CHARACTERIZATION OF THE CHICKEN ANTIBODY RESPONSE AGAINST INFECTIOUS BURSAL DISEASE VIRUS (IBDV), IBDV IMPACT ON B-CELLS AND FULL-LENGTH GENOMIC SEQUENCING FOLLOWED BY ANTIGENIC AND PHYLOGENETIC ANALYSIS OF IBDV STRAINS WITH DIFFERENT PATHOGENICITIES

by

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(Under the Direction of Holly Sellers)

ABSTRACT

The aim of the first project was to elucidate the effect of infectious bursal disease virus (IBDV) on the serum immunoglobulin levels and B-lymphocyte subpopulations using flow cytometric analysis. In the bursa, two B-cell subpopulations designated as A and B were identified based on cell size and granularity. The IgM^+, B-cell subpopulation B was reduced following IBDV vaccination and challenge. Both subpopulations were phenotyped using B-cell surface expressed antigens. Age-related changes were demonstrated such as a decrease in the proportion of subpopulation A and an increase of subpopulation B when compared with the total analyzed bursal cells. In addition, they express $Lewis^x$, IgM, Bu1b, MUI36, and 78 differentially but not MHCII surface antigens. The reduction of subpopulation B did not reduce the total serum immunoglobulins nor did it affect IgG^+ and IgA^+, B-cells in the spleen. The IBDV resistant subpopulation A most likely consists of immature cells which act to repopulate the bursa following IBDV infection.

The second project was designed to sequence and characterize the full-length genomes of four IBDV strains with different pathogenicities. Only previously described hydrophilic, major A and minor 1 peaks are located within the newly predicted VP2 antigenic regions. At the VP2 processing site the Edgar cell culture adapted (CCA) and chicken embryo adapted (CEA) were more similar to the very virulent (vvIBDV) strains. Lukert, Edgar CCA, and Edgar CEA have overall sequence characteristics of the classical strains and were closely related to each other.

Analysis of the VP1, VP3, and VP4 proteins revealed 9109 has characteristics of classical type virus but within the same proteins shares unique amino acids with vvIBDV strains. At the 3' and 5' noncoding regions of segment A 9109 has similarities to the vvIBDV strains. Within the VP2 protein several predicted antigenic regions and deduced amino acids were conserved between this isolate and variant E.

The amino acid (aa) sequences of VPX protein aa 202-451 and 210-473 but not the VP2 protein are the best representatives of the entire IBDV genome.

INDEX WORDS: Bursa, B-cell subpopulations, Chicken, ELISA, Flow cytometry, Humoral immune response, Infectious Bursal Disease Virus, Phylogenetic analysis, Serum immunoglobulins

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DEDICATION

I would like to dedicate this work to my parents and my brother whose unquestionable love and support made one dream a reality. This work is also dedicated to Susan Weidner - a very special woman in my life. THANK YOU.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER IV

CHAPTER V

CHAPTER I

INTRODUCTION

Purpose of the Study

Infectious bursal disease (IBD) is a highly contagious immunosuppressive disease primarily affecting young chickens. The disease has worldwide distribution with significant impact on the commercial poultry industry. The immunosuppression is accompanied by high levels of specific anti-infectious bursal disease virus (IBDV) antibodies. Chickens respond poorly to immunization against other agents, and they are predisposed to opportunistic secondary infections such as *Escherichia coli* and *Salmonella spp*., and are highly susceptible to other viral diseases such as Newcastle disease, fowl pox, and infectious laryngotracheitis.

There is significant antigenic, immunogenic, and pathogenic variation between IBDV strains which determines disease outcome. Some IBDV strains cause an immunosuppressive, subclinical form of disease with less than 5% mortality, while others, such as very virulent strains, can cause a clinical form with up to 100% mortality. The clinical signs and the level of immunosuppression can also vary significantly. As vaccination is the primary means for control, most efforts for protection against IBDV by the commercial poultry industry are focused on developing efficient vaccination programs. Successful immunization requires reliable IBDV field and vaccine strain characterization.

The first project was designed to elucidate the IBDV induced immunosuppression, specifically the effect of IBDV on serum immunoglobulin levels and B-lymphocyte subpopulations using flow cytometric analysis. The mechanism by which IBDV induces cellular

and humoral immunosuppression is one of the key reasons for developing effective vaccine. The virus is ubiquitous in the environment and is extremely resistant. The primary target of IBDV is immature IgM^+, B-lymphocytes in the bursa of Fabricius, the primary lymphoid organ of chickens. Following IBDV infection in young chickens, the level of lymphocyte depletion within the bursal follicle varies and depends on the IBDV field strain, extent of viral exposure, and maternal antibody levels. Although the bursa remains a functional lymphoid organ, B-lymphocytes have reduced proliferating responses following IBDV infection. In addition, the temporary reduction of B-lymphocytes in the bursa has a systemic effect on both the B-cell repertoire and serum immunoglobulins.

The second project was designed to sequence the complete genomes of three IBDV strains and one field isolate and perform molecular characterization. Phylogenetic analysis and prediction of antigenic regions within the VP2 protein was performed. Different techniques have been used for typing IBDV isolates. *In vitro* virus neutralization, antigen-capture enzyme immunoassays, and *in vivo* reciprocal cross-challenge tests are highly reliable although expensive and time consuming. Reverse transcriptase-polymerase chain reaction (RT-PCR) followed by restriction fragment length polymorphism (RFLP) allows rapid analysis of small fragments of IBDV genome. The RT-PCR/RFLP genotyping technique allows quick and reliable definition of multiple field isolates using the major protective and neutralizing antibody inducing VP2 protein. The VP2 contains a hypervariable region which varies significantly between strains. Unfortunately, the information obtained from VP2 analysis does not fully characterize some newly emerging IBDV field isolates. Currently a limited number of full-length IBDV genomic sequences are available and the role of the regions outside of the hypervariable domain of VP2 has not been studied extensively.

The findings of these studies may improve current techniques developed for rapid IBDV field isolate characterization and determining the antigenic properties of field isolates. This information can be used in determining the efficiency of the current vaccine that is used by the commercial poultry industry.

CHAPER II

LITERATURE REVIRIEW

Part 1: Infectious bursal disease virus

1.1 History and epidemiology

Infectious bursal disease (IBD) was first reported in 1961 as "avian nephrosis" (37) reflecting the observed predominant kidney damage in chickens. The disease is highly infectious with up to 100% serological conversion. The clinical signs included watery diarrhea, anorexia, and death. On post-mortem examination severe dehydration, hemorrhages in the leg muscles, urate deposits in kidneys, and enlarged bursa of Fabricius can be observed. The first cases were noticed in 1957, in Gumboro, Delaware, USA and the disease later named "Gumboro disease". In 1962 Winterfield isolated the virus named as "Gray agent" in embryonating eggs (247) and later Hitchner suggested the term "Infectious bursal disease" (73). During the next few years, IBD was diagnosed in many other regions within the USA (119), Europe, Australia, New Zealand (26), Middle East, Africa, Far East, and Antarctica (53, 56, 60, 180, 236, 238). In 1995 a resolution for control of IBDV was accepted at the 63rd general session of the International committee of office international des epizooties (164).

In 1984, reports of four IBDV isolates designated as A, D, G, and E originated from broiler flocks in Delmarva Peninsula demonstrating significant mortality contributed to secondary infectious such as *Escherichia coli* (199). The isolates induce rapid bursal atrophy with minimal inflammatory response. The vaccines available at that time did not provide

protection against challenge with these isolates. The new virus isolates were termed as “antigenic variants”. Since then antigenic variants have been isolated throughout United States (213, 215).

