed to the immune system. In addition, it needs to be mentioned that amino acids located in the minor hydrophilic peak (aa 253, aa 284) are responsible for the ability of the virus to infect both cell culture and B-lymphocytes, or cell culture only [34, 43]. Most amino acid exchanges were observed in hydrophilic peak B, followed by hydrophilic peak A, minor hydrophilic peak 1, and minor hydrophilic peak 2. Amino acids located in the hydrophilic regions where most of the exchanges occurred were N213, T222 (peak A); K249, S251, S254 (minor peak 1); I286 (minor peak 2), S317, D318, A321, G322, and E323 (peak B). Interestingly, amino acids T73, N77, S168, L180, I187, M193, and N299 were also frequently exchanged although they were not located in any of the hydrophilic regions of the viral capsid protein VP2. These findings clearly showed that certain regions of the viral protein were under selection pressure. Amino acids N213, T222, K249, S251, S254, I286, N299, S317, D318, A321, G322, and E323 are located in the projection domain and thus exposed to the outside of the protein. The remaining amino acids (T73, N77, S168, L180, I187, M193) were located in regions that are located in the shell domain and the border between the shell and the projection domain (for an explanation of the location of the domains, see Fig. 2.5). The amino acid exchanges that are located in the projection domain were previously thought to be responsible for changes in the antigenicity of IBDV [11, 33]. In contrast, the amino acids located in the shell domain are important for the stability of the VP2 homotrimer [11] due to their likely function as contact points between the single VP2 molecules to form the homotrimer (aa L74, N77: loop S_{C'C''}; M193: hairpin P_{AA'}; N213, Q215: loop S_{BC}; T269: loop S_{FG}; for location of the

functional domains, see Coulibaly et al. [11]). In general, there was no clear pattern with regard to the presence of certain amino acids that could be related to differences in antibody binding.

To further illustrate the high variability of the amino acid sequences, a comparison of all amino acid exchanges with the E/Del sequence was performed (Fig. 2.3). This comparison was based on the groups with appropriate Mab reactivity patterns. The viruses that showed an E/Del-like Mab reactivity pattern (Mab R63 and 67 positive) showed aa exchanges in 111 out of 326 amino acids analyzed (34%). Twenty-eight of the amino acid exchanges involved two different amino acids, and four amino acid exchanges involved three different amino acids at certain positions. The viruses that showed no reaction with any of the Mabs had amino acid exchanges at a total of 181 positions (50%). Out of these 181 positions, 42 had two different amino acids, 17 had three different amino acids, and five had four different amino acids. Viruses that reacted only with Mab 67 showed 64 exchanged aa positions. Out of these, 10 positions had two, two positions had three, and two positions had four different amino acids. A similar result was observed for viruses that showed reactivity with only Mab 63 (65 aa exchanges, four aa exchanges with two different amino acids), with Mabs 10 and 57 (34 aa exchanges, 2 aa with two different amino acids), with Mabs 10, 57, and 67 (6 aa exchanges), and with Mabs 10, R63 and B69 (42 aa exchanges, three positions with two different amino acids). The last of these were interesting because they belong to the classical antigenic type and had only three sequences with a comparably large number of aa exchanges. Interestingly, every antigenic subtype showed amino acid exchanges in all four previously determined hydrophilic regions except the group of viruses that showed reactivity with Mabs R63 and 67. These viruses showed no exchanges in hydrophilic peak B, except for two viruses that showed aa exchanges at position 312 (JF748919 [Ile to Lys] and JF749048 [Ile

to Thr]) located at the N-terminus of this hydrophilic peak. This indicated that this region is most important for the E/Del antigenic type and suggests that evaluation of this region could be used as a diagnostic tool, since any change observed in this region would indicate that this virus does not belong to the E/Del antigenic type. The other amino acid of interest was aa 222. Here, a great variety of aa exchanges were observed, indicating that this region (hydrophilic peak A) is under a high degree of selective pressure. The same holds true for amino acid 254, 280, 317, and 318, which are all located in hydrophilic regions, except aa 280, which is located just outside of the N-terminus of the minor hydrophilic peak 2.

Also, we found amino acid exchanges outside of the hydrophilic regions that were responsible for the change in antigenic makeup. To illustrate this, *in silico*-translated amino acid sequences of nucleotide sequences that were phylogenically closely related were compared (Fig. 2.4, see also Fig. 2.2). Although a number of amino acid exchanges (GenBank accession number X54858) were observed for both of the isolates in comparison to the E/Del sequence (a1, [JF748899] and a2 [JF7489000], Fig. 2.2), nine additional amino acid exchanges were observed in the isolate that did not react with any of the Mabs. Only one of these amino acid exchanges was observed in the hydrophilic peak B (Ile312Met). Another example showed that exchange of one amino acid from Tyr141 (b1 [JF748922], Fig. 2.2) to His141 (b2 [JF7491221], Fig. 2.2) led to the absence of any reactivity with the Mabs used. It needs to be mentioned that aa 141 is located outside of any of the hydrophilic regions described. It was also notable that 15 aa exchanges in comparison to the E/Del sequence in the isolate JF748922 (b1, Fig. 2.2) did not result in a change in the Mab reactivity pattern. In the third example (c1 [JF749101], c2 [JF749103], Fig. 2.2), the exchange of the amino acids within hydrophilic peak B (Asp318Asn,

Ala321Glu, Glu323Asp) were probably responsible for the changed Mab reactivity pattern as described by Letzel et al. [33]. However, the exchange of aa 49 (Thr to Ala) probably prevented the binding of any of the Mabs. The location of aa 41 is in the shell domain of the viral protein VP2 [11], and it is located outside of the hydrophilic peaks.

Examination of viral structural components related to antigenicity:

Amino acid exchanges were highlighted in the crystal structure of the VP2 monomer using the Pymol program (<http://www.pymol.org>) to determine the locations of aa exchanges in the protein structure (Fig. 2.5) of viruses that did not react with any of the Mabs and those that reacted with Mabs R63 and 67. These groups were selected because they represent the two largest groups of viruses with different antigenic subtypes. The base, shell, and projection domain were marked for a better and more specific spatial discrimination. The analysis was performed using a comparative approach, with identification of aa exchanges present only in the same group. Due to the frequency of the dots, it became immediately apparent that there were domain-specific differences. The number of aa exchanges in viral sequences that were only present in the E/Del subtype group (positive for Mabs R63 and 67) was almost identical between the shell domain 12.5% (19/152) and the projection domain 10% (18/174). Interestingly, the numbers of specific aa exchanges were higher in the group that did not react with any of the Mabs (Shell domain: 30% [47/152], projection domain: 33% [57/174]). This finding indicates that a similar selection pressure, due to existing immunity based on vaccination programs and existing field viruses, occurred in both domains of the VP2 molecule. As mentioned above and visualized by this examination, only the hydrophilic peak B contained aa exchanges in viruses that did not react with any of the Mabs.

Discussion:

The determination of factors that influence the antigenicity of viruses is an important tool for diagnosis, and more importantly, for vaccine development. In IBDV, VP2 is the only protein of the virus that is able to induce neutralizing antibodies in chickens [5] and is thus the host-protective antigen. Although VP3 contains group- and serotype-specific epitopes [47], no convincing evidence has been provided for its ability to induce neutralizing antibodies. Within VP2 is a region that shows high diversity among different IBDVs and is called the variable region [4]. Since the only component of the IBDV capsid is VP2 [11], we focused our analysis on a large portion of the capsid protein (aa 27-347) including the variable region. The genetic basis for protective immunity against IBDV has been extensively evaluated, with the focus on VP2 [3, 14, 48, 51], using a small number of viruses. Using the IBDV reverse genetics system [46], a more precise analysis of regions and, more specifically, amino acids within VP2 that are responsible for the antigenicity was possible [33]. The analysis of a large number of samples using a comprehensive approach (nucleotide sequence, amino acid sequence, antigenicity) as described in this study was performed with the goal of identifying certain amino acids that correlate with changes in antigenicity of the virus as expressed by the Mab reactivity pattern. The data analysis indicated that there was no correlation between the antigenic makeup of the viruses as characterized by their Mab reactivity pattern and their location in the phylogenetic tree. These data further support the conclusion that use of phylogenetic analysis of nucleotide sequences, which is basically a grouping based on similarities, is no longer sufficient by itself for characterizing IBDVs and might lead to incorrect conclusions regarding the relatedness of the subtype to the antigenic properties of the virus. It needs to be mentioned that the viruses that did not react with any of the Mabs are not necessarily antigenically similar to each other; they are

only antigenically different from known IBDV subtypes. Most of the sequences were not related to any of the known IBDV subtype viruses (e.g., D78, E/Del, GLS), and this correlates with the findings of Jackwood and Sommer-Wagner [25], in which two out of four of viruses analyzed did not group with any known viruses. The results described in this study show that most of the viruses did not group with any of the known IBDVs, and this indicates a tremendous genetic drift in the field. The molecular-virological assay described here connects sequence analysis with an immunological assay, which would allow analysis of the consequences of certain amino acid exchanges within the VP2 region as it relates to the antigenicity, which could not otherwise be explained [20]. The surprising results prompted us to analyze our data in more detail. The use of the previously published crystal structure [11] was of immense help in fitting the aa exchanges into the structure of the VP2. A similar but rather restricted approach, focused solely on the influence of certain amino acid exchanges on the viral antigenicity of the VP2 molecule, identified important regions for the antigenicity of the VP2 molecule [33]. We extended this study using field isolates. To our surprise, there was no clear pattern between changes in the aa sequence and changes in the antigenicity, with one exception. Only when changes between aa 312 and 324 occurred was the E/Del antigenotype absent, indicating the importance of this region for the antigenicity of IBDV belonging to the E/Del subtype. Heine et al. [16] identified eight amino acids (Asn213, Thr222, Lys249, Ser254, Ala270, Ile286, Asp318, Glu323) that were specific for the E/Del virus analyzed in comparison to different classical IBDV strains. Jackwood et al. [20] stated that threonine at position 222 and serine at position 254 are hallmark amino acids for the E/Del subtype. However, the aa sequences found in the group of the 96 viruses that did not react with any of the Mabs encoded Asn213 (77 samples), Thr222 (87 samples), Lys249 (94 samples), Ser254 (52 samples), Ala270 (96 samples), Ile286 (93 samples), Asp318 (16

samples), and Glu323 (56 samples). On the other hand, viruses have been identified that showed neither amino acid at the appropriate position, but reacted with both Mabs (R63, 67). These findings indicate that these particular amino acids cannot be used as indicators for certain IBDV antigenic types.

The location of the mutations observed in the VP2 structure clearly indicates that certain regions in the VP2 shell and projection domain were under more selective pressure than others. Surprisingly, only one region could be related to a certain antigenotype, hydrophilic peak B. A recent finding [26] has indicated that even when the aa sequence in hydrophilic peak B has not changed, the antigenicity of the virus can change. Our findings show that amino acid exchanges outside of the known hydrophilic regions can lead to changes in antigenicity, as demonstrated in this study using monoclonal antibodies. These findings contrast with published data [33] and indicate a broader involvement of amino acids in the formation of neutralizing epitopes. This was surprising, since the aim of the study was to identify amino acids located in the VP2 sequence that could be used as diagnostic markers. Thus, sequence analysis, regardless of whether a nucleotide or aa sequence is used, can only indicate that changes have occurred and at what frequency. Further testing needs to be performed, by ELISA, based on a certain panel of Mabs [60], by the reverse genetics system in conjunction with a panel of Mabs [19], or by cross-neutralization assays in embryonated eggs or vaccinated chickens.

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Table 2.1: List of the investigated virus isolates

State ^a	Monoclonal antibody panel pattern ^b: All negative (96 sequences)
AL	JF748901 - 02 ^{c 07d} , JF748941 - 46 ⁰⁸ , JF748952 - 53 ⁰⁸ , JF748958 ⁰⁸ , JF748968 - 69 ⁰⁸ , JF748992 - 94 ⁰⁸ , JF749167 ¹⁰ , JF749170 ¹⁰ , JF749174 ¹⁰
CA	JF749059 ⁰⁹
DE	JF749007 - 08 ⁰⁸
FL	JF749089 ⁰⁹ , JF749091 ⁰⁹ , JF749155 - 56 ¹⁰
GA	JF748876 ⁰⁷ , JF748881 - 83 ⁰⁷ , JF748885 - 87 ⁰⁷ , JF748900 ⁰⁷ , JF748904 - 05 ⁰⁷ , JF748991 ⁰⁸ , JF748995 - 97 ⁰⁸ , JF749012 ⁰⁸ , JF749027 - 30 ⁰⁸ , JF749036 - 38 ⁰⁹ , JF749041 - 42 ⁰⁹ , JF749051 - 52 ⁰⁹ , JF749056 - 58 ⁰⁹ , JF749068 - 74 ⁰⁹ , JF749083 - 85 ⁰⁹ , JF749094 ⁰⁹ , JF749097 - 101 ⁰⁹ , JF749117 ⁰⁹ , JF749122 ⁰⁷ , JF749149 - 50 ¹⁰ , JF749152 - 54 ¹⁰ , JF749157 - 59 ¹⁰ , JF749161 - 65 ¹⁰
MS	JF748950 ⁰⁷ , JF748954 ⁰⁷ , JF748956 - 57 ⁰⁷ , JF749088 ⁰⁹ , JF749172 - 73 ¹⁰
SC	JF749119 ¹⁰ , JF749135 ¹⁰ , JF749137 ¹⁰
	Monoclonal antibody panel pattern: positive for Mab's R63 and 67 (100 sequences)
AL	JF749143 - 44 ¹⁰
FL	JF749092 ⁰⁹ , JF749138 - 39 ¹⁰
GA	JF748878 - 79 ⁰⁷ , JF748888 - 91 ⁰⁷ , JF748893 ⁰⁷ , JF748895 - 99 ⁰⁷ , JF748918 - 22 ⁰⁷ , JF748924 - 31 ⁰⁷ , JF748932 - 35 ⁰⁸ , JF748962 ⁰⁸ , JF748973 - 79 ⁰⁸ , JF749011 ⁰⁸ , JF749013 - 15 ⁰⁸ , JF749013 - 26 ⁰⁹ , JF749031 - 35 ⁰⁹ , JF749043 - 50 ⁰⁹ , JF749053 - 55 ⁰⁹ , JF749066 - 67 ⁰⁹ , JF749081 ⁰⁹ , JF749112 - 16 ⁰⁹ , JF749123 ¹⁰ , JF749132 ⁰⁹ , JF749142 ¹⁰ , JF749151 ¹⁰
MS	JF748909 ⁰⁷ , JF748936 ⁰⁷ , JF748980 - 84 ⁰⁷ , JF749169 ¹⁰
SC	JF748959 - 61 ⁰⁸ , JF749003 - 05 ⁰⁷ , JF749140 ¹⁰
	Monoclonal antibody panel pattern: positive for Mab 67 (44 sequences)
GA	JF748873 - 75 ⁰⁷ , JF748877 ⁰⁷ , JF748963 - 64 ⁰⁸ , JF748998 - JF749002 ⁰⁸ , JF749060 - 61 ⁰⁹ , JF749063 - 65 ⁰⁹ , JF749075 - 76 ⁰⁹ , JF749078 - 80 ⁰⁹ , JF749082 ⁰⁹ , JF749096 ⁰⁹ , JF749105 - 11 ⁰⁹ , JF749118 ⁰⁹ , JF749124 - 26 ¹⁰ , JF749129 - 31 ¹⁰ , JF749133 - 34 ¹⁰
DE	JF749006 ⁰⁸ , JF749008 ⁰⁸
SC	JF749120 ¹⁰ , JF749141 ¹⁰
MS	JF749087 ⁰⁹
	Monoclonal antibody panel pattern: positive for Mab 57 (23 sequences)
AL	JF748937 - 40 ⁰⁸ , JF748947 - 49 ⁰⁸ , JF748966 - 67 ⁰⁸
GA	JF748971 - 72 ⁰⁸ , JF748988 ⁰⁸ , JF749039 - 40 ⁰⁸ , JF749095 ⁰⁹ , JF749127 ¹⁰
MS	JF748906 - 08 ⁰⁷ , JF748951 ⁰⁷ , JF748955 ⁰⁷ , JF748086 ⁰⁹
SC	JF749121 ¹⁰
	Monoclonal antibody panel pattern: positive for Mab R63 (18 sequences)
AL	JF749168 ¹⁰
FL	JF749090 ⁰⁹
GA	JF748894 ⁰⁷ , JF748903 ⁰⁷ , JF748910 - 17 ⁰⁷ , JF748923 ⁰⁷ , JF748965 ⁰⁸ , JF749102 ⁰⁹ ,

MS	JF749145 ¹⁰ , JF749147 ¹⁰ JF749166 ¹⁰
Monoclonal antibody panel pattern: positive for Mab's 10 and 57 (8 sequences)	
DE	JF749010 ⁰⁸
GA	JF748892 ⁰⁷ , JF748970 ⁰⁸ , JF748990 ⁰⁸ , JF749077 ⁰⁹ , JF749160 ¹⁰ , JF749171 ¹⁰
SC	JF749136 ¹⁰
Monoclonal antibody panel pattern: positive for Mab's 57 and 67 (7 sequences)	
GA	JF748880 ⁰⁷ , JF748884 ⁰⁷ , JF748885 ⁰⁸ , JF749062 ⁰⁹ , JF749103 ⁰⁹ , JF749104 ⁰⁹ , JF749128 ¹⁰
Monoclonal antibody panel pattern: positive for Mab's 10, 57, and 67 (3 sequences)	
GA	JF748986 ⁰⁸ , JF748987 ⁰⁸ , JF748989 ⁰⁸
Monoclonal antibody panel pattern: positive for Mab's 10, R63, and B69 (3 sequences)	
GA	JF749146 ¹⁰ , JF749148 ¹⁰
FL	JF749093 ⁰⁹

^a State of the USA where the virus was isolated shown in the states abbreviation code

^b Reactivity after transfection with a panel of monoclonal antibodies 10 (Snyder et al., 1992), 57(Snyder et al., 1992), R63 (Snyder et al., 1988), 67 (Vakharia et al, 1994) , B69 (Snyder et al., 1988)

^c NCBI genbank accession number

^d Year of isolation (e.g. 2007 = 07)

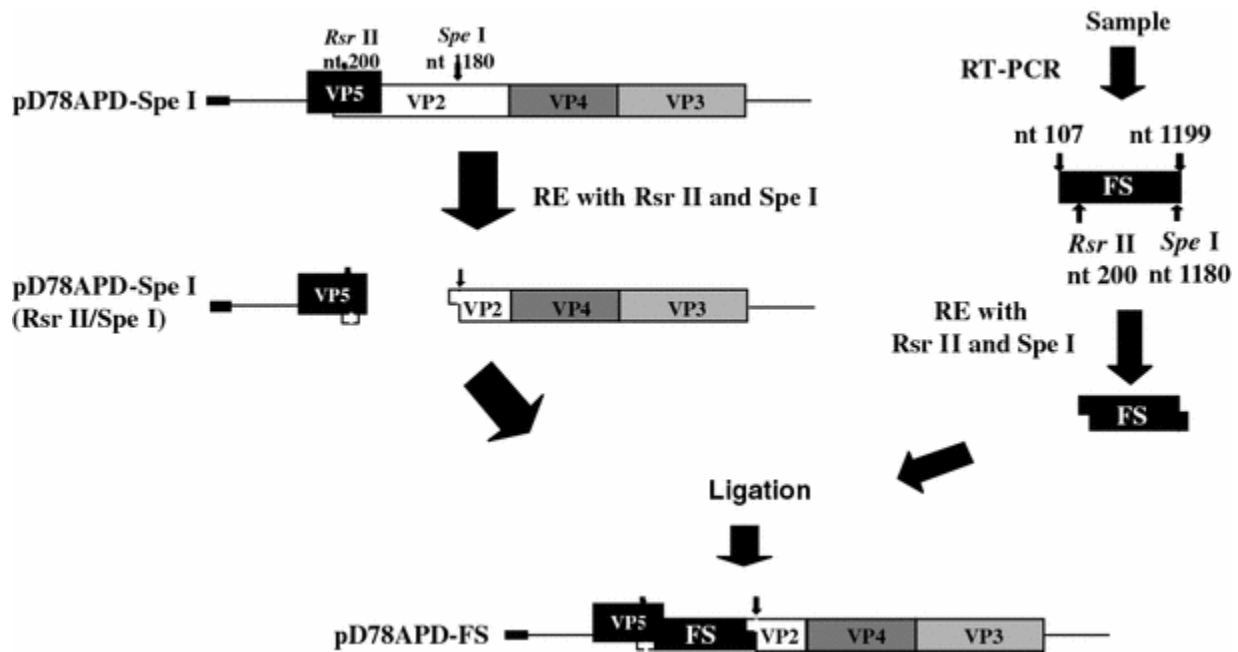


Fig. 2.1 Generation of chimeric segment A plasmids. The restriction enzyme cleavage sites Rsr II (nt 200) and the newly engineered Spe I (nt1180) were used to substitute the appropriate coding region of VP2 of pD78APD-Spe I (19) with the appropriate coding region of IBDV field samples (FS) to generate plasmids which encode for a chimeric segment A polypeptide (pD78APD-FS). The numbering of the nucleotides is in accordance with Mundt and Müller (1995).

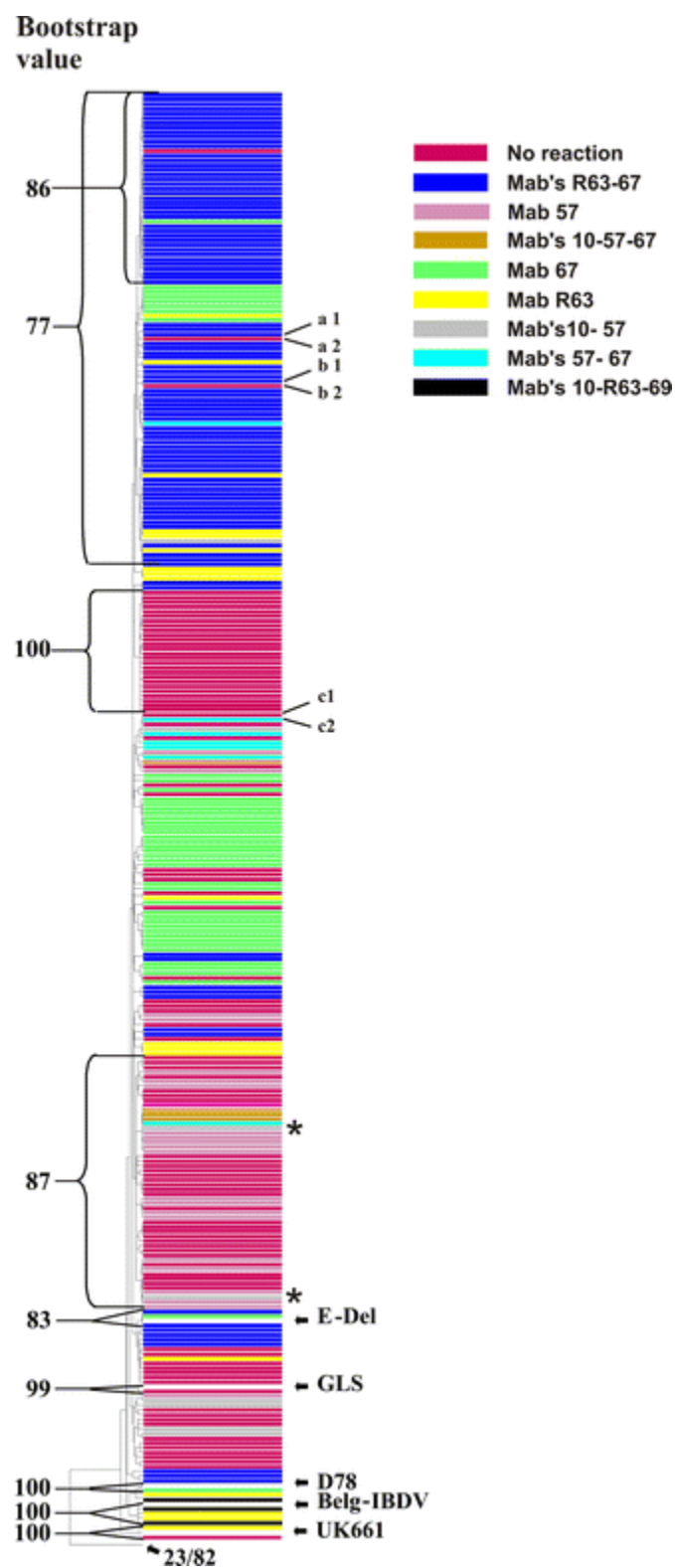


Fig. 2.2 Phylogenic analysis of VP2 encoding sequences of IBDV. The evolutionary history of the sequences was inferred using the minimum evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test using 1000 replicates (Bootstrap value) are shown for significant groups at the left of the tree. Nucleotide sequences encoding for the appropriate coding region of VP2 (nt 200 – 1181) used as standard sequences were E/Del (Genbank accession number X54858), GLS subtype (GLS, AY368653), subtype Bel-IBDV (Letzel et al, 2007), the classical subtype (D78, Mundt and Vakharia, 1996), the very virulent subtype (UK661, van Loon et al, 2003) and serotype 2 strain 23/82 (Schnitzler et al., 1993). The panel pattern were encoded by certain colors and overlaid above the appropriate encoding nucleotide sequence. Two sequences which showed the GLS antigenic subtype were marked by an asterisk. Sequences which were phylogenically related but belonged to antigenic different subtypes were marked (a1, a2; b1, b2; c1; c2).