In 1987 “very virulent” strains were isolated in Europe (27, 17, 232) which can cause mortality up to 70% in some of the commercial egg laying pullet flocks (218, 234, 235). These strains have been isolated in other parts of the world (49, 163) but not in United States.

1.2 Host susceptibility and transmission

Currently two serotypes are known. Chickens (*Gallus gallus*) can be infected with serotype I IBDV and develop clinical signs. Turkeys (*Meleagris gallopavo*) are often asymptomatic carriers of serotype 1 (192) and 2 (88, 94, 144). Neutralizing antibodies for serotype 1 have been demonstrated in Pekin duck (*Cairina moschata*) (144), guinea-fowl (*Numida meleagris*) (2), pheasants (*Phesanus colchicus*) (130), and ostrich (*Struthio camelus*). Neutralizing antibodies for serotype 2 (64) were demonstrated in tern, puffin, crow, and penguin (60, 166, 245).

There are significant differences in the susceptibility of inbred chicken lines with increased susceptibility in the layer lines and a higher resistance of the meat lines (21, 160). Only horizontal transmission has been described. Virus is shed in the feces from 48 hours up to 14 days following infection (243). Other carriers of IBDV are mosquitoes (81) and mealworms (*Alphitobius* sp.) (141). Humans can not be infected with IBDV (175).

1.3 Resistance to chemical and physical agents

Without proper disinfection the virus can survive on the premises for more than 4 months. The virus is extremely resistant to disinfection and pH changes between pH 2-12. The virus remains viable following incubation for 5 hours at 56 C and 30 min at 60 C. The infectivity can be reduced by treatment with 0.5% formalin for 6 hours or 1% formalin for 1 hour (9).

1.4 Economic significance

Direct losses are due to the mortality ranging from 10% to 30% in chickens between 20-30 days of age (d.a.) (238) and increase of mortality from 6% to 30% over 45-55 day broiler growing cycle was reported (205).

Indirect losses are due to virus induced immunosuppression and secondary opportunistic bacterial and viral infectious. Profit reduction with 10% (145) and 8% decline in feed conversion, slower growth rate, and increase of condemnation of carcasses from 1 to 3% due to the septicemic airsacculitis and cellulitis were also observed (205).

1.5 Etiology

IBDV is a member of the *Birnaviridae* family (42, 157) in the genus *Avibirnavirus*. Other members of this family include infectious pancreatic necrosis virus (IPNV), oyster virus (OV), and Drosophila X virus (DXV) (157). It is a small virus with 60-70 nm diameter (236, 237), non-enveloped, and composed of 32 capsomers with single-shelled icosohedral capsid (16).

The IBDV genome is a bi-segmented, (designated as A and B), double stranded (ds)RNA (102). Segment A contains approximately 3260 nucleotides (nts) and contains two open reading frames (ORF). The shorter ORF1 (438 nts) encodes a cytotoxic VP5 (17kDa) protein (129) which is most likely involved in viral release (155). The ORF1 precedes and partially overlaps the longer ORF2 (104, 105, 106, 153). The ORF2 (3,039 nts) is monocistronic and encodes a 109 kDa precursor polyprotein (NH_3-VPX-VP4-VP3-COOH). The precursor polyprotein is auto-catalytically cleaved at amino acids (aa) Ala^{512}-Ala^{513} for VPX-VP4 and Ala^{755}-Ala^{756} for VP4 (28-30.5 kDa)-VP3 (32-34 kDa) proteins (5, 83, 108, 122, 149, 201).

VP2 and VP3 are structural proteins, which form the external surface of the capsid in trimeric subunits. VP3 is an RNA-binding protein (117) found primarily in the inner core of the

capsid (16, 129). The VPX protein containing 1-512 amino acids (aa) is processed into VP2 (40-45 kDa) containing 1-441 aa protein (83).The VPX is processed within the virion and is associated with maturation and release of the virus (109). VP2 is a conformationally dependent, hydrophobic protein and contains major antigenic sites responsible for induction of neutralizing antibodies (52). VP3 contains group specific antigenic regions (8, 136, 169) but alone fails to induce neutralizing antibodies (179). The C-terminus of the VP3 is positively charged, interacts with dsRNA (16, 83), and forms complexes with VP1 and/or VP2 (129, 219).

VP4 is a viral protease that processes the polyprotein to VPX, VP3, and VP4 (5). A catalytic triad of a serine protease is formed by H546, D589, and S652 (19) and was characterized as a "serine-lysine" catalytic dyad (13). Protease cleavage sites are at dibasic residues 453 and 723 (83, 201).

The smaller genome segment B (2827 nts) encodes for VP1 (90kDa), the viral RNA-dependent, RNA-polymerase (RdRp) (42, 106, 216). It is found in small amounts within the virion, both as a free (108) and as a genome linked protein (VPg) (107). The VPg circularizes both segments (152) and plays a major role in the encapsidation process (129). It is likely that VP1 is attached to the genome segments through guanidine residues at the 5' terminus of the segments as was reported for IPNV (42).

Inverted repeats were described at the 5' and 3' ends of segments A and B. The 5' terminal sequence in both segments consists of a 32-nucleotide consensus sequence and 3' terminal sequences in both segments end with the conserved GCGGU pentamer. The inverted repeats at the 3' terminus on segment A and 5' terminus on segment B may form stem and loop secondary structures important for viral replication, packaging, transcription (106). In segment

A, there are differences in the 5' noncoding region in the VP5 proteins between serotype 1 and 2, which may be important for viral replication (156).

1.6 Virus replication

The mechanism for IBDV replication is not well understood. *In vitro* studies showed that the RNA polymerase synthesizes viral single-stranded RNA by a semi-conservative, strand displacement mechanism. The reaction results in two products 24S mRNA and 14S RNA (10, 216). 24S mRNA is believed to be the viral RNA serving as a template. VP1 catalyses the guanylation of the template strand and serves to prime viral RNA synthesis. The viral transcription and replication occurs within the host cell without uncoating (216) and the host cellular proteins are not shut down (7).

The virus can replicate in primary cell cultures such as bursal lymphoid cells, chicken embryo kidney, and fibroblast cells (134, 146), as well as, several mammalian cell lines such as VERO and BGM-70. The IBDV polyprotein is detected in bursal lymphoid cells *in vitro* approximately 90 min following infection (149).

1.7 Clinical aspects

The incubation period is very short and the clinical signs can vary depending on the conditions in the farm, age of the birds, and viral strain. Chickens are most susceptible between 4 and 6 weeks of age (w.a.). Generally clinical signs are dehydration, lethargy, anorexia, ruffled feathers, and white diarrhea (37, 133).

There are three known IBDV pathotypes within serotype 1: classical, variant, and very virulent. Based on the immunopathogenicity the classical strains are further subdivided into mild, intermediate, and virulent strains (189). Two clinical and one subclinical form have been described. The classical form is caused by classical strains of IBDV (53) has incubation period

between 2 and 4 days and is characterized by low mortality, acute onset of depression, watery diarrhea, and stained feathers around the vent with urates (37, 133). In the US the disease is primarily subclinical resulting in immunosuppression. Field challenge is likely a result of mild or variant strains of IBDV, such as Delaware variant E (97, 213). The clinical form of the disease is characterized by an acute onset, high mortality, and obvious clinical signs. It is described in Europe, Africa, and Asia and is caused by the very virulent strains of IBDV (27, 218, 235). There are differences in the viral replication patterns between IBDV strains such as Ehime/91, J1, and K in the bursa, spleen and bone marrow which affect the severity of the clinical signs (227). Age-related coagulation disorders coincide with the mortality and lesion severity. Insignificant changes in birds infected with field IBDV isolates at 17 d.a. were observed compared to severe effects on birds infected at 42 d.a. (211).

Postmortem examination of chickens infected with classical serotype 1 IBDV reveals dehydration of the subcutaneous fascia and musculature, hemorrhages on the pectoral and thigh muscles and in the mucosa of the proventriculus at the junction with the gizzard. Renal lesions with accumulation of urates in the tubules may be present following coinfection with nephropathogenic infectious bronchitis virus strains (37, 133). The changes in bursa of Fabricius vary depending on the IBDV strain and stage of the infection (133, 143). In the first 3-4 days following infection with classical strains, the bursal size and weight progressively increase and the tissue is hyperemic and edematous. The bursa is covered with a yellow transudate and has a white striation. The bursa gradually returns to its normal size and by the 8^{th} day following IBDV infection atrophies to about one-third of its normal size (37, 133). Histological lesions are predominantly located in the bursa of Fabricius and thymus (206). Necrosis of lymphocytes in the bursal follicles can be detected within one day following IBDV infection and by the third day

heterophil infiltration and hemorrhages can be observed (28). Acute hepatitis and aplastic anemia may also occur (163).

The vvIBDV strains cause severe lesions in the cecal tonsils, thymus, and bone marrow (133). Bursal lesions caused by very virulent "9011" strain are even more severe: cortical lymphocyte necrosis and depletion of medullary lymphocytes with interstitial inflammation and hyperplasia of epithelial reticular cells. The thymus, spleen, liver, and bone marrow are also affected (70). Highly virulent HPS-2 strain causes severe lysis of heterophil myelocytes with pyknotic nuclei (86). Cortical lymphocytes necrosis and depletion of lymphocytes in the thymus are more severe from HPS-2 strain than those caused by virulent strain GBF-1 (87).

1.8 Diagnosis, IBDV isolation and propagation

Differential diagnosis. Clinical diagnosis of the acute form is based on the clinical symptoms and post-mortem examination. The differential diagnosis includes velogenic viscerotropic Newcastle disease, chicken infectious anemia virus, nephro-pathogenic forms of infectious bronchitis, Marek's disease, mycotoxicosis, avian coccidiosis, and stunting syndrome (119). Microscopic lesions in the bursa and other organs such as the thymus (87), spleen, and bone marrow are important for establishing a diagnosis (125).

Virus isolation and propagation. IBDV can be isolated from most lymphoid tissues during the earliest stages of infection. The bursa of Fabricius is the primary target organ and organ of choice for virus isolation (132, 133) between the 2^{nd} and 10^{th} days following IBDV infection (247). Initial isolation is possible in 9-11 days of age specific pathogen free (SPF) embryonated eggs via inoculation in the chorioallantoic membrane (CAM) or the yolk sac (198). IBDV is in highest concentration in the embryo's liver (143). While variant strains do not cause mortality, embryos inoculated with classical strains can result in mortality within 3 to 5 days

following inoculation. Necrosis and hemorrhages in the liver and kidney, a parboiled appearance of the heart, pale spleen and small necrotic foci may be present. Although field isolates can be difficult to adapt, most of the strains grow in primary avian embryo cell cultures (29, 146) or continuous cell lines of mammalian origin such as rabbit kidney (RK-13), baby Grivet monkey kidney (BGM-70), fetal rhesus monkey kidney, and African green monkey kidney (VERO) cells (96, 103, 134, 237).

Detection and characterization of viral antigens. The viral antigens can be detected using immunofluorescence (4), immunoperoxidase (30), agar-gel precipitation, and virus-neutralization (48) tests but results can vary (234, 235). Quantitative tests used are: agar-gel immunodiffusion (38), and enzyme-linked immunosorbent assay (ELISA) (138). Several neutralizing serotype specific monoclonal antibodies R63 and B69 (212, 214) and BK9 and MCA 57 (233) are used against VP2 protein with antigen capture (AC)-ELISA. Neutralizing antibodies 5H6 and 7C9 against VP2 protein are used in serum-neutralization tests and ELISA (232, 234, 235). ELISA using antibodies against the VP2 protein expressed with the baculovirus-expression system was used for evaluation of the immune response (98). In addition, an ELISA test with recombinant VP2 protein expressed in the baculovirus-expression system was used for evaluation of the immune response (98).

Serology. Chicken flocks exposed to IBDV seroconvert. Although the commercial serological tests are useful for evaluating specific seroconversion, they are unable to differentiate seroconversion induced by pathogenic and attenuated IBDV strains used as a vaccine (40, 118).

Molecular techniques. Currently, reliable IBDV strain characterization is possible with the virus-neutralization assay (96, 144). The technique requires *in vitro* virus propagation and not all strains can be propagated *in vivo* (144). Most of the current molecular techniques used for

IBDV typing utilize amplification of the VP2 hypervariable region by reverse transcriptase (RT) polymerase chain reaction (PCR) (249) followed by restriction fragment length polymorphism (RFLP) analysis (85, 137). The most widely accepted is the amplification of 743 nucleotides within VP2 gene using RT-PCR followed by digestion with either *BstN I-Mbo I or BstN I-Sty I* restriction enzymes (92, 93). Although serotype 1 strains can be grouped into six RFLP patterns (92, 98) several strains can not be characterized using this procedure. The VP2 fragment between amino acid 206 to 350 an *Acc I-Spe I* fragment (6, 104) encodes epitopes for virus-neutralization (5) and has high variability between variant E and Delaware (67), DS326, GLS, (158), and Cu-1 (170) strains. Within this fragment there are two hypervariable regions. While the first fragment, aa 212-224, stabilizes the protein conformation, the second, aa 314-324, binds to the neutralizing monoclonal antibodies (mAbs) (67, 204). Although the results from the RT-PCR/RFLP technique are rapid, the assay has to be adapted for tissue rather than for cell-culture (93). In addition, the focus of the analysis has to be at the amino acid level as they are subject to higher selection pressure then the nucleotides (98).

Nucleotide sequencing and analysis of conserved genomic segments (217, 222, 248, 249) or hyperveriable regions of VP2 (128) can also be used for molecular genotyping. The simultaneous presence of four amino acids: Ala^{222}, $Ile^{256, 294}$, and Ser^{299} within the hypervariable region was suggested as a determinant unique to vvIBDV strains (17, 24, 49, 250). Although DNA probes have been used for detection of IBDV, the technique can not differentiate IBDV subtypes (236, 237).

1.9 Prevention and control

IBDV is extremely resistant and ubiquitous in the environment. Eradication of the virus from the poultry premises is almost impossible. Chickens are infected by direct, bird to bird, and

indirectly via contaminated fomites. The viral burden can be controlled with enhanced bio-security measures and efficient flock immunization programs. The vaccine has to satisfy several requirements: the protection has to be established within 10 days following vaccination, it shouldn't cause clinical lesions, or decrease bursal weight (50, 224).

Humoral immunity is an essential part of the protection against IBDV. There is a close relationship between titers of neutralizing antibodies and protection (98, 234, 235). The half-life of the antibodies against IBDV is between 3 and 5 days and is important for evaluating the time of vaccination (40, 131). Formaldehyde inactivated IBDV vaccines have less than 0.01% detectable antigen conformation change compared to glutaraldehyde treated vaccines (25).