	30	40	50	60	70	80	90	100	110	120	130	
E/Del	PASIPDDTLEKHTLRSETSTYNLTVDGTGSLIVFFPGFPGSI VGAHYTLQSNNGYKFDQMLLT AQNLPASYNRYCRLVSRSLTVRSSTLPGGVYALNGTINAVTFQGSLS											
No reaction	-----YIPQE-P-PL-G---D---V--P-P---L---APAA---HIP--D-DHNLHHI--ATRDP--IHSHRK-L-PT-I-PGA-----C--DDAM-PL---GPD											
No reaction	-----N---K---Y---G-----P---S---W-Y---H-----H-----N-N											
No reaction	-----R-----R-----A-----R-----G											
63/67+	--PT--A--N---LK-LA-SS-G-G---T-P-----HI--NS--S---PP---P---SH-G-ACP--GPGA---H-P--M---A---N											
63/67+	--M-----R-----H-----H-----N-----A-----C-----R											
67+	--F-----A-----S-G---P---L---V---I--GS-----NH-----											
63+	--L-N---PK---S---R---GD-S---H-----E-PN--A-----P---N--											
63+	-----K-----D-----M-----											
10/57+	-----K-----D-----S-----N-----P---T---L---											
10/57/67+	-----K-----D-----S-----N-----P---T---L---											
10/R63/B69+	---L---K---AK---F-----N---H---H---H---P-AR-----K-----N											
	Hydrophilic peak A											
	140	150	160	170	180	190	200	210	220	230	240	
E/Del	ELTDVS YNGLMSATANINDKIGNVLVGEVTVLSLPTS YDLGYVRLGDPPIAIGLDPKMTATCDSSDRPRVYITIT <u>AADNYQFSSQYQTGGV</u> ITILFSANIDAITSLSVGG											
No reaction	R---A-HSWS---PHV---TWS-P-RGR-P-PN---P-HP-HA-P-GGM--MR-ELEIATARG-GGGHKAC--I---DCL-PLH-Q--AAT--SLPDFTT---GG--											
No reaction	---G---RV-----K---T---N---V---L---VW-----I-----S-H---S-I-----											
No reaction	-----A-----G-----A-----R-----Y-----A-----											
No reaction	-----R-----R-----Y-----A-----											
63/67+	-P-Y-GH---T--ST-I--D--I-----P-----PC--M--V-PE-ELE--G-G-G--C-TA-V-SC-S--KQ-AA--HV--GG--											
63/67+	-Q---F---V-E-S--A---G-----M-----IA---T-YH---A-----I-----											
63/67+	-----T-----T-----P-----GA-H-VA--D-KL---P-----T-----											
67+	K-NA---T--S---S---T-P-R-G---T--R---GA-H-VA--D-KL---P-----T-----											
63+	--AN-----LFT---GP---R---L-T--G---D-K---CKL---S---S---I--											
63+	-----P-----A-----											
10/57+	-----H---RA---R-E---L---S---D---PT---Q---											
10/57/67+	-----H---RA---R-E---L---S---D---PT---Q---											
10/R63/B69+	--KH---Y-----D---L-----RI--											
10/R63/B69+	-----P-----											
	Minor peak 1 Minor peak 2 Hydrophilic peak B											
	250	260	270	280	290	300	310	320	330	340	350	
E/Del	<u>ELVFKTSVQSL</u> VLGATIIYLIGFDGTAVITRAVAANN <u>GLTAGIDNLMFPN</u> ILNVPTNEITQPIITSIKLEI <u>VT</u> <u>TSKSDGQAGE</u> QMSWSASGSLAVTIHGGNYPGALRPVTL											
No reaction	--MSQ-NIHNPIMR--TH-M-LH-S-A--TS-V-ST--MT-TH-PVALDPAT-PSVTIR--VS--MA-RNNN--ESD-----D-PA--R--C-S-----											
No reaction	--E-R-G-----A-----S---V--D---F-----A-----F-----RG--DE-----T-TM-----V-----											
No reaction	-----I-----I-----IS--V-----T-----T-----P-----											
No reaction	-----T-----T-----V-----											
63/67+	-FEY--G-HN---PVC--S--R-----S--A--VG--T--YPE--S-T-R-V-P-IPGK-----L-----VAM---CS--P---											
63/67+	-----T-----S-----Y-----L-----E--T-----											
63/67+	-----N-----H-----											
67	-PMLQPN-HG-----M--G-ST-V---S--T-T--L--AV--S-----I-----RG-TTED---TR-----I--											
67	--L-R--RN-----T-----T---N-----TN-----G-----											
67	-----R-----T-----											
67	-----K-----I-----											
63+	---H---HG-A-N--T-----T--SI--SDY-P-T-T--A--I---SG-----I-G--R-NG-RTED-----R-G-T-----											

Fig 2.3 Cumulative amino acid exchanges in the analyzed VP2 encoding region of IBVDV.

Nucleotide sequences were in silico translated and subsequently grouped based on their antigenic subtype. Next the amino acid sequences were aligned using Clustal W and compared with the in silico translated nucleotide sequence of E/Del (Genbank accession number X54858). All observed amino acid exchanges shown in the single letter code were grouped based on the observed antigenic subtype. Amino acids which were not exchanged were marked by a dash. The hydrophilic regions [hydrophilic peak A and B (Azad et al, 1987), minor peak 1 and 2 (van den Berg et al, 1986)] were underlined and bold typed. The numbering of the amino acid sequences is shown in accordance to Mundt and Muller (1995).

	30	40	50	60	70	80	90	100	110	120	130	
E/Del	PASIPDDTLEKHTLRSETSTYNLTVDGDTGSLIVFFPGFPGSI VGAHYTLQSNNGNYKFDQMLLTQNLPA S YNYCRLVSRSLTVRSSTLPGGVYALNGTINAVTFQGSLS											
JF748899 (63/67, a1)	-----L-R-----S-----*-----S-----*-----*-----											
JF748900 (no R, a2)	-----P-K-----N-----P-----T-----A-----*-----*-----D-----G-----											
JF748922 (63/67, b1)	-----A-R-----S-----S-----G-----C-----M-----											
JF749122 (no R, b2)	-----A-R-----S-----S-----G-----C-----M-----											
JF749101 (no R, c1)	-----*-----D-----											
JF749103 (57/67, c2)	-----A-----*-----											
	Hydrophilic peak A											
	140	150	160	160	180	190	200	210	220	230	240	
E/Del	ELTDVSYNGLMSATANINDKIGNVLVGEVTVLSLPTSYDLGYVRLGDPDIPAIGLDPKMWATC DSSDRPRVYITITADNYQFSSQYQTGGVTITLFSANIDAITSLSVGG											
JF748899 (63/67, a1)	-----E-S-----M-V-----											
JF748900 (no R, a2)	-----*S-----A-----*V-----											
JF748922 (63/67, b1)	-----GH-----V-----A-----M-M-E-----G-C-----SC-----A-----											
JF749122 (no R, b2)	-----GK-----V-----A-----M-M-E-----G-C-----SC-----A-----											
JF749101 (no R, c1)	-----											
JF749103 (57/67, c2)	-----											
	Minor peak 1			Minor peak 2			Hydrophobic peak B					
	50	260	270	280	290	300	310	320	330	340	350	
E/Del	ELVFTSVQSLVLGATIVLIGFDGTAVITRAVAANGLTAGIDNLMPTNLTPIPTNEITQPIITSIKLEIVTSKSDGQAGEQMSWSASGSLAVTINGGNYPGALRPVTL											
JF748899 (63/67, a1)	-----HN-----S-----*											
JF748900 (no R, a2)	-----HN-----S-----MA-----M-----											
JF748922 (63/67, b1)	-----G-G-----V-----S-----											
JF749122 (no R, b2)	-----G-G-----V-----S-----											
JF749101 (no R, c1)	-----N-E-D-----											
JF749103 (57/67, c2)	-----N-E-D-----											

Fig. 2.4 Amino acid exchanges in the VP2 encoding region outside of the hydrophilic regions cause change of the antigenic subtype. VP2 encoding nucleotide sequences of IBDV isolates (see the genbank accession numbers) which were phylogenetic not significant different (a1, a2; b1, b2; c1, c2; see also Fig 2) were in silico translated and subsequently aligned using Clustal W. The amino acid sequences were compared with the in silico translated nucleotide sequence of E/Del (Genbank accession number X54858) and differences were shown in single letter code. Amino acids which were not exchanged were marked by a dash. The appropriate reactions were either positive for Mab's R63 and 67 (63/67) and for Mab's 57 and 67 (57/67) or negative for all MAB's analyzed (no R). The hydrophilic regions [hydrophilic peak A and B (Azad et al, 1987), minor peak 1 and 2 (van den Berg et al, 1986)] were underlined and bold typed. The numbering of the amino acid sequences is shown in accordance to Mundt and Muller (1995). Amino acids which were different between the phylogenetic not significant different nucleotide sequences were highlighted by an asterisk.

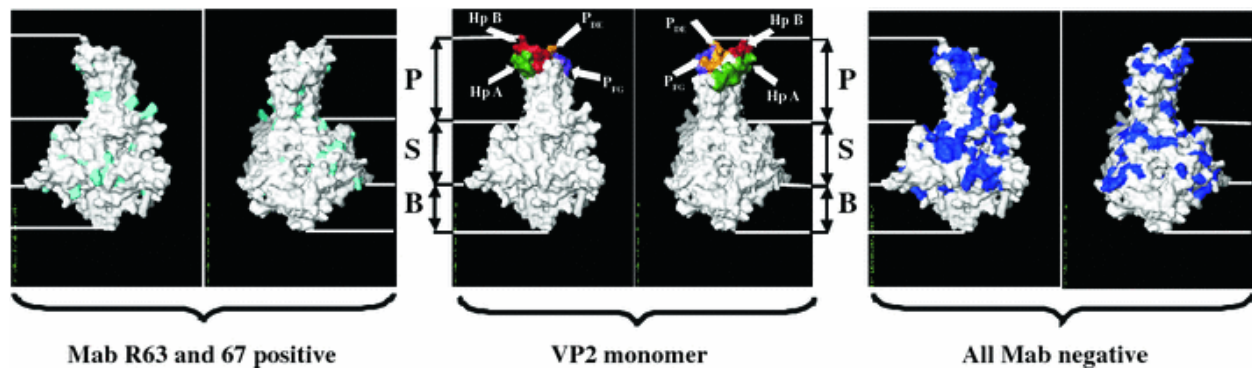


Fig. 2.5 Location of amino acid exchanges observed in comparison to the E/Del sequence. The monomer of the VP2 molecule was subdivided into the base (B), shell (S), and projection (P) domain (Coulibaly et al., 2005). The location of the hydrophilic peaks (Hp) A and B were highlighted as well as the loops in the projection domain PDE and PFG which essentially resemble the minor hydrophilic peaks localized in VP2 (Coulibaly et al., 2005). Amino acid exchanges which were only observed in the VP2 sequences of IBDV which were positive with Mab R63 and 67 were highlighted green while amino acid exchanges which were only observed in those sequences which did not react with any Mab were highlighted in blue.

CHAPTER-3

DEVELOPMENT OF MOLECULAR TOOL FOR ANTIGENIC CHARACTERIZATION OF
INFECTIOUS BURSAL DISEASE VIRUSES AT THE GLOBAL LEVEL

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Abstract:

Infectious bursal disease is a highly contagious and immunosuppressive disease in young chickens caused by infectious bursal disease virus (IBDV). IBDV is ubiquitous in poultry flocks worldwide and vaccination is used in an attempt to control the disease. Selection of efficacious vaccines is based on the antigenic subtype of circulating IBDVs and is critical in controlling the disease. The IBDV reverse genetics system was adapted for analysis of IBDV at the global level. FTA cards from different continents were used to safely transport IBDV genetic material to the laboratory. IBDV RNA was isolated and cDNA, encompassing the complete VP2 region responsible for the antigenicity of IBDV, was amplified using specific primers. The VP2 product was cloned into the IBDV reverse genetics system and the antigenicity determined by subsequent analysis of reactivity with a panel of monoclonal antibodies. Results revealed new antigenic subtypes were present in poultry flocks on different continents indicating a parallel antigenic evolution of IBDV. This system allows for global antigenic characterization of IBDV without importation of infectious virus

Keywords: Infectious bursal disease, antigenicity, FTA cards, reverse genetics, molecular techniques.

Introduction:

Infectious bursal disease (IBD) is an acute and contagious disease affecting chickens. IBD is worldwide in distribution and causes significant economic losses to the poultry industry (21, 43, 46). The disease was initially described as avian nephrosis by Cosgrove (1962), but was later designated as IBD due to morphologic and histologic changes observed in the bursa of Fabricius (BF, 31). Young chickens, 3-6 weeks of age, are most susceptible to clinical disease (16, 30, 50); however, 2-15 weeks-old chickens were also shown to be affected (49). IBD is caused by infectious bursal disease virus (IBDV) and belongs within the family *Birnaviridae* to the genus *Avibirnavirus* (19). IBDV targets B-lymphocytes (29, 35, 42) causing lymphoid depletion which leads to immunosuppression. Muller (1986) showed that immature, proliferating B-lymphocytes were the primary population of cells depleted following infection with IBDV. Consequently, this leads to suppression of the humoral immune response (2, 25, 26, 73, 76). There are two serotypes of IBDV, serotype 1 and 2, which can be differentiated by cross neutralization assays (53). Serotype 1 IBDVs are considered pathogenic (87) in chickens to varying degrees and several pathotypes have been described as variant IBDV (varIBDV), classical IBDV (cIBDV) and very virulent IBDV (vvIBDV) (10, 77, 91). Classical IBDV and vvIBDV are considered different pathotypes based on the outcome of clinical disease, (10, 91) but are antigenically related to each other (11, 68). In contrast, varIBDVs are antigenically and clinically different from known cIBDV and vvIBDV (72, 74). The two IBDV subtypes (varIBDV and vvIBDV) are likely descendents of the cIBDV since they are more related to each other than to serotype 2 IBDV. Serotype 2 IBDVs have been isolated from turkeys, fowl and duck (53) and shown to be apathogenic in chickens (34) and their origin is unknown.