Live virus vaccines are made from attenuated strains and are classified as mild, intermediate, and hot. The designation of the type of the vaccine is base on the level of attenuation and lesions produced in SPF chickens (165). Hot vaccines cause lesions similar to the nonattenuated strains but do not cause mortality. Mild vaccines are typically used for breeders and intermediate vaccines for broilers and breeders (236, 237). Inactivated vaccines are used in hen's prior to lay in cases where they have been vaccinated with live vaccines or have been exposed to IBDV (236, 237). The vaccination of hens with inactivated oil emulsified vaccines provides the offspring with passive immunity. In the case of highly pathogenic strains, maternal antibodies did not provide maximum protection even if the laying hens were boostered at the point of lay. The offspring needs to be vaccinated prior to 38 d.a. with live vaccine with the appropriate strain, which even in the presence of the high levels of maternal antibodies can establish infection (234).

The existence of very virulent and variant strains requires development of specific vaccines (65, 66, 196). While vaccines developed from the IBDV antigenic variants induce

protection against both classical and variant strains, the vaccines from classical strains do not protect against challenge of variant strains (133, 197).

Recombinant vaccines. Different systems for expression of IBDV proteins have been tested with variable success. The host protective VP2 protein expressed in yeast system does induce virus-neutralizing antibodies in vaccinated SPF chickens (135). Baculovirus (109), herpes virus of turkey (HVT), and vaccinia virus (55, 129) systems have been used for expression of different IBDV genomic regions or the full-length IBDV genome using reverse genetic techniques (3, 14, 15, 39, 126, 154, 155, 251). A DNA vaccine expressing either VP2 or VP2-VP4-VP3 (58), immune complex vaccine, and hyperimmune IBDV chicken serum (IBDV-Icx vaccine) have also been tested.

Part 2: Molecular basis for IBDV antigenic and pathogenic variability

Currently two IBDV serotypes have been identified. Serotype 1 strains such as SK140, IN, P2, Cu1, Cu1wt can cause immunosuppression in chickens and serotype 2, such as OH (105) and 23/82, can cause subacute infections in turkeys. In turkeys, serotype 1 is found only in vaccinated flocks and serotype 2 as a natural infection (94). In turkeys, infection with serotype 2 did not affect humoral immune response to sheep red blood cells and *Salmonella heidelberg*, T-dependent and independent antigens, respectively (95). Although serotype 2 can infect chickens and seroconversion occurs it is not pathogenic for broilers (88). Close antigenic relationship between strains from the two serotypes may exist such as Cu1 and 23/82 serotype 1 and 2, respectively (8). Serotype cross-reactivity was observed with immunofluorescent antibody test but not by the virus-neutralization test (94, 144).

As determined by virus-neutralization and/or cross-neutralization tests, strains within serotype 1 are antigenically heterogeneous (94, 97, 144, 148). The minimal region for neutralizing monoclonal antibody (mAb) binding is within the VP2 protein variable region, between amino acids at position 206 and 350 (6). Within VP2 there are two hydrophilic amino acid domains between 212-224 and 314-324 (204) and changes within these domains can lead to new antigenic variants (67, 93, 204). Analysis of several sequences suggested that the variable regions in VP2 are responsible for antigenic variation (170, 204, 229, 231).

Antigenic drift was described in 1984 in strains from serotype one. Several strains such as variant E (Delaware) (199), GLS, and DS326 (18) were isolated in USA from vaccinated broiler flocks. In the USA serotype 1 variants were designated as "pathogenic variants" as antigen-capture enzyme immunoassays (AC-ELISA) demonstrated that only two of the VP2 protein epitopes are conserved between them and the classical strains isolated prior 1985. These strains cause immunosuppression without clinical signs. Six subtypes were described by serum neutralization assay (97, 212, 214,) and neutralizing antibodies (214) but only one was demonstrated to be a "true" variant in cross-protection studies (199). Six strains isolated in France causing 3.5 to 4 time's higher mortality have been characterized with cross-agar gel precipitation (AGP) and cross-virus neutralization. They are related to strain 52/70, serotype 1 and show antigenic shift (48). Comparative analysis of immunosuppression and pathogenic characteristics between serotype 1 isolate IM and variant A demonstrated considerable pathogenic variation within these isolates. Both isolates caused reduction of antibody levels and lymphocyte mitogenic response. Bursal atrophy and the thymus were affected only by IM isolate (209). Strains isolated after 1985 have loss of the mAbB69 epitope (212, 214) and number of other neutralizing antibody epitopes within the hyperveriable antigenic domain (5, 8). Delaware

variants lack the mAbB69 binding epitope unique for USA field isolates but have gained BK9. The GLS strain has lost mAb epitope B69 and R63 but has gained mAb57 and DS326 strain has lost B69, R63, and 179 epitopes (215). Variants GLS and DS326 have mAb57 epitope, which differentiate them from variant E (Delaware) (190). All European strains possess B69 and R63 epitopes and none have BK9 or mAb57 (233). Amino acid Gln^{249} in VP2 protein is critical for mAb binding. Substitutions at amino acids Glu^{321} in GLS and Ile^{286}, Asp^{318}, Glu^{323} in E/Del and Glu^{311} and Gln^{320} in DS^{326} are important in binding mAb57, mAb67, and mAb179 (229).

In 1987 "very virulent" (vvIBDV) strains were isolated in Europe (27, 48, 218, 227, 233, 235) which lack the antigenic variation described for the US variants. The very virulent strains are antigenetically related to the Faragher 52/70 reference strain for serotype 1 on the basis of monoclonal antibody binding patterns (170, 233, 235) and cross-neutralization assays (48). The origin of vvIBDV such as UK661 (19) and OKYM strains (250) on the basis of segment B phylogenic analysis is not clear. The data obtained with a panel of VP2 neutralizing monoclonal antibodies and immunoprecipitation-immunoblotting techniques suggest that IBDV did not undergo antigenic shift in Europe and Africa. Isolates may escape vaccine protection by simultaneous mutations in two or three epitopes within VP2 (170). Two antibodies, mAbs 3 and 4 against conformation-dependent overlapping neutralizing antigenic domains in VP2 can be used in the AC-ELISA test for differentiation of vvIBDV strains from the classical European pathogenic strains (47). Analysis of VP2 sequences from "very virulent" isolates indicates unique amino acids at position Ala^{242}, Ile^{246}, and Ile^{294} (6), which can be useful for phylogenic analysis (24, 49, 250). In 1992 Japanese strains were isolated that cause higher than 10% mortality with fast spread of the disease and rapid recovery. Atrophy of the bursa of Fabricius and thymus is accompanied with inflammatory response, which is in contrast with the minimal

inflammatory response in the bursa caused by US variants (213, 227). Several vvIBDV isolated in Japan (127), as well as, US variants have a serine rich heptapeptide sequence SWSASGS, between VP2 amino acid positions 326 and 332. Less virulent strains lack this motif (67, 229). European strains have the same VP2 amino acid sequences as vvJapanese isolate "9011" (18).

Multiple alignments with different strains suggest that simultaneous mutations in different regions are the most probable cause for strain variations. It is not possible to predict the most important amino acid residues without complete knowledge of the three-dimensional structure of the viral proteins (19, 179, 250, 253). Viral structure and sequences are important either for the survival of the virus or for the strain and type specificity. Most of the changes that occur in the genetic sequences and/or regulatory proteins may influence the viral replication cycle, host specificity, and the virulence (158).

Part 3: Immunosuppression and host immune response

3.1 Bursa of Fabricius and normal B-cell ontogeny

The bursa is a major site for B-cell division, gene conversion, (194, 195, 223), and clonal differentiation (45, 177, 178, 184, 194) containing more than 85% IgM^+, B-cells (63, 46, 184, 186). In chickens, cell progenitor commitment to B-lymphocyte lineage occurs during a short period of time (184) and immunoglobulin gene rearrangement is not a permanent process (244). B-cell development consists of a pre-bursal, bursal, and post-bursal phase (185, 225). Bursectomized and irradiated chickens at one day of age (d.a.) develop agammaglobulinemia and do not have circulating antibodies (34). Between 21 and 49 d.a. each of the ~10^4 bursal lymphoid follicles (168) has 1-3 x 10^5 cells originating from 1 to 10 (177, 178) stem cells with specific

VDJ rearrangement (76, 244). Diversification of the immunoglobulin classes is in the IgM→IgG→IgA (36) order. Some of the IgM^+ cells in the bursa switch to IgG synthesis and become IgM^+/IgG^+ double positive cells (36, 114) but most of the IgG is exogenous, trapped as an IgG-antigen complex (46). Some of the IgG can also originate from plasma cells around the bursal follicles (34, 36).

Self renewed, post-bursal stem cells populate the peripheral lymphoid organs (23, 35, 225) before initial signs of involution at approximately 56 d.a. in the bursa (12, 31, 59). At 21 d.a. the renewal of two of the three B-cell subpopulations in the peripheral blood is still bursa-dependent (171, 172, 174). In addition, the bursal B-cell subpopulations have a different abilities to populate bursal compartments and peripheral tissues (172, 225, 226) and they differentially express surface and cytoplasmic IgM (63) and MHCII antigens (51). Although there are differences between genders and bird species, in general, following rapid growth of the bursal follicles occurs within the first 28 d.a., signs of functional involution are observed around 56 d.a. with complete bursal involution at approximately 200 d.a. (12, 31, 59).

Earlier histopathological evaluation of the bursa suggests the existence of bursal IBDV resistant B-lymphocytes expressing surface determinants $Bu1b^+/Lewis^{x-}$ (176, 240, 241) or $MUI36^+$ cells at the bursal cortico-medullary junction (89, 182, 183, 246). *In vitro* studies suggest for IBDV resistant immature (11, 150), competent B-cells (7), and small blood lymphocytes (22). $Bu1b^+$ cells repopulate bursal follicles depleted by IBDV before the functionally active $Lewis^{x+}$ cells are detected (89). The Bu1 is an early B-cell surface antigen, expressed before Ig rearrangement (200) and has been used as a marker for B-cell clone differentiation (77, 78, 177, 178). This antigen is not restricted to cell cycle, immunoglobulin, major histocompatibility complex (MHC), and B-cells. It is also expressed on Mø and

monocytes (176, 240, 241) but not on plasma cells (77, 78). The MUI36 antigen is a B-cell determinant and is not Ig, MHCII, or B-cell restricted. Initially MUI36^{+} cells are distributed evenly in the bursal follicle and later restricted on plasma cells. The expression of Lewis^{x} during the bursal phase of avian B-cell differentiation is a stage-dependent process. It was suggested that similar to sialyl Lewis^{x} the terminal oligosaccharide Lewis^{x} is important for cell adhesion and homing. The Lewis^{x} initial expression coincides with the initial immunoglobulin gene conversion and subsequently is down-regulated with the bursal lymphocytes maturation (121, 139, 140). In addition, although the IBDV cell receptor is not identified, it was suggested to contain N-glycosylated protein (166).

Major histocompatibility complex has been linked to the immune response against IBDV and it was suggested that MHCII to restricts T-cell dependent secretory antibody response against this virus (84, 100). MHCII is expressed on antigen presenting cells and is involved in presentation and recognition by T-cells (228). MHCII expression coincides with IgM expression and increases during the bursal embryonic phase of B-cell differentiation. It is not B-cell restricted and is also expressed on Mø and monocytes (51). MUI78 is a MHCII restricted antigen also expressed on Mø-like and dendritic cells. The MUI79, a Mø marker (182, 183), was not expressed in both populations.

Bursal regeneration is necessary following IBDV infection for normal antibody response (43, 111). The IBDV effect on the bursa resembles a bursectomy, which performed in different ages chickens affects different immunoglobulin classes. B-cell commitment is irreversible and an age-dependent process in an IgM→IgG→IgA order (36, 46, 114). As IgM^{+} cells leave the bursa prior to IgG^{+} cells and IgG expression will be inhibited if bursectomy is performed prior to IgM→IgG switch (35, 230). Bursectomy at an early embryonic stage leads to complete

agammaglobulinemia, at a later stage it leads to lack of IgG but above normal IgM synthesis (35). IgA^+ cells seed the peripheral organs after IgG^+ cells and a bursectomy prior to embryonic day 18^{th} will lead to a lack of IgA synthesis (115, 116, 124). The seeded non-bursal sites are a functional dead end for the progenitor B-cells but they can help restore humoral function in B-cell depleted animals (79, 80, 184) as the cecal tonsils may partially replace the bursal microenvironment (20, 123). The bursal lymphocyte number is dependent on B-cell proliferation, apoptosis, and migration of mature cells to the peripheral lymphoid organs (120, 172, 173). Although IgM^+, B-cells in the bursa have undergone Ig V(D)J recombination the gene V conversion (142, 194) and the hyperconversion process continues until the bursa involutes (139, 140, 193). IBDV hinders this process and most likely decreases the B-cell repertoire when chickens are infected at an early stage but not after 21 d.a. (57, 82).

Previously, it was reported that in specific-pathogen-free (SPF) chickens IgG^+ and IgA^+ plasma cells were detected as early as 14 d.a. and IgM^+ at 5 d.a. (99). The germ-free environment hinders B-cell development in peripheral lymphoid organs (74, 114) due to inadequate antigenic stimuli. Bursa isolated at nineteen days of embryonation from environmental antigen leads to decreased numbers of IgG^+, B-cells and reduces B-cell proliferation (44, 46 202). In SPF chickens, the cytoplasmic IgM, μ chain peak is delayed and the surface μ chain has lower expression (182) than in normal chickens.

3.2 Humoral immune response

IBDV infected chickens are predisposed to secondary infections such as *Escherichia coli* and *Salmonella spp*., and are highly susceptible to other viruses such as Paramyxovirus and Herpes virus (54, 62). Immunosuppression caused by IBDV can be assessed by chicken humoral response to *Brucella abortus* (59), sheep blood cells (T-dependent antigen), and *Salmonella*

heidelberg (T-independent, O-antigen). Humoral immunity is an essential part of the protection against IBDV. Immunosuppression is accompanied by high levels of anti-IBDV antibodies as a result of mature B-cell stimulation and susceptibility of the immature lymphocytes to the virus (236, 237). There is a close relationship between titers of the neutralizing antibodies and the level of protection (98, 234). Neutralizing immune response against IBDV was evaluated with simultaneous infection of B-cells with IBDV and reticuloendotheliosis virus. Results from this flow cytometry study suggested that neutralizing antibodies probably did not inhibit virus attachment to the cell, rather inhibited penetration or uncoating of the virus (191). The tertiary structure of the secreted immunoglobulin molecules may also be altered (91).

The pathogenic effect is directly related to the number of B-cells in the bursa. Clinical signs are severe when broilers or layers are challenged even in the presence of maternal antibodies at the maximal development stage of the bursa between 3 and 6 w. a. (163, 234, 235). There is a relationship between the time of infection and the seeding of the bursa with B-cells. Chickens infected at one day of age with IBDV isolate 12/96 showed no mortality and 50% immunodeficiency. Chickens infected with the same isolate at 3 w.a. showed 50% mortality and insignificant immunodeficiency (91). The lack of complete immunodeficiency can be explained by the "B-cell seeding pattern". In the first few days after hatch, some B-cells migrate from the bursa to the peripheral lymphoid organs. In addition, small numbers of B-cells migrate to the peripheral lymphoid organs prior to hatch (35, 90).