IBDV particles range from 55 to 65 nm in diameter with icosahedral symmetry and contain a double-stranded RNA genome (43, 58) with two segments (A and B). Segment A encodes a polyprotein of approximately 110 kDa with 1,012 amino acids (aa) (32). This polyprotein is proteolytically cleaved into three polypeptides, the immature pVP2 (VPX) (48 kDa), VP3 (33kDa) and VP4 (29 kDa) (4), by its own viral protease VP4 (7). The pVP2 (512 aa) is further processed, through maturation, into the N-terminal encoded mature VP2 (441 aa) and four peptides named after their length (pep46, pep7a, pep7b pep11 (4, 18, 23). The mature VP2 is the only structural protein forming the capsid, contains determinants for cell tropism (9, 17, 64), and represents the only IBDV protein responsible for the induction of protective immunity (4, 6, 24). Interestingly, neutralizing epitopes characterized by the binding of monoclonal antibodies (MAb) were conformationally dependent (4, 6, 83). Some of these MAb recognized the epitopes in an antigen capture ELISA (cELISA) and indirect immunofluorescence (48, 84). A second open reading frame, partially overlapping the polyprotein gene (80) encodes the nonstructural and nonessential protein VP5 (62, 63). Segment B encodes for the viral RNA dependent RNA polymerase VP1 (81, 85).

Clinical signs alone cannot be used as a presumptive diagnostic tool for IBDV since varIBDVs do not cause clinical disease but are rather subclinical. Thus, laboratory tests are performed to confirm the presence of IBDV. Initially, IBDV was detected by agar gel precipitation test (AGPT) and also by virus neutralization test performed either in eggs or cell culture (86, 87). Using of a panel of MAb, a cELISA was developed to antigenically characterize IBDV using bursal homogenate (84). Advancements in molecular technologies has enabled the detection and amplification of the nucleic acid by RT-PCR, encoding for regions which form the neutralizing epitopes located in VP2. Restriction endonucleases based profiles resulted in the grouping of

IBDVs into the different subtypes as described above (36, 37, 38). Other molecular tests, such as quantitative RT-PCR (39, 44, 55), were developed for detection and classification of IBDV. The disadvantage was that these methods could not provide information for characterizing the antigenicity of viruses. The use of Flinders Technology Associates (FTA) cards for detection of IBDV from diagnostic samples using RT-PCR has been previously described (52, 56, 69). Furthermore, FTA cards have been used to detect various human diseases caused by bacteria such as leprosy (3) as well as in detecting viruses of animal diseases, e.g. IBDV (56), fowl adenovirus 9 (57), rabies virus (67), foot and mouth disease virus (65), Marek's disease virus (15), avian influenza virus (1, 45), porcine reproductive and respiratory syndrome virus (51) and protozoal diseases caused by *Blepharisma japonicum* (27), *Leishmania* (41) and fungal diseases like fungal keratitis (54). The advantage of FTA cards is that pathogens will become inactivated by the proprietary technology within the card and thus the nucleic acids can be easily collected and transported across borders without concerns or restrictions of importing infectious agents. The disadvantage is that only the analysis of the genetic information is possible. Due to the availability of modern technologies, such as reverse genetics, the genetic information can be translated into proteins within a transfected cell, and processed in a way that mimics its protein structure on the virus. Using reverse genetics as a diagnostic tool (20, 33) antigenic characterization of IBDV can be performed without importing infectious virus. In this report we described the establishment of a system for the analysis of IBDV antigenicity using reverse genetics on FTA cards submitted from different parts of the globe. This approach will enable us to understand more about the genetic background for antigenic differences of IBDV.

Materials and methods

Cells and FTA cards. Transfection experiments were performed in the chicken embryo fibroblast cell line, DF-1 (28). DF-1 cells were grown in Dulbecco's Modified Eagles's Medium with 4.5g/l glucose (DMEM-4.5, Thermo Scientific, Waltham, MA, USA) and supplemented with 10% fetal bovine serum (FBS, Mediatech, Manassas, VA, USA) in the presence of penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were grown at 37⁰C with 5% CO₂. FTA cards (Whatman, Newton, MA) used for this study were submitted as diagnostic cases to Poultry Diagnostic & Research Center, The University of Georgia (Athens, Georgia, USA).

Construction of recombinant segment A and B. Both segments of the D78 vaccine strain (Intervet, Millsboro, DE, USA) were amplified by reverse transcription polymerase chain reaction (RT-PCR) and a reverse genetics system for diagnostic purposes was recently established for both segments of the IBDV strain D78 (33). The cDNA for segment A was modified to obtain a plasmid with a unique Spe I cleavage site (pD78Ap-SpeI). This plasmid was previously used for diagnostic samples to determine the antigenicity of IBDV (20, 33)

Extraction of total RNA from FTA cards. FTA cards were submitted with BF impressions and used for RNA isolation. The spotted region on the FTA card was carefully incised with a sterile blade, so that at least of 2- 3 mm of the tissue impression was removed for RNA isolation. The incised portion of the FTA card was placed in a sterile 1.5 ml microcentrifuge tube and incubated with 0.5 ml digestion buffer [0.5% SDS, 1 mg/ml of proteinase K (New England Biolabs, Ipswich, MA) at 37⁰C for 1 hour. The sample of FTA card was removed and after a brief centrifugation (1 min at 16000 xg), the supernatant was purified using the High pure RNA isolation kit (Roche, Mannheim, Germany) as recommended by manufacturer's protocol.

Selection of primer pairs for amplification and analysis of RT-PCR products. The experiments were designed to obtain the necessary genetic information from the FTA cards. To this end, two primer pairs were used. The first primer pair was previously described (IBDV FP1 and IBDV RP4-SpeI, see 33). A second pair of primers was designed based on sequences from the NCBI database and sequences which were used for the previous characterization of several field samples (20). For analysis, sequences were selected which were described from several regions of the world to obtain as broad of coverage as possible for FTA card submissions. The location of the primers needed to fulfill several requirements: i) the nucleotide sequences for the primers need to be highly conserved ii) the amplified RT-PCR fragments are similar in size iii) the primer pair must overlap in a region where a unique restriction enzyme is located. These primers (IBDVFP1-FP, IBDVPciI-RP, see table 3.1) were used in three combinations: IBDVFP1/IBDVPciI-RP, IBDVPciI-FP/IBDV RP4-SpeI, IBDVFP1/IBDV RP4-SpeI. The combination of the first primer pair (IBDVFP1/IBDVPciI-RP) resulted in an RT-PCR fragment (FRAG1) of 734 basepairs (bp, nucleotide 104 – 838). While the second (IBDVPciI-FP/IBDV RP4-SpeI) and third primer pairs (IBDVFP1/IBDVRP4-SpeI) resulted in RT-PCR fragments of 669 bp (FRAG2, nucleotide 531 – 1200) and 1096 bp (Frag 12, 104-1200), respectively. The RT-PCR fragments overlapped in a region where the cleavage site for the restriction enzyme Pci I was located (nucleotide 716) and was necessary for the subsequent cloning strategy. Enumeration for the nucleotides followed the full length sequence for segment A, of IBDV strain P2, as described before (60). The location of the primers and respective RT-PCR fragments are depicted in figure 3.1.

Amplification and cloning of the RT-PCR fragments. The three primer pairs described above were used to amplify cDNA from RNA obtained from bursal samples, cell culture supernatants,

and FTA cards. The bursal samples and cell culture supernatants were processed and viral RNA was extracted from the FTA cards as previously described (20). The amplification and cloning for Frag 12 was performed as described earlier (20, 33). The cloning of Frag 1 and Frag 2 into pD78A-SpeI was performed in a two fragment approach. To this end, the two primer pairs (IBDVFP1/ IBDV-PciI-RP, IBDV-PciI-FP IBDV RP4-SpeI) were used in RT-PCR described previously (33) with one exception. The elongation step was shortened to 1 min. The reaction products obtained were separated on a 1% gel, the reaction product with the appropriate length was eluted from the gel using the QIAquick Gel Extraction Kit (Qiagen, USA). The Frag 1 was incubated with the restriction enzymes Rsr II and Pci I, while Frag 2 was incubated with the restriction enzymes Pci I and Spe I. The appropriate reaction products were gel eluted again and both fragments were ligated at once into the appropriately cleaved pD78A-SpeI. The ligation reaction was transformed into competent E.coli from the strain Top10 F (Invitrogen) and recombinant plasmids were selected by using the restriction enzymes Rsr II and Spe I. Recombinant plasmids selected were sequenced with the four primers used for the RT-PCR using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequence reactions were purified using Performa(R) DTR Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD, USA) and the sequence reactions were analyzed at Georgia Genomic Facility using the ABI PRISM 3730 GENETIC ANALYZER (Applied Biosystems) at the University of Georgia, Athens, GA.

Sensitivity assay for cell culture adapted IBDV and field IBDV along with their FTA card counterparts. To test the sensitivity, the cell culture adapted E/Del-like strain 8903 (Intervet, Millsboro, DE, USA) with a TCID₅₀ of $10^{8.75}$ /100 µl was used. The virus was propagated in DF1 cells as previously described (33). A wild type E/Del virus (Intervet, Millsboro, DE, USA) was

propagated in specific pathogen free (SPF) chickens (Merial, Gainsville, GA, USA). To this end, twenty, 21-day-old SPF chickens were inoculated with the E/Del virus with 10^2 EID₅₀. Three days after infection, the BF were removed and a tissue homogenate was prepared as recently described (20). The samples were centrifuged at 13000x g for 10 min at 4°C. The supernatant was filtered through a 450 nm filter (Millipore, Billerica, MA, USA) and 200 µl aliquots were stored at -80 °C. For the determination of the chicken infectious dose fifty (CID₅₀), five 21-day-old SPF chickens were orally infected with 100 µl of 10-fold virus dilutions up to a 10^{-8} fold dilution. The chickens were raised in Horsefall-Baur isolation units with HEPA filtered supply and exhaust air. Feed and water were supplied *ad libitum*. Seven days after infection, the birds were humanly euthanized and the BF was collected, fixed in 10% phosphate buffered saline (PBS) buffered formalin and processed for hematoxylin-eosin staining following standard procedures. During microscopical examination, the bursal sections were scored on a scale of 1-4, with a score of 1 based on no B lymphocyte depletion of the bursal follicles. Only a score of 1 was regarded as unaffected. Based on the examination, the CID₅₀ was determined as $10^{5.5}$ following the method of Reed and Muench (1938).

In the next experiments, the virus 8903 and E/Del was diluted tenfold up to a dilution factor of 10^{-9} . One hundred microliters from the undiluted and each of the dilutions were processed for RNA purification as described above. In parallel, the same amount was pipetted onto an FTA card which was incubated overnight at room temperature. The next day nucleic acids were extracted as described above and the appropriate RT-PCRs were performed using 5 µl of the eluate obtained.

Transcription, Transfection and analysis of antigenicity. The recombinant plasmids with the pD78A-SpeI backbone and pD78B-PD were linearized with BsrGI and PstI, respectively. The T7 RNA polymerase (Takara) reaction was performed individually for each segment as previously described by Mundt and Vakharia (1996). The plus-sense transcripts of segment A and B were co-transfected into DF1 cells, seeded in 48 well cell culture plates, using *TransIT®-mRNA Kit* (Mirus Bio LLC, Madison, WI, USA). Six wells were transfected with each transfection mixture. The transfected cells were fixed 16 hours after transfection with ice-cold 96% ethanol. An indirect immunofluorescence assay was performed with MAbs 10, 57, R63, B69, (78, 79), 67 (83), and rabbit VP1 antiserum (7) as described by Letzel et al., (2007). The binding of the primary antibodies were detected using species-specific fluorescein-isothiocyanate (FITC) - conjugated goat anti-mouse or goat anti-rabbit antibodies (Jackson ImmunoResearch, WestGrove, PA). The fluorescence was analyzed using an inverted Zeiss microscope Axiovert 40 CFL (Zeiss GmbH, Jena, Germany).