The initial site of IBDV replication following oral challenge is in gut-associated macrophages and associated lymphoid cells, where no tissue destruction occurs. Following transient viremia, the virus is disseminated via the blood to all organs including the bursa of Fabricius. A second replication cycle takes place in the bursa, 10-11 hours post-inoculation and

is marked with significant changes within the bursal follicles (150). Within the bursa, viral antigen is found mostly around medullar-cortical boundaries and intrafollicular stroma. B-cell depletion in bursal follicles with initial necrosis is accompanied by T-cell infiltration. The level of bursal follicle repopulation within immature IgM^+, B-cells is IBDV strain dependent. Seven weeks post-inoculation, 40% of the follicles are repopulated in chickens inoculated with virulent IM strain and 80% when intermediate vaccine strain IBDV-Vac was used. The capacity for serum immunoglobulin production is not strain dependent and is restored within 6 weeks following IBDV infection (110). The functional bursal restoration is accompanied by the activation of chB1 gene expression, an indicator for active bursal B-lymphocytes and expression of surface Lewis carbohydrate, a marker for B-lymphocytes hyperconversion (89). The IBDV secondary antibody response is T-cell dependent and may be linked to the major histocompatibility complex II (100).

Primary target cells for virus replication are immature B lymphocytes in the bursa of Fabricius (28). In bursectomized, chickens older than 3 w.a., the level of viral replication is significantly lower in the liver, spleen, and thymus. The concentration of the virus is about 1000 times less than in normal chickens even after an infectious dose of 10^8 PFU is used for challenge. The lower level of replication is due to the insufficient number of susceptible cells. Host defense mechanisms can control viral replication and the infection at that point is not apparent (101). There is a correlation between the number of surface IgM^+, B-cells and the severity of IBDV infection (71, 166). Serotype 1 and 2 strains have different B-cell receptors and both receptors are expressed in chicken embryo fibroblasts (162). IgM^+ or IgG^+, B-cells in IBDV challenged birds decrease faster in the spleen (42%) than in blood (22%) (72). A decrease of IgG expressing B-cells may be indirectly related to the initial infection of IgM expressing B-cells by IBDV. In

these cells an isotope switch from IgM to IgG will not occur. In an IBDV cell-susceptibility study using T-cells, IgM^+ (μ-specific), IgG^+ (γ-specific) B-cells, and "null cells" showed that only IgM expressing B-cells are susceptible to the GBF-1 strain of IBDV (159). An i*n vitro* study using the Cu1 strain of IBDV suggests that viral replication is in small lymphocytes, lymphoblasts, non-stimulated lymphoid blood cells, and monocytes. Virus dissemination occurs in cell-bound monocytes (22). Virus replication is significantly higher during the proliferating stage of immature IgM^+, B-cells (151). Lymphoid cells in the bursa follicles are destroyed through both apoptosis and necrosis. The VP2 and VP5/NS proteins are involved in apoptosis (55, 251). An increase in apoptotic cell death observed within three days post-inoculation (219, 239), through direct and indirect mechanisms, was suggested by the presence or absence of IBDV antigen within the cells (161) and IBDV-positive and negative bursal follicles. In the bursa, the IM strain induces a significantly higher level of apoptosis than variant E or B2 strains. Although apoptosis in the thymus was observed, IBDV antigen was not found (221) and there is no evidence that the thymus lesions are caused by direct viral replication (206). Pathogenic changes in the bursa are also due to uncontrolled cytokine production (236, 237). More pathogenic strains induce a stronger interferon (INF) response (61) which activates chicken macrophages (41). An increase in macrophages plays an important role in the immunosuppression caused by IBDV (167). During IBDV infection, macrophages enhance expression of cytokines such as interferon type I, interleukins (IL)-6, and IL-8, and reactive species such as nitric oxide (111). Recent data suggested interference with INF-α and INF-γ transcription even when the infection is at subclinical form (181).

In addition, it was suggested that the recombinant INF-α inhibits IBDV replication both *in vivo* and *in vitro* (147). Several avian cytokines were tested as immunoadjuvants in IBDV

vaccination. Recombinant IFN-α, INF-β, and IFN-γ, expressed in *E. coli* was inoculated with inactivated D78 IBDV strain. They failed to induce a humoral immune response (203). Several other avian cytokines were tested as vaccine's adjutants, which stimulate T-cells proliferation (68, 69).

3.3 Cell mediated immune response

Cell mediated immunity and immunosuppression has been demonstrated in birds infected with IBDV alone (207, 208) or simultaneously with chicken anemia virus (32, 33). Administration of crude thymus extract in one day of age chickens prior, during, and following IBDV vaccination showed significant improvement in the immune response. Lymphoid hyperplasia in the bursa is observed in IBDV-vaccinated, thymus extract-treated group and only necrosis is observed in the control, IBDV-vaccinated, non-thymus-treated group. Protection in the thymus-treated group is 100% *vs.* 80% in the non-thymus treated group. Total protein, albumin, and globulin are also increased in the thymus-treated group (1).

The role of helper T-cells is demonstrated in T-cell compromised chickens challenged with an inactivated IM strain of IBDV. Ninety one percent of the T-cell compromised chickens did not have protection compared to the -non-T-cell compromised control group. Only 5% of the T-cell compromised chickens produce neutralizing antibodies in contrast to 58% in the control group. In the compromised group, passive administration of antibodies does not provide any protection against IBDV, which suggests that antibodies alone did not provide adequate protection (187, 188). Cyclophosphamide treated chickens are unable to produce antibodies. These birds challenged with the IM strain of IBDV could control the virus with the cell mediated immune response. Purified splenocytes respond poorly to concavaline A following challenge

with IBDV. The response to re-infection does not show suppression which is indicative of strong immunological cell mediated memory (252).

T-cell proliferation is inhibited by the virulent IM strain of IBDV. Expression of macrophage secreted cytokine, type I INF, chicken myelomonocytic growth factor (cMGF)-homolog of mammalian IL-6, and 9E3/CEF4-homolog of mammalian IL-8, is upregulated. Nitric oxide production is also elevated in cultured macrophages (86, 210). Suppressed activity of either bursal T-cells or supernatant from cultured T-cells from IBDV infected chickens is demonstrated by a slower mitogenic response of normal splenocytes. The majority of T-cells during IBDV infection are either $CD8^+/CD4^-$ or $CD8^-/CD4^+$, but the TCR phenotype of these cells has not been established yet (113).

One or two days following intraocular inoculation, $CD3^+$, T-cells accumulate between the cortex and medulla and in intrafollicular tissue in the bursal follicles and persists up to 13 days following the infection. Fewer $CD4^+$, T-cells are observed in the bursal follicles compared to $CD3^+$, T-cells. It is possible that $CD4^+$, T-cells stimulate B-cells to produce anti-IBDV antibodies in the bursa and other organs. Specific $CD8^+$ cytotoxic T-cells are found in the bursal follicles and in fewer numbers in cecal tonsils. $CD3^+/TCR2^+$ positive cells appear on the site where the viral antigens are present and disappear following viral clearance (220). The number of $CD4^+$TCR $\alpha,\beta\ 1^+$ and $CD8^+$TCR $\alpha,\beta\ 1^+$ cells increases in the bursa following IBDV infection (242). T-cells are needed for IBDV control during the acute phase of infection. Significantly higher levels of IBDV antigen are found in the bursa in T-compromised chickens when infected with IBDV. In T-cell compromised chickens, the number of apoptotic bursal cells is fewer, and the expression of IL-2 and IFN-γ is less than in the non-T-cell compromised group. Intrabursal T-cells are detected 4 days post-inoculation, and at 7 days, they compromise 65% of the bursal

cells, only 7% of which are B-cells. T-cell markers Ia and CD25, as well as, IL-6-like factor significantly increase in this organ. The bursal T-cell population is IBDV-dose dependent (112, 113). In T-cell compromised chickens inoculated with the intermediate vaccine Bursine-2, bursal cells were restored faster than in non-T-cell compromised chickens. The tumor necrosis factor, IL-2, and IFN-γ were upregulated in IBDV infected non-T-cell compromised chickens. Semi-quantitative real time PCR demonstrated a high concentration of viral genome in T-cell compromised chickens (187, 188).

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CHAPTER III

IDENTIFICATION OF TWO BURSAL B-CELL SUBPOPULATIONS WITH DIFFERENT FLOW CYTOMETRY PROFILES FOLLOWING INFECTIOUS BURSAL DISEASE VIRUS INFECTION[1]

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Abstract

Infectious bursal disease virus (IBDV) is an immunosuppressive virus which primarily infects IgM^+, B-cells in the bursa of Fabricius. It has been speculated that these are immature B-cells that provide for the chicken humoral immune response. Flow cytometric analysis was used to measure B-cell subpopulation kinetics in the bursa and spleen following IBDV infection. In the bursa, two B-cell subpopulations, designated as A and B, were identified based on cell size and granularity. The IgM^+, B-cell subpopulation B was significantly reduced following IBDV vaccination and challenge. Both subpopulations were phenotyped using B-cell surface expressed antigens. They demonstrated age-related changes such as a decrease of the proportion of subpopulation A and an increase of subpopulation B when compared with the total number of analyzed bursal cells. In addition, they express $Lewis^x$, IgM, Bu1b, MUI36, and 78 differentially but not MHCII surface antigens. The reduction of subpopulation B did not reduce the levels of total serum IgA, IgG, and IgM immunoglobulins nor did it affect IgG^+ and IgA^+, B-cells in the spleen.

We have identified IBDV-resistant and susceptible bursal IgM^+, B-cell subpopulations. The IBDV resistant subpopulation A B-cells most likely consists of immature cells which act to repopulate the bursa following IBDV infection.

Key words: IBDV; bursa B-cells subpopulations; flow cytometry; ELISA; humoral immune response; chicken

Abbreviations: Ab, antibody; d.a., days of age; d.p.c., days post challenge; d.p.v., days post vaccination; ELISA, enzyme linked immunosorbent assay; IBDV, infectious bursal disease virus; PBS, phosphate buffered saline

1. Introduction

Infectious bursal disease virus (IBDV) is a member of the *Birnaviridae* family (Dobos et al., 1979) that causes humoral and cellular immunodeficiency in chickens (Giambrone et al., 1977; Thompson et al., 1997; Sharma et al., 1994, 2000). In the bursa of Fabricius, IBDV infection results in follicular lymphoid depletion (Ley et al., 1983), a temporary reduction of proliferating (Muller, 1986) immature IgM^+, B-cells (Cheville, 1967; Hirai and Calnek 1979; Hirai et al., 1979 a.b.; Sivanandan and Maheswaran, 1980 a.b.; Nakai and Hirai, 1981; Ramm et al., 1991; Rodenberg et al, 1994; Vasconcelos and Lam, 1995; Nieper and Muller, 1996; Ojeda et al., 1997; Ogawa et al., 1998) and thus reduces the B-cell repertoire. Following IBDV infection, the bursa remains the functional primary lymphoid organ wherein bursal follicles are restored and B-cells are functionally active (Ivan et al., 2001) but with reduced proliferating responses (Peters et al., 2004).

The bursa is a major site for B-cell division, gene conversion, (Thompson and Neiman, 1987; Reynaud et al., 1987, 1989), and clonal differentiation (Pink et al., 1985; Ratcliffe et al., 1986; Reynaud et al., 1987; Ekino, 1993) containing more than 85% IgM^+, B-cells (Grossi et al., 1977; Ratcliffe et al., 1986, 2002; Ekino et al., 1995). In chickens, cell progenitor commitment to the B-lymphocyte lineage occurs during a short period of time (Ratcliffe et al., 1986) and immunoglobulin gene rearrangement is not a permanent process (Weill et al., 1986). B-cell

development consists of a pre-bursal, bursal, and post-bursal phase (Toivanen and Toivanen, 1973; Ratcliffe, 1989). Bursectomized and irradiated chickens at one day of age (d.a.) develop agammaglobulinemia and do not produce circulating antibodies (Cooper et al. 1966). Between 21 and 49 d.a. each of the ~10^4 bursal lymphoid follicles (Olah and Glick, 1978) have 1-3 x 10^5 cells originating from 1 to 10 (Pink et al., 1985 a.b.) stem cells with a specific VDJ rearrangement (Weill et al., 1986; Houssaint et al., 1976). Diversification of the immunoglobulin classes is in the order of IgM→IgG→IgA (Cooper et al., 1972). Some of the IgM^+ cells in the bursa switch to IgG synthesis and become IgM^+/IgG^+ double positive cells (Kinkade and Cooper, 1971; Cooper et al., 1972). Self renewed post-bursal stem cells populate the peripheral lymphoid organs (Cain et al., 1969; Cooper et al., 1969; Toivanen and Toivanen, 1973) before initial signs of bursal involution at approximately 56 d.a. (Bickford et al., 1985; Franchini and Ottaviani, 1999; Ciriaco et al., 2003). At 21 d.a. the renewal of two of the three B-cell subpopulations in the peripheral blood is still bursa-dependent (Paramithiotis and Ratcliffe, 1993; Paramithiotis et al., 1994, 1996). In addition, the bursal B-cell subpopulations have different abilities to populate bursal compartments and peripheral tissues (Toivanen and Toivanen, 1973; Toivanen, 1974; Paramithiotis and Ratcliffe, 1994) and they differentially express surface and cytoplasmic IgM (Grossi et al., 1977) and MHCII antigens (Ewert et al., 1984).

The effect of IBDV on B-cells in the bursa and spleen (Hudson et al., 1975; Hirai et al., 1979 a.b., 1981; Cloud et al., 1992; Rodenberg et al., 1994; Corley and Giambrone, 2002; Ivan et al., 2001) and on total serum immunoglobulins depends (Hudson et al., 1975; Ivanyi, 1975; Ivanyi and Morris, 1976; Giambrone et al., 1977; Hirai et al., 1979) on immunogenicity and pathogenicity of the strains (Rodriguez et al., 2002 a.b.c.; Rautenschlein et al., 2003), as well as, the time of infection (Ivanyi and Morris, 1976). The secondary immune response is normal

following IBDV infection at one day of age but the primary response has a marked suppressed level of IgG and enhanced IgM total serum immunoglobulins. In addition, the specific anti-IBDV response is non-existent following infection at one day of age, but moderate to high when chickens are infected at 21 d.a. (Ivanyi and Morris, 1976).

The precise IBDV cell target has not been identified *in vivo. In vitro* studies suggest that IBDV-resistant cells may be immature (Beug et al., 1981; Muller, 1986), competent B-cells (Becht, 1980) or small blood lymphocytes (Burkhardt and Muller, 1987). Earlier histopathological evaluation of the bursa suggests the existence of bursal IBDV resistant B-lymphocytes expressing surface determinants Bu1b (Pink and Rijnbeek, 1983; Veromaa et al, 1988 a.b.; Ivan et al., 2001) or MUI36 (Wison et al., 1988; Ramm et al., 1988, 1991). In addition, there is no clear understanding of IBDV affect on the total serum immunoglobulin levels.