Analysis of nucleotide and amino acid sequences. The nucleotide sequences obtained were analyzed using DNASTAR Lasergene 8 (DNASTAR, Inc., Madison, WI, USA) software. Furthermore, partial sequences of VP2 were *in silico* translated using a translation tool available online (www.expasy.org/tools/dna.html). Nucleotide and amino acid sequence identity and divergence were calculated using Clustal W alignment (www.ebi.ac.uk/clustalw). The nucleotide query obtained from each FTA card was blasted against a nucleotide database using blastn (NCBI, Bethesda, MD). In addition, the amino acid sequences obtained were similarly evaluated against the protein database by blastp (NCBI, Bethesda, MD).

Results

Analysis of the sensitivity of the assay. In first experiments, the sensitivity of the assays was tested using cell culture supernatant of DF1 cells infected with the E/Del –like strain 8903 and with samples from bursal homogenates from birds infected with a non-tissue culture adapted E/Del strain (Table 3.2). Both viruses were quantified in their respective system. The results showed that the RT-PCR for Frag12 was able to detect the equivalent of 100 TCID₅₀ of IBDV strain 8903 while both fragments (Frag1 and Frag2) were amplified by RT-PCR when approximately one infectious virus particle was present in the analyzed volume. When taken into account that only 20 % of the resulting eluate following RNA purification was used for RT-PCR, only one fifth infectious virus particles was detected. This was independent of the starting material pipetted onto the FTA cards (cell culture supernatant or homogenized bursal samples). Using the bursal homogenate, viral RNA was detected by RT-PCR generating the longer fragment, Frag12, in samples containing 10² CID₅₀. Surprisingly, when the bursal material was added to the FTA card matrix, the sensitivity increased 100 fold. The sensitivity increased in the bursal samples tested directly, when the shorter fragments were amplified to the level of 1 CID₅₀/100 µl. A possible scenario for the results obtained during the course of the study is given in figure 3.1B. Frag 12, Frag1 and Frag 2 were always amplified when either cell culture supernatant or bursal homogenate was used for the RNA purification. In contrast, when using RNA purified from FTA cards sometimes the longer fragment, Frag 12, was not amplified, but Frag 1 and Frag 2 were always amplified from the same RNA. Since both cDNA fragments (Frag 1 and Frag2) were overlapping, the combined use of the appropriate restriction enzymes (see Materials and Methods and below) resulted in the generation of a cDNA fragment which was also obtained after use of the primer pair IBDVFP1/IBDVRP4-SpeI.

Analysis of the antigenicity of bursal samples outside and inside USA. The cDNA amplified from FTA cards was subject to an appropriate incubation with restriction endonucleases. The obtained fragment (Frag12) or fragments (Frag1, Frag 2) were ligated in the RsrII/SpeI cleaved pD78A-SpeI. The recombinant plasmids were then linearized followed by a T7 transcription reaction. The cRNAs obtained from the T7 transcription of segment A and B plasmids were co-transfected in DF1 cells. The antigenicity of the different IBDV VP2s was characterized based on their reactivity with a panel of Mabs 24 h after transfection by indirect immunofluorescence (Table 3.3). Samples originating from USA, Costa Rica, Peru, and The Netherlands were analyzed. The reactivity panel pattern using the MABs revealed different antigenic subtypes. The panel patterns were classified as previously described (20, 33). Samples obtained from USA, Costa Rica, and The Netherlands reacted with MAb R63 and MAb 67 which was regarded as the E/Del subtype. The panel pattern positive for MABs 10, R63, and B69 (classical IBDV/vvIBDV) was observed after analysis of samples from USA and The Netherlands. Interestingly, three of the six samples from The Netherlands reacted with the MABs R63, 67 and B69 which is the same pattern previously described for a Belgium isolate (Letzel et al., 2007). It was also observed that three samples reacted only with MABs 67 (USA, Peru) and one sample obtained from the USA reacted with two MABs specific for either GLS (MAb 57) or E/Del (MAb 67). One sample from the Netherlands reacted only with MABs R63. Interestingly, four samples did not react with any of the MABs and indicates an unknown antigenic subtype described previously. These samples were obtained from Peru and The Netherlands and indicated that these IBDVs are also present outside of the USA and suggests the evolution of IBDV is not directed by different conditions during husbandry (20, 33). Unfortunately, more information about the samples was not available.

Genomic analysis of the nucleotide and amino acid sequence. The nucleotide and *in silico* translated amino acid sequences were analyzed for their similarity using blastn and blastp, respectively. The analysis of the sequences revealed that eleven samples (GAEM 1851, GAEM 1871, GAEM 1901, GAEM 1902, GAEM 1921, GAEM 1932, GAEM 1961, GAEM 1962, GAEM 1964, GAEM 1972, GAEM 2021) correlated with the classification based on the MAb panel pattern observed. In contrast, ten samples showed a difference in the classification based on MAb panel pattern and that based on blastn/blastp analysis. Samples that showed a Belg-IBDV MAb panel pattern (GAEM 1941, GAEM 1944, GAEM 1945, 48) were classified as the classic subtype (blastn) or E/Del subtype (blastp) which indicates that the ancestor was a classical virus but antigenic change was directed to E/Del antigenicity. Other samples also diverged in their classification based on MAb panel pattern and sequence similarity (GAEM 1942, GAEM 1943, sample (GAEM 1943)). Most of the samples that did not react with any MAbs, were grouped with a group of varIBDVs and originated from South America (GAEM 1911, GAEM 1951, GAEM 1971, GAEM 2012). One sample obtained from The Netherlands (GAEM 1946) also did not react with any MAb, but was grouped with cIBDV (blastn) and varIBDV (blastp). Taken together, IBDVs which do not react with any of the MAbs used in the described assays are present outside of the USA and thus indicate antigenic changes of IBDV on a global scale. Furthermore, it was shown that samples can be analyzed by this method using RNA transported on FTA cards without importing infectious virus across country borders. Furthermore, it was shown that the classification of IBDV just based on similarities with known nucleotide or amino acid sequences are not sufficient anymore since IBDV antigenic subtypes became more diverse.

Discussion

Aside from good biosecurity practices, vaccination is a very important tool for the control of IBDV in chickens. Infection with IBDV can compromise the immune system of chickens and eventually increases the susceptibility of birds to other viral diseases (13, 22, 71, 90), and secondary bacterial infections (75, 89). Proper vaccination of breeder hens can provide passive protection for up to the first three weeks of age. At that time, chickens are most susceptible to the infection and thus selection of the appropriate vaccine and its use in a vaccination program is very important due to the antigenic differences between IBDV isolates. One of the causes of vaccination failure is the potential for variant field isolates to differ antigenically from the commercial vaccine strains used in the field. This is not an uncommon situation in the field and has supported production of autogenous IBDV vaccines for use as a tool in controlling the disease. Due to the worldwide increase of intense poultry production, changes in IBDV antigenicity may arise faster and thus the antigenic match of the vaccine virus to circulating field viruses might vary. The use of very sensitive molecular tools to detect and classify IBDV subtypes has been described (37, 47, 82, 88). IBDV grouping, based on patterns obtained following restriction enzyme (RE) digestion of a cDNA fragment within a region of VP2 (37) which is also known as variable region (5), has been used. Since this pattern is a result of short nucleotide sequences recognized by the RE, the exchange of different nucleotides could lead to mis-grouping of viruses that are considered the same antigenic subtype (48). Thus, grouping of IBDV based solely on nucleotide and amino acid sequences jeopardizes the actual antigenic classification of the virus as concluded previously (40). In addition, the VP2 fragment generated for the coding region might contain differences in nucleotide sequences, but translate into the same amino acid sequence. This statement was supported by the findings that location of an

IBDV sequence (nucleotide as well as amino acid) in a phylogenetic tree did not reliably correlate to the determined antigenic profile (20).

Due to the nature of IBDV being an RNA virus which does not possess a proof reading mechanism during viral RNA replication, the probability of antigenic changes in the face of neutralizing antibodies is likely. Also, the emergence of a different pathotype of IBDV might occur. Both events have been documented in the past at almost the same time as variant strains in the US (72, 74) and vvIBDV in Europe (8, 12) were detected in the mid-eighties. Surveillance for the detection of antigenic variants of IBDV plays an important role for the development of vaccines and for testing the efficacy of currently used vaccines. Also importation of antigenically different strains due to trade might play a role (14, 40). The detection of IBDV with a new antigenic makeup was described (20, 33, 48). Whether such IBDV strains become established needs to be evaluated. The use of antigen-capture ELISA based on monoclonal antibodies was used to characterize antigenicity of IBDV (66, 84). These assays are very efficient and can be used for screening purposes but are not commercially available. In addition the import of infectious viruses, which would be necessary for this kind of assay, is rather complicated due to appropriate restrictions aimed at preventing the transfer OIE list A infectious agents across country borders. The use of FTA card technology facilitates transport of material through a proprietary inactivation reagent embedded within the card. The directed amplification of the VP2 region, encoding for the neutralizing antibody epitopes, from RNA obtained from the FTA card and the subsequent cloning into the reverse genetics system of IBDV (61) allows analysis of IBDV antigenicity without transporting infectious virus across borders. The amplification of the 1100 bp fragment by RT-PCR is rather uncomplicated when the starting material is bursal tissue or cell culture supernatant. The challenge was to optimize this system to allow amplification,

cloning, and transfection of the VP2 cDNA fragment, encoding for neutralizing epitopes, from RNA extracted from FTA cards. The key to success was identifying a restriction enzyme which was highly conserved within IBDV sequences and located within both cDNA fragments (Frag 1 and Frag 2) amplified by RT-PCR and supporting an efficient ligation of the two fragments into the Rsr II/Spe I cleaved pD78A-SpeI. The reaction conditions for the found Pci I restriction enzyme were also highly compatible with the restriction enzymes Spe I and Rsr II. All of these components allowed an efficient ligation into the IBDV segment A backbone. The specificity of the RT-PCR was not tested since the experiments were designed to amplify and subsequently clone the cDNA fragment. In an unlikely event of a false positive amplification, the nucleotide sequence obtained would ultimately reveal this event. The sensitivity was sufficient since the amplification was possible from most cards (> 95 %) supporting the appropriate assumption in the field and reason for submitting the sample. It was not possible to determine if the remaining cards were falsely negative since the gold standard for IBDV is virus isolation and it was not possible to do this from the FTA card. As mentioned above, the beauty of the established system is that companies and labs around the globe that are not set up to do this kind of analysis can submit FTA samples taken from any source, e.g. cell culture supernatant, bursal samples, allantoic fluids, for the antigenic characterization of IBDV. Accurate antigenic characterization of new field isolates will facilitate the appropriate selection of IBDV vaccines, and, if/when needed, aid in vaccine development. In this study, the use of a reverse genetics system for antigenic characterization of IBDV was shown to be feasible using FTA cards from diagnostic case submissions. The results obtained by reverse genetics provided more precise antigenic characterization of IBDV than phylogenetic analysis of VP2 nucleotide and amino acid sequences. The use of FTA cards combined with the IBDV reverse genetics system facilitates the

transport of material between countries and can serve as a global standard for antigenic characterization of IBDV benefiting the poultry industry worldwide.

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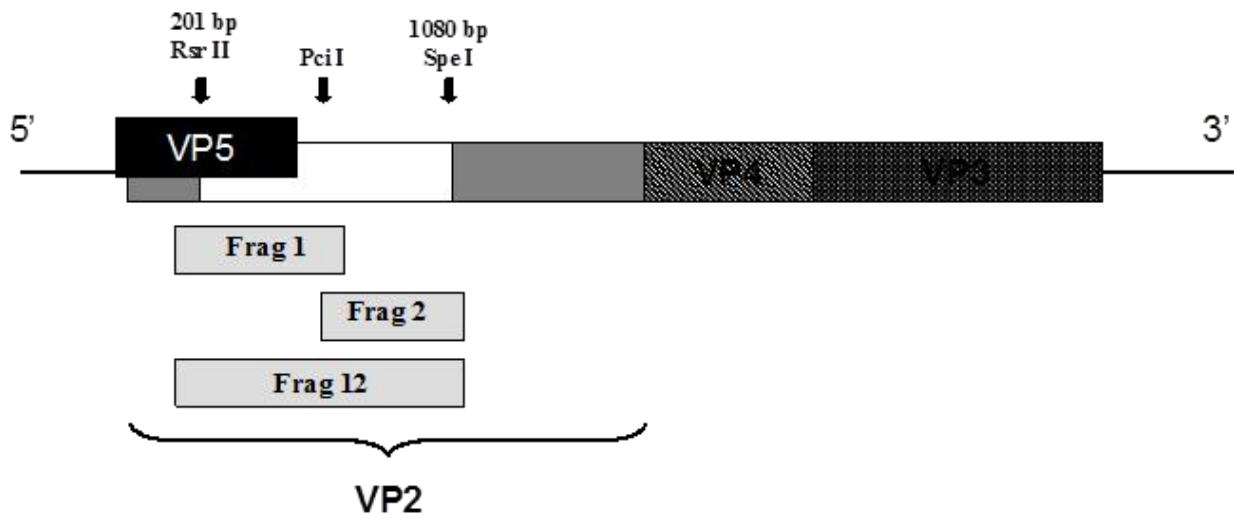
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A



B

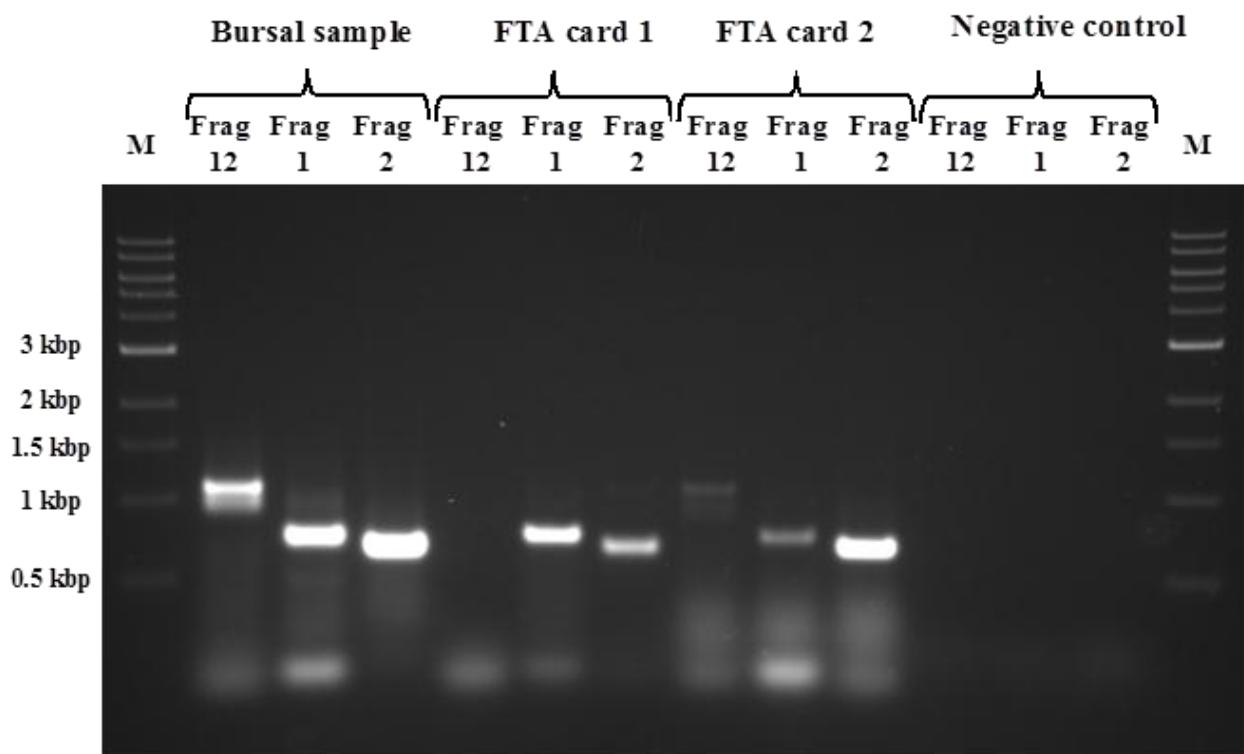


Figure 3.1: Schematic representation of the location of IBDV VP2-specific cDNA fragments and results of their amplification by RT-PCR. (A) Schematic depiction of the genomic organization of IBDV segment A. The location of the coding region for the single viral proteins (VP) and the 5' - and 3' noncoding region (NCR) were indicated. RT-PCR with the primer pairs IBDVFP1/IBDVRP4-SpeI, IBDVFP1/ IBDV-PciI-RP and IBDV-PciI-FP/IBDVRP4-SpeI resulted in cDNA fragments Frag12, Frag1, and Frag 2, respectively. (B) RT-PCR fragments after amplification and separation on a 1% agarose gel. Different possible scenarios were depicted. RT-PCR with RNA obtained directly from a bursal sample, from an FTA card 1 and another FTA card 2. Note that by using the FTA card only, Frag 1 and 2 were continuously amplified while Frag 12 was not always visible. A DNA ladder (M) was used for the size estimation of the amplified cDNA fragments.

Table 3.1: Oligonucleotides used for RT-PCR

Name	Primer Sequence	Orientation	Location ^a
IBDV-PciI-RP	GTGATGGCATCAATGTTGGC	antisense	819-838
IBDV-PciI-FP	GTGAACTGACAGATGTTAGC	sense	531-550

^a The positions where the primers bind (nucleotide number) are in accordance with the published sequence of strain P2 (Mundt and Muller, 1995).

Table 3.2. Sensitivity assay of RT-PCR for detecting viral RNA extracted from virus suspensions or FTA cards using IBDV strains 8903 and E/Del

Dilution factor	8903 ^a				E/Del			
	Frag 12		Frag 1 and Frag 2		Frag 12		Frag 1 and Frag 2	
	VCSN ^b	FTA	VCSN	FTA	VCSN	FTA	VCSN	FTA
10 ⁰	+ ^c	+	+	+	+	+	+	+
10 ⁻¹	+	+	+	+	+	+	+	+
10 ⁻²	+	+	+	+	+	+	+	+
10 ⁻³	+	+	+	+	+	+	+	+
10 ⁻⁴	+	+	+	+	+	+	+	+
10 ⁻⁵	+	+	+	+	-	+	+	+
10 ⁻⁶	+	+	+	+	-	+	+	+
10 ⁻⁷	-	-	+	+	-	-	-	-
10 ⁻⁸	-	-	+	+	-	-	-	-
10 ⁻⁹	-	-	-	-	-	-	-	-

^a The titer of the virus suspensions used in this assay for 8903 virus was 10^{8.75}

TCID₅₀/100 µl and for the E/Del virus was

10^{5.5} CID₅₀/100 µl.

^b virus containing supernatant

^c positive: +, negative: -

Table 3.3. Antigenic characterization of FTA cards based on their reactivity with a panel of monoclonal antibodies

Lab-No	Country	MAb ^a					Antigenic Subtype	Blastn ^b			Blastp ^b		
		10	57	R63	67	B69		Genbank Acc No	Country	Antigenic Subtype	Genbank Acc No	Country	Antigenic Subtype
GAEM 1851	USA	^c	-	-	+	-	Variant	M64285.1	USA	var A	AEJ08497.1	USA	variant
GAEM 1871	USA	+	-	+	-	+	Classical	DQ355820.1	Vietnam	classic	ABC86602.1	Vietnam	classic
GAEM 1901	Costa Rica	-	-	+	+	-	E/Del	JF749092.1	USA	E/Del	AEJ08354.1	USA	E/Del
GAEM 1902	Costa Rica	-	-	+	+	-	E/Del	JF749092.1	USA	E/Del	AEJ08354.1	USA	E/Del
GAEM 1911	Peru	-	-	-	-	-	Unknown	M64285.1	USA	var A	AEJ08497.1	USA	variant
GAEM 1921	USA	-	-	-	+	-	Variant	M64285.1	USA	Var A	AEJ08453.1	USA	variant
GAEM 1932	USA	-	+	-	+	-	Variant	JF749092.1	USA	E/Del	AEJ08253.1	USA	variant
GAEM 1941	Netherlands	-	-	+	+	+	Belg IBDV	DQ187988.1	Brazil	Classic	AEJ08423.1	USA	E/Del
GAEM 1942	Netherlands	-	-	+	-	-	Classical	DQ187988.1	Brazil	Classic	AEJ08423.1	USA	E/Del
GAEM 1943	Netherlands	-	-	+	+	-	E/Del	DQ187988.1	Brazil	Classic	AEJ08423.1	USA	E/Del
GAEM 1944	Netherlands	-	-	+	+	+	Belg IBDV	DQ187988.1	Brazil	Classic	AEJ08423.1	USA	E/Del
GAEM 1945	Netherlands	-	-	+	+	+	Belg IBDV	DQ187988.1	Brazil	Classic	AEJ08423.1	USA	E/Del
GAEM 1946	Netherlands	-	-	-	-	-	Unknown	DQ187988.1	Brazil	Classic	AEJ08423.1	USA	E/Del
GAEM 1951	Peru	-	-	-	-	-	Unknown	M64285.1	USA	Var A	AEJ08497.1	USA	Variant
GAEM 1961	USA	+	-	+	-	-	Classical	EU162087.1	USA	Classic	ABA54270.1	China	Classic
GAEM 1962	USA	-	-	+	+	-	E/Del	JF749118.1	USA	Variant	AEJ08423.1	USA	E/Del
GAEM 1964	USA	+	-	+	-	+	Classical	EU162087.1	USA	Classic	ABA54270.1	China	Classic
GAEM 1971	Peru	-	-	-	-	-	Unknown	M64285.1	USA	Var A	AEJ08497.1	USA	Variant
GAEM 1972	Peru	-	-	-	+	-	Variant	M64285.1	USA	Var A	AEJ08497.1	USA	Variant
GAEM 2012	Colombia	-	-	-	-	-	Unknown	X54858.1	USA	E/Del	AEJ08497.1	USA	Variant
GAEM 2021	Colombia	+	-	+	-	+	Classical	EU162087.1	USA	Classic	ABA54270.1	China	Classic

^a Neutralizing monoclonal antibodies (Mabs 10, 57, R63, 67 and B69) used for indirect immunofluorescence

^b method used for comparison in the NCBI database

^c positive (+) and negative (-) in the indirect immunofluorescence after transfection in cell culture

CHAPTER 4

DEVELOPMENT OF A NOVEL *IN VIVO* EXPERIMENTAL MODEL TO DIFFERENTIATE ANTIGENIC VARIATIONS AMONG INFECTIOUS BURSAL DISEASE VIRUSES

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Abstract:

Infectious bursal disease virus (IBDV) is a double stranded RNA virus causing infectious bursal disease (IBD) in chickens. IBDV targets proliferating B-lymphocytes in the bursa of Fabricius (BF). IBDV undergoes antigenic drift likely due to selection based on existing immunity in the field. Thus, characterizing the antigenicity of IBDV plays an important role in selecting the right vaccine candidates. In this study, we developed an *in vivo* experimental model to differentiate antigenic variations in IBDV based on the antigenically predominant vaccine strain in the USA, the E/Del strain. Our objective was to determine the degree of antigenic similarity of a field isolate to the E/Del strain. First, hyper-immune serum to E/Del-type virus was generated in SPF chickens. Next, a standard volume of the hyper-immune serum was serially diluted and injected in SPF birds via intravenous, subcutaneous, and intramuscular routes. The chickens were bled at different time points in order to evaluate the dynamics of virus neutralization titers (VNT). Based on the results, chickens were injected with different serum dilutions by the subcutaneous route. Twenty four hours later, chickens were bled and then challenged with 100 CID₅₀ of the E/Del virus and a new variant field isolate. A VN assay was conducted to determine the VNT. Chickens were necropsied 7 d.p.i. to remove the BF and histopathological analysis was conducted to determine the bursal lesion score (BLS) which was used to determine the breakthrough titer in the *in vivo* chicken model. The advantage of this system was that the breakthrough titer value was determined based on the lesions in the primary target of IBDV, the BF. This model displays the most sensitive model for virus infection with non-tissue culture adapted IBDV.

Key words: IBDV, antigenic variations, *in vivo* experiment, bursal lesion score, virus neutralization assay.

Introduction:

Infectious bursal disease (IBD) is an immunosuppressive disease of young chickens and is a threat to the poultry industry worldwide (1, 14, 15, 22, 53, 58). The causative agent, infectious bursal disease virus (IBDV), affects the bursa of Fabricius (BF) due to a lytic infection of proliferating lymphocytes of the B-cell lineage (18, 29, 35, 46). The disease was first described by Cosgrove (1962) as avian nephrosis and later recognized as IBD due to the pathologic/histologic changes in the (BF) (16, 23). Chickens between 3-6 weeks of age are highly susceptible for clinical IBD (8, 22, 40) when protective levels of neutralizing antibody levels are absent. IBDV is a double stranded, nonenveloped, icosahedral virus (9) with diameter ranging from 55 – 65 nm (19, 49). IBDV belongs to the family *Birnaviridae*, genus *Avibirnaviridae*. IBDV is a bisegmented virus with two segments, A and B (10, 36, 45). Segment A encodes two open reading frames (ORF) encoding the viral proteins. ORF 1 encodes for the viral polyprotein which is autoproteolytically cleaved by its viral protease to initially three viral proteins (VP), the premature pVP2, VP4 and VP3 (4, 38). A second ORF (2, 65) precedes and partially overlaps the first ORF and encodes for the viral protein VP5 (47), essential for virus replication in cell culture (48), but attenuated IBDV once the expression was omitted (74). Segment B encodes for the VP1 which represents the viral RNA dependent RNA polymerase (66, 69). There are two serotypes of IBDV, serotype 1 and 2. Serotype 1 is pathogenic to chickens while Serotype 2, isolated from turkeys, ducks and fowl (42), is apathogenic in chickens (27) and turkeys (34). Both serotypes were differentiated by cross neutralization assays (42) and ELISA (50).

Based on pathogenicity, IBDV broadly d into three are classical, very virulent (vv),and variant strains, resulting in three pathotypes of disease. Classical IBDV strains cause a strong

inflammatory response in the (BF) and chickens infected with virulent classical IBDV show clinical signs (8, 35). In contrast, variant IBDV strains result in bursal atrophy in the absence of any inflammatory changes (55, 60). The subclinical disease caused by variant IBDVs may barely be noticed and sometimes only an increase in the incident of respiratory diseases might be observed (55, 59). More dramatic are vvIBDV which cause a strong and fast depletion of the B lymphocytes the BF which is also associated with petechial hemorrhage in the muscles and a hemorrhagic BF (3). IBDV strains described in the early 1960s from United States of Americas (US) belong to the classical subtype. IBDV strains isolated in the US in the mid 1980s were described as variant strains due to change in their pathogenic phenotype and later determined to be antigenically different from earlier viruses (57, 59, 61, 62, 63). The vvIBDVs were first described in European countries (5) and were later found in almost all countries, except Australia and New Zealand. All three pathotypes of IBDV induce immunosuppression leading to an increase in the opportunity for secondary pathogens to evade the host and cause multisystemic diseases (6, 13, 44, 51, 54, 75). There are several variant strains of IBDV, such as Md (59), E/Del (57), variant A (56), IN (28), GLS, DS 326 (64) and AL2 (67), circulating in U.S. commercial poultry operations. Based on the variability of IBDV strains in the field, immunization with vaccines which are antigenically similar to the circulating IBDV strains plays a key role in IBDV control. Breeder hens are commonly vaccinated with a series of live and killed vaccines in an effort to induce antibodies which are transferred to the offspring via the eggs (71, 72, 73). Vaccination programs differ between poultry operations and are highly dependent upon the virus challenge circulating in the field.

Identification of circulating field strains of IBDV plays an important role in prevention and control. Field surveillance studies are necessary in the poultry flocks within commercial

operations to select the most appropriate vaccines for use in vaccination programs as well as to identify possible vaccine candidates (11). Various diagnostic tools were developed for identification of IBDV in poultry flocks. Initially, isolation and identification of IBDV by electron microscopy was used to confirm the presence of the virus (19, 21). Later, diagnosis of IBDV was based on the agar gel diffusion test (20) and also by virus neutralization assays (70). Advanced molecular methods, such as RT-PCR, supported rapid diagnosis of IBDV (7, 37, 43). RT-PCR followed by RFLP was used for genotyping IBDV (30, 31, 32, 41) and intended to diagnose IBDV based on comparison of RFLP patterns from unknown IBDVs to patterns of known viruses. However, these methods did not provide the most important information for determining the antigenicity of the viruses. Antigenic characterization of the IBDVs was necessary to determine whether changes in field viruses would result in a diminished protection from current vaccines. For this purpose, an antigen-capture ELISA was developed that provided a system for differentiation between different antigenic subtypes of IBDV based on a certain reaction patterns using a panel of neutralizing monoclonal antibodies (MAb, 68). In depth analysis of amino acid exchanges causing differences in the antigenicity of IBDV lead to the conclusion that IBDV antigenicity is more complex than expected (12, 26, 39). It also became clear that amino acid exchanges outside of the projection domain might result in antigenic differences (12). Using this approach several IBDVs, lacking reactivity with any neutralizing MAb and indicating a different antigenic makeup (12, 26) were detected and isolated (IBDVn-Var). Attempts to characterize the viruses in cross neutralization assays in embryonated eggs were subjective since the viruses were not lethal in embryos, as observed with some IBDV strains, and embryo lesions were variable. Thus we developed an *in vivo* model to evaluate the antigenicity of IBDVn-var strains. Since E/Del-like IBDV are the most common variant strains

circulating in the US, a system based on E/Del neutralizing antibodies was developed with a read-out system focused on lesions in the BF. Using this system, we were able to demonstrate that one virus field isolate was antigenically different from E/Del IBDV. The *in vivo* experimental model described will serve as a defined platform for biological evaluation of IBDV field isolates, lacking an antigenic signature in our *in vitro* characterization model, with known vaccine strains.

Materials and methods

Cells. The virus neutralization assay was performed in the DF-1 chicken embryo fibroblast cell line (17) grown in Dulbecco's Modified Eagle Medium with 4.5g/l glucose (DMEM-4.5, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Mediatech, Manassas, VA, USA), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were incubated at 37°C with 5% CO₂.

Virus propagation in SPF chickens. The viruses used in this experiment were E/Del (kindly provided by Ruud Hein Intervet, Millsboro, DE) and one IBDV field isolate from Alabama (IBDVn-var, Genbank accession number JF748992) previously described (12). For virus propagation, three-week-old SPF chickens (Merial, Gainesville, GA) were orally infected with E/Del and IBDVn-var in separate Horsfall-Bauer-type isolation units in a forced air positive pressure system (Poultry Diagnostic and Research Center, The University of Georgia). The SPF chickens were maintained in isolators and given feed and water *ad libitum* for the duration of the study. All chicken experiments were approved by the University of Georgia animal care and use committee (AUP number A2010 04-064-Y2-A1). The chickens were humanely euthanized and necropsied 96 hours after infection. The BF's were harvested and homogenized in viral transport

medium as described by Durairaj et al (2011). The virus stocks were aliquoted and stored at -80°C until further use.

Virus Titrations. Both viruses were titrated in nine- day-old embryonated SPF eggs (Merial, Gainesville, GA) and three-week-old SPF chickens to determine the embryo infectious dose₅₀ (EID₅₀) and chicken infectious dose₅₀ (CID₅₀), respectively. The determination of the EID₅₀ was performed by inoculating 100 µl per dilution into five embryonated eggs via the chorio allantoic membrane route (CAM) (24). The inoculated embryonated eggs were maintained in an incubator at 37°C with 55% relative humidity and candled daily to check for viability. Dead embryos were removed and stored at 4°C until seven days after inoculation when the remaining embryonated eggs were evaluated for the presence of lesions characteristic of IBDV (retarded growth, green-spotted liver, enlarged spleen). For the determination of the CID₅₀, the SPF chickens were divided into groups of five chickens each. Chickens were bled prior to inoculation and the sera were tested by virus neutralization assay to confirm absence of pre-existing IBDV antibodies. Seven groups per virus were infected by the intra-ocular route with serially diluted viral inoculum of E/Del or IBDVn-var. One group was not inoculated and served as negative controls. The chickens were maintained in Horsfall-Baur-type isolation units and given feed and water *ad libitum*. Seven days after infection, the chickens were humanely euthanized, and BF's were collected and preserved in 10% neutral buffered formalin for microscopic evaluation. The lesions in the BF were scored based on the degree of B lymphocyte depletion using a bursal lesion score (BLS) from 1-4, where a score of 1 = up to 10% of the bursal follicles show depletion, score of 2 = 10%-30% of the bursal follicles show depletion, score of 3 = 31%-70% of the bursal follicles show depletion and a score of 4 = > 70%-of the

bursal follicles show depletion. Any BLS greater than 1 was regarded as positive. Calculations of the titers were performed according to the method of Reed and Muench (1938).

Generation of hyperimmune serum in chickens. Three-week-old SPF chickens were orally infected with 100 μ l of the E/Del-like virus, 8903 (kindly provided by Ruud Hein, Intervet, Millsboro, DE), with a titer of $10^{1.5}$ TCID₅₀. Three weeks later, 100 μ l of the E/Del-like 8903 strain ($10^{2.0}$ TCID₅₀) was administered by the intramuscular route. Three weeks later, chickens were intramuscularly injected with an oil-emulsion vaccine containing one volume part beta – propiolactone inactivated 8903 virus containing 10^7 TCID₅₀/ml prior to inactivation and one part incomplete Freund's adjuvant. The inactivated virus was passaged twice in DF 1 cells to confirm successful inactivation. Five weeks later, chickens were exsanguinated under aseptic conditions. The clotted blood samples were centrifuged by 700x g at 4°C, and serum individually harvested and heat inactivated at 56°C for 30 minutes. The virus neutralizing antibody titer (VN-titer) was determined (see below) and serum samples with a VN-titer $> 2^{13}$ were pooled, aliquoted and final VN-titer was determined for the pool. The serum samples were stored at -80°C.

Determination of virus neutralizing antibody titer. The virus neutralization (VN) assay was performed to determine levels of E/Del-specific neutralizing antibodies in the chicken sera obtained. First, the TCID₅₀ for the E/Del like strain 8903 was determined following standard methods. For the VN test, 50 μ l of FBS-free 4.5 DMEM was added to all wells of a 96-well tissue culture plate. Next 50 μ l of the serum was added to the first column (2^{-1}) and serially two-fold diluted through the twelfth column of the plate (2^{-12}). In case the VN titer was greater than 2^{12} a second plate was used to continue the dilutions. Next, 50 μ l of virus containing 100

TCID₅₀ was added to each serum dilution and incubated for 1 h at 37°C. During that time, DF1 cells were trypsinized, resuspended in 10% FBS-containing 4.5-DMEM and adjusted to a cell density of 5×10^5 cells/ml. One hundred microliters of DF1 cells were added to all wells of the virus-serum-suspension and incubated for 5 d at 37°C and 5% CO₂. After incubation, cells were scored for the presence of a CPE. The end-point of the VN test for a serum sample was determined to be the reciprocal of the highest dilution, expressed in log₂, in which there was no visible CPE. During each test, the diluted virus was back titrated to determine the true TCID₅₀. If the TCID₅₀ was greater than a 0.25 log₁₀ difference from 100 TCID₅₀, the VN test was regarded as invalid and repeated.

Administration of hyperimmune serum. Two three-week-old SPF chickens per group were used for this study. Three hundred microliters of the hyperimmune serum was administered to each chicken via either the intramuscular (IM), intravenous (IV) or subcutaneous (SC) routes and two chickens were used as negative control. One chicken from each group was kept in a Horsefall-Baur unit where water and feed was provided *ad libitum*. Chickens were bled at 24, 48, 72 and 168 h post injection via the brachial vein on the side opposite of the inoculation. The clotted blood samples were centrifuged at 700x g for 5 min and the serum was harvested. The serum samples were heat inactivated at 56°C for 30 minutes and the VN-test was performed as described above to study the dynamics of VN antibody titer.

Differentiation of IBDV strains in vivo. Three-week-old SPF chickens were divided into 21 groups of 5 chickens per group. Each chicken was wing banded for identification. Serum was diluted up to a dilution of 1:128. Ten groups were used for each virus during the experiments; Group 1 (serum undiluted), group 2 (1:2 diluted), group 3 (1:4 diluted), group 4 (1:6 diluted),

group 5 (1:8 diluted), group 6 (1:16 diluted), group 7 (1:32 diluted), group 8 (1:64 diluted), group 9 (1:128 diluted), group 10 (phosphate buffered saline). Twenty four hours after injection, the chickens were bled via the brachial vein on the opposite wing and blood samples were processed for the VN-test as described above. One hour later, one group from each serum dilution level was challenged with either 100 CID₅₀ of E/Del or 100 CID₅₀ IBDVn-var. One group of SPF chickens did not receive serum and was left unchallenged. Seven days after challenge, chickens were humanely euthanized and BF was collected for microscopic evaluation to determine the BLS.

Histopathology. Bursal tissues were collected at the time of necropsy on an individual basis to allow the matching of each serum sample to the appropriate chicken. The bursal sample was placed in 10% neutral buffered formalin, and paraffin embedded. Sections of the paraffin embedded BF were hematoxylin and eosin stained following standard histologic procedures. The stained sections were microscopically examined for the presence of bursal lesions. Based on the depletion of the B lymphocytes, the BLS was determined for each BF based on the scoring system described above.

Results

Determination of viral titers. Initially, cross neutralizations assays in SPF embryos inoculated via the CAM were performed to determine the antigenic relatedness of IBDVn-var with E/Del. However, IBDVn-var was not embryo lethal and identification of embryo lesions was minimal and somewhat subjective. Although results from the initial cross neutralization studies in embryos indicated IBDVn-var was antigenically different from E/Del, additional testing was needed to provide more definitive and reliable data. Thus the determination of the CID₅₀ was undertaken for determining the titer of the viruses. The read out system was the

presence of lesions in the BF, thus providing the most sensitive system for determining infection in each dilution. The infectious titer was determined using material generated from the BF. This would provide a standard and comparable system for evaluating infection at later timepoints, during titration and challenge. Although the EID₅₀ was not used during the study, the calculated titers for the E/Del bursa material was $10^{4.1}/100\ \mu\text{l}$ for EID₅₀ and $10^{4.5}/100\ \mu\text{l}$ for CID₅₀. Interestingly, titration of IBDVn-var resulted in a titer of $10^{3.5}/100\ \mu\text{l}$ (EID₅₀) and $10^{4.5}/100\ \mu\text{l}$ (CID₅₀). Thus if the calculation for the infectious dose would have been based on 100 EID₅₀, the infectivity for the BF would have been underestimated by a factor of 10 for IBDVn-var and a higher dose would have been used for infection in comparison to the E/Del virus.

Generation and administration of hyperimmune serum. The final VN titer of the pooled hyperimmune serum from chickens vaccinated with the E/Del like strain 8903 as described above was 2^{14} . This serum was used for all subsequent experiments. An important experiment was performed to determine the most appropriate route of serum administration. Three routes of application (IM, SC, IV) were tested for their practicality and reliability. From the user's point of view, the easiest route was the intramuscular application. The subcutaneous route of application had the same practicality and reliability. The intravenous route was not easy to perform and failed several times during experiments (data not shown). Based on the VN titers measured at several time points after serum administration, it was observed that following intramuscular application, the VN titer decreased relatively fast (Table 4.1). As expected, the VN titer was highest after IV application in comparison to both IM and SC application. The SC administration resulted in a higher titer than the IM, but lower than the IV route of administration, however, the decline of the SC VN titer was comparable to the IV application. After taking into consideration

all aspects of the serum administration, the SC route was chosen since it was easy to perform, reliable and resulted in VN titers which lasted for a relatively long period of time.

Comparison of E/Del and IBDVn-var in the in vivo model. The experiments were performed using three-week-old SPF chickens. The chickens were SC injected with E/Del specific antiserum at different dilutions. The VN titer prior to dilution was 2^{14} when 100 TCID₅₀ of the E/Del like strains 8903 was used for the VN test. In a separate experiment, the identity of the tissue culture adapted 8903 was confirmed by indirect immunofluorescence using the panel of monoclonal antibodies (10, 57, R63, 67, B69) which resulted in a positive reaction with only R63 and 67 as previously described (26). This confirmed the E/Del like antigenic subtype. Twenty four hours later the chickens were bled and the VN titers were determined using the E/Del like strain 8903 (Table 4.2). As expected the VN titers declined in parallel with the dilution of the serum. Furthermore the VN titers ranged from one titer (group 9) to five different titers (group 5) and indicated variable uptake of the antibodies administered into the bloodstream. The highest VN titer observed was 1024 in three chickens injected with either the undiluted or 1:2 diluted serum. The control chickens were free of any IBDV strain 8903 neutralizing antibodies. The result of the challenge infection with IBDV strain E/Del and the IBDVn-Var was evaluated using the BLS at day seven following challenge. In the group of the chickens infected with only virus and no serum, a BLS of 4 was detected in every chicken indicating a sufficient infectious dose of virus. Chickens that did not receive serum or virus, had a BLS of 1. Chickens that received serum preparations and were challenged were regarded as protected if the BLS was between 1 and 2. The results showed that E/Del challenged chickens were fully protected up to a VN titer of 128 (see Table 4.3). A few chickens were protected when

the VN titer was ≤ 64 . Even at a VN titer of 8, two out of six chickens were fully protected as indicated by a BLS of 1.

Results from the challenge infection with IBDVn-var were different. Only chickens with a VN titer of ≥ 512 were fully protected. Fifty - 60% percent (VN titers of 256 and 128 respectively) of birds were protected from challenge infection which indicated that IBDVn-var was able to break through VN titers at levels where chickens were still fully protected when challenged with the homologue E/Del strain. The data clearly showed that only one chicken out of 30 was protected from IBDVn-var challenge at VN titer levels of ≤ 32 while six out of 30 chickens were still protected at these titers after challenge with E/Del.

Discussion

IBDV is ubiquitous in poultry operations worldwide. Mutations in the viral progeny can arise during replication as the viral replicase likely lacks a proof reading mechanism, responsible for repair of misincorporated nucleotides. In addition, changes in the antigenic makeup can occur due to the presence of neutralizing antibodies and may result in IBDVs that differ from vaccines in either their antigenicity or virulence or both. The results of this selection process are not predictable. Consequently this requires the continuous characterization of field isolates for their pathogenic potential and antigenic makeup. The development of neutralizing MAb's which were used to classify different IBDV antigenic subtypes (61, 62, 63, 64) and their subsequent use in an antigen capture ELISA was an important step for antigenic characterization of field isolates (68) and served as an effective surveillance tool. More rapid techniques were developed and the use of RT-PCR followed by restriction enzyme digestion (RFLP) of the amplified cDNA fragment (31) became mainstream for typing IBDV. Using this approach it was possible to distinguish between different IBDV subtypes, but did not provide essential information regarding the

virulence or antigenicity of viruses (30, 33). In some cases, the data generated by RT-PCR/RFLP led to the incorrect designation of IBDV subtype. One example was the detection of two IBDV isolates which were grouped into the vvIBDV group based on RFLP. Surprisingly, one isolate caused 70-80% mortality, typical for vvIBDV, while the other isolate caused only 10% mortality (25).

Various antigenic strains of IBDV have been described in the US based on their reactivity with MAbs: E/Del like IBDV reacted with MAbs R63 and 67, GLS-like IBDV reacted with MAbs 10 and 57, and classical IBDV reacted with MAbs 10, R63, and B69 (61, 62, 63, 64). In addition, an IBDV isolated in Belgium reacted with MAbs 10, R63, 67, and B69, a combination previously not described (39). Furthermore, a new group of viruses were described that did not react with any of the MAbs using a diagnostic reverse genetics system for IBDV (12, 26). It is important to keep in mind that virus isolates represent only a snap shot of IBDVs evolving in the field. Based on an evolutionary advantage, they might become established in an environment or in the case of an evolutionary disadvantage they may go unnoticed. Thus, surveillance is a necessary tool for monitoring IBDV evolution in the field. The recently described IBDVs that did not react with any of MAbs have also been identified in South America and Europe (data not shown, paper in preparation). Consequently these IBDV isolates need to be characterized either *in vitro* or *in vivo*. One virus isolate from Alabama, belonging to the group of IBDVs that do not react with any of the MAbs, was evaluated *in vitro* and *in vivo* (12). Virus titrations in SPF chicken embryos repeatedly resulted in no embryo mortality and minimal embryo lesions that were inconsistent between virus dilutions, making it difficult to quantitate the virus *in vitro* (data not shown). This particular virus appeared to be less embryo adapted than observed for other IBDV field isolates. Ultimately the virus was titrated in chickens and provided the read out system for

subsequent *in vivo* virus neutralization assays. The use of susceptible IBDV antibody-free chickens represents the most sensitive and reliable system for detection and quantification of IBDV. In the *in vivo* model described, the administration of IBDV hyperimmune serum using the SC route was reproducible and IBDV antibody titers detected in the serum were comparable to those obtained following intravenous administration. The results showed that independent of the serum dilution, IBDV antibody titers were present in a gradient in the chicken serum. The VN titer levels were not always in agreement with the dilution of the serum used in a particular group. This may be a result in differences in the uptake of IBDV antibodies into the blood stream. Due to this finding, the data was arranged by chicken and corresponding VN titer followed by the addition of BLS score. The results obtained showed that the E/Del was neutralized, as expected, at E/Del-specific VN titer levels, whereas IBDVn-var was able to break through even higher VN titers and induced bursal lesions. These results confirmed the assumption that a virus lacking reactivity with any of the neutralizing MAb in the *in vitro* assay was also antigenically different from E/Del since the same IBDV titers for infection were used. This finding does not show that IBDVn-var was also different from classical as well as GLS-like IBDVs, but allows the assumption. Hyperimmune sera specific for classical IBDV (e.g. D78) or GLS-like IBDV (GLS-05) are being prepared and will be used similarly in this *in vivo* system. In summary, an *in vivo* experimental model was developed that can be used for the biological evaluation of IBDV field isolates in unvaccinated susceptible chickens. The use of mono-specific anti-IBDV sera for passive immunization will allow the determination of the break through titers between IBDV isolates in a titer-dependent manner. The *in vivo* experimental model is a highly sensitive method for evaluating IBDV infections since the results are based on lesions in the BF which represents the target organ for IBDV.

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Table 4.1. Virus neutralization titer after different applications methods and time points

Chicken Wing band ID	Route of application	Hours after serum administration			
		24 h	48 h	72 h	168 h
494	IM ^a	256 ^b	32	64	128
475	IM	128	32	32	32
487	IV	512	128	128	64
489	IV	512	256	64	64
490	SC	256	64	64	32
493	SC	256	256	64	128
486	control	< 8	< 8	< 8	< 8
491	control	< 8	< 8	< 8	< 8

^a intramuscular (IM), intravenous (IV), subcutaneous (SC)

^b virus neutralization titer

Table 4.2. Virus neutralization titer at day of challenge infection

Group	Serum dilution ^a	Virus neutralization titer								
		1024	512	256	128	64	32	16	8	< 8
1	Undiluted	2 ^b	7	1	0	0	0	0	0	0
2	1:2	1	5	4	0	0	0	0	0	0
3	1:4	0	0	5	4	0	1	0	0	0
4	1:6	0	0	1	2	4	1	2	0	0
5	1:8	0	0	0	1	2	2	4	1	0
6	1:16	0	0	0	1	0	3	1	5	0
7	1:32	0	0	0	0	0	0	1	8	1
8	1:64	0	0	0	0	0	0	1	2	7
9	1:128	0	0	0	0	0	0	0	1	9
10	No serum	0	0	0	0	0	0	0	0	10

^a Serum dilution factor prior to subcutaneous administration

^b Number of chickens in the corresponding serum dilution group

Table 4.3. Protection of chickens after challenge infection with two different
IBDV strains

VN-Titer ^a	Challenge virus			
	E/Del		IBDV _n -var	
	protected/total	BLS	protected/total	BLS
1024	1/1 ^b	1	2/2	1,1
512	6/6	1,1,1,2,2,1,	6/6	1,1,1,2,2,2
256	5/5	1,1,1,1,1	3/6	1,1,4,4,1,1
128	3/3	1,1,1	3/5	4,4,2,2,1
64	2/5	4,4,4,2,1	1/1	1
32	3/5	1,4,4,1,1	0/2	4,4
16	1/4	4,1,4,4	1/5	1,4,4,4,4
8	2/6	4,1,1,4,4,4	0/11	4 (11x)
<8	0/15	4 (15x)	0/12	4 (12x)

a Virus neutralization titer at the day of challenge/infection

b Number of chickens protected /total number of chickens in
corresponding VN titer group

CHAPTER-5

CONCLUSION

IBDV is ubiquitous in commercial poultry operations. IBDV can persist on poultry farms for extended periods of time. RNA viruses, such as IBDV, lack proof reading mechanisms during RNA replication which results in a higher mutation rate in the virus progeny. Intensive vaccination programs and immune pressure due to the presence of circulating IBDV in the poultry field, forces IBDV to undergo antigenic drift. As a result, new emerging variant strains of IBDV evolve in day to day commercial poultry operations. This provides a movable target and challenging environment in designing vaccine strains. Since VP2 is the sole protein responsible for inducing neutralizing antibodies, our research focuses on amplifying and cloning the VP2 fragment from the field samples into a reverse genetics background. The antigenic subtype is based on the reactivity with a panel of MAbs and correlated to determined nucleotide and amino acid sequences. More than 300 samples were collected from different commercial poultry operations within the US and were analyzed in this study. Phylogenetic analysis of the nucleotide sequences was also conducted. The results revealed limited correlation between the antigenic subtype of the virus and their position in the phylogenetic tree. Thus, grouping of IBDV based on nucleotide sequences alone will probably lead to a false classification of the investigated field isolate. The results suggests that reverse genetics can be used a diagnostic tool for antigenic characterization of IBDV. Also, in this study, we identified a group of viruses which did not react with the panel of MAbs (IBDV_n-var) and may be antigenically different from previously described IBDV strains.

Since IBDV is ubiquitous in poultry flocks, we were interested in studying the antigenicity of IBDV across the globe. The availability of FTA cards and the reverse genetics technique allowed the realization of this project. The VP2 encoding fragment was amplified using a two primer pair approach. The results obtained from this study further supported the accuracy of the reverse genetics system along with a panel of MAbs for antigenic characterization of IBDV versus the solely reliance on phylogenetic analysis of VP2 nucleotide sequences. In this study, we also identified new variant strains of IBDV including viruses that lacked a MAb reactivity pattern (IBDVn-Var). In parallel a global antigenic drift occurring in IBDV.

For a virus to be considered as a vaccine candidate, it has to be evaluated in a biological system. Embryonated eggs may not serve as a suitable biological system, since some IBDV are less adapted to the embryos. This mostly holds true for IBDV isolates from the field. The read out system for IBDV in embryos is subjective and in general not well defined. Based on this knowledge and also for a more defined characterization of IBDV field isolates we have developed an *in vivo* experimental model in chickens. In this experimental model, the read out system is well defined since the results are based on the microscopic evaluation of bursa of Fabricius which is the target organ for IBDV. In this system we evaluated a field isolate, previously characterized as an IBDVn-var, against E/Del, the common IBDV vaccine strain virus. In this method, a specific IBDV sera against E/Del was used for passive immunization of chickens. This system allows for determination of the break through titers based on a titer-dependent manner. Based on results of the break through titer and bursal lesion score, it clearly revealed that IBDVn-var is antigenically different from E/Del virus. This *in vivo* experimental model is a highly sensitive method for evaluating IBDV, since the results are based on microscopic evaluation of bursa of Fabricius, the target organ for IBDV.