Since IBDV causes a reduction of immature IgM^+, B-cell subpopulations IgA^+, IgG^+, and IgM^+, B-cell subpopulations are likely affected. Our objectives were to identify and phenotype the B-cell subpopulations susceptible to IBDV and those responsible for bursal regeneration.

B-cell subpopulation kinetics in IBDV infected chickens in the bursa (primary) and spleen (secondary) lymphoid organs using flow cytometric analysis were measured. In the bursa, two B-cell subpopulations, designated as A and B, with different flow cytometry profiles and kinetics were observed. Only subpopulation B IgM^+, B-cells was significantly reduced following challenge. The two subpopulations were phenotyped using B-cell surface expressed antigens. In addition, we determined the effect of IBDV on the total serum immunoglobulin levels.

2. Materials and methods

2.1. Animals and viruses

Day old female specific-pathogenic-free (SPF) Leghorn chickens were wing banded and housed in separate forced air, positive pressure (FAPP) isolation units (PDRC, University of Georgia) with feed and water given *ab libitum*. Chickens were arbitrarily allotted into four experimental groups (Table 3.1): (I) non-vaccinated non-challenged, (II) vaccinated non-challenged, (III) vaccinated challenged and (IV) non-vaccinated challenged. At 3 days of age (d.a.) twenty seven chickens, designated groups II and III received $10^{4.4}$ infectious dose (ID_{50}) of intermediate IBDV vaccine strain S-706 (Merial-Select Laboratories, Gainesville, GA) ocularly. At 24 d.a. eighteen chickens, designated groups III and IV received $10^{1.8}$ ID_{50} Edgar, chicken embryo adapted (CEA) IBDV strain (124-ADV-9501, National Veterinary Services Laboratories) orally.

2.2. Antibodies

The following primary antibodies were used for flow cytometry: mouse anti-chicken, heavy chain specific IgA (clone A-1, α), IgG (clone G-1, γ), and IgM (clone M-1, μ) (Chen et al., 1982) antibodies (Abs) coupled to fluorescein isothiocyanate (FITC) and mouse anti-chicken Bu1a (clone 21-1A4) and Bu1b (clone 5-11G2) Abs against B-cell surface alloantigens (Veromaa et al., 1988 a.b.) coupled to R-phycoerythrin (PE) (Southern Biotechnology Associates, Inc., Birmingham, AL) and mouse anti-chicken MHC class II-conjugated with FITC

(Vainio et al., 1988 a.b.) (Serotec Inc., Raleigh, NC). The Abs were used at dilutions recommended by the manufacturer. The anti-Lewis^x Ab, MC 480 (Developmental Studies Hybridoma Bank, University of Iowa, 1.2 mg/ml) was used at 1:500 dilution and stained with FITC conjugated AffiniPure F(ab')^2 fragment goat anti-mouse IgM, μ chain specific at dilution 1:100 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Mouse anti-chicken MUI36, 78, and 79 Abs (IgG2a) were kindly provided by Dr. Richard Boyd (Monash University, Australia) and used at dilutions 1:300, 1:30, and 1:30, respectively and stained with FITC conjugated anti-mouse IgG (Jackson ImmunoResearch).

2.3. Preparation of cell suspensions and B-lymphocytes identification and phenotyping

The bursa and spleen were aseptically collected from 3 chickens at 3, 10, 17, 24, 31, 38, and 45 d.a. and placed in Dulbecco's modified Eagle's medium and stored on ice. Single cell suspensions were prepared using 70 μm mesh screen. Cells were counted using the trypan blue exclusion method with a hemocytometer as previously described (Drake et al., 1972). B-cell populations were identified using unpurified, unfixed, single-cell suspensions from the bursa and spleen as previously described (Burgess and Davison, 1999). Briefly, 1x10^6 cells were washed twice with PAB buffer containing 1x phosphate-buffered saline, 0.1% sodium azide, and 0.5% Bovine serum albumin, incubated for 30 min on ice with 2 μl of anti-chicken Bu1a or Bu1b, washed twice with PAB buffer, incubated for additional 30 min with 1μl of anti-chicken IgA, IgG, or IgM, and washed a second time twice with PAB buffer. Single cell suspensions from spleen and bursa from each time point were collected and stained with IgA, IgG, and IgM.

Bursal subpopulations were phenotyped using bursal cell suspensions collected from 3 female SPF Leghorns, every 7 days during two time periods: 3, 10, 17, 24, 31, 38, 45, and 90,

97, 104, 111, 118, and 125 d.a. The cell suspensions were stained with one of the following primary antibodies: MHCII, IgM, and Bu1b in PAB buffer as described above (Lee et al., 1996). The antibodies anti-Lewis^x, MUI36, 78, and 79 were used as previously described (Masteller et al., 1995 a.b.). Briefly, $1x10^6$ cells were resuspended in 100 µl Dulbecco's modified Eagle's medium containing 2% fetal bovine serum, 10 mM HEPES, 0.1% sodium azide, incubated with the appropriate antibody on ice for 30 min, and washed twice. Goat anti-mouse $F(ab')_2$ IgM-FITC and IgG-FITC antibodies were utilized as secondary antibodies for anti-Lewis^x and MUI36, 78, and 79 antibodies, respectively. Samples were incubated on ice for 30 min and washed twice with the medium. All centrifugation steps were run at 300 g for 5 min. A total of 20,000 events were collected per sample. The cell preparations were analyzed with the FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Ca.) and Summit™ Software (Dako Cytomation, Carpinteria, CA).

2.4. Histopathology

Bursa samples were taken at 7, 14, and 21 days post challenge (d.p.c.) for histopathological examination. Briefly, bursas were fixed in 10% neutral buffered formalin. All sampled tissues were routinely processed into paraffin and 3-µm sections were cut and stained with hematoxylin and eosin as previously described (Thompson and Samuel, 1966).

2.5. ELISA

Serum was collected from three chickens per group at 3, 10, 17, 24, 31, 38, 45 d.a. Specific IBDV antibody levels were measured with the IBDV-XR (IDEXX Laboratories, Inc., Westbrook, ME) ELISA kit. Total serum IgG, IgA, and IgM levels were measured with a commercially obtained ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX). Sera from all groups were also tested against infectious bronchitis virus, Newcastle disease virus, reovirus, and chicken anemia virus using ELISA kit (IDEXX).

3. Results

3.1. Effect of IBDV on B-lymphocytes

In the bursa, two B-cell subpopulations with different granularity and cell size, designated as A and B, respectively were observed following staining with IgM and Bu1b antibodies (Fig 3.1A). The two identified subpopulations differed in granularity and cells size. A similar staining was observed with bursa cells stained with Bu1a and IgA^+ and IgG^+ cells. The B-cells from spleen were presented as a single population.

Subpopulation A IgA^+ cells, increased in the vacc/non-chall group at 7 days post vaccination (d.p.v.) and in the non-vacc/chall group between 7-21 d.p.c. (Fig. 3.2A). Subpopulation B IgG^+ cells, increased only in the non-vacc/chall group between 7-21 d.p.c. (Fig. 3.2B). Subpopulation B IgM^+ cells were decreased slightly at 7 days post vaccination (d.p.v.). While the same subpopulation was not reduced in the vacc/chall group in the non-vacc/chall group it was decreased at 7, depleted at 14, and slightly increased at 21 d.p.c. (Fig. 3.2C, 3.1B,

and 3.1B.1). Subpopulation A IgM^+ cells did not change overtime between the four groups (Fig. 3.2D).

In the spleen the percent IgM^+ cells in non-vacc/chall group following challenge increased at 7 d.p.c. then decreased at 14 and 21 d.p.c. in comparison to non-vacc/non-chall group. In the spleen no differences were observed for IgG^+ and IgA^+ B-cells between the four groups.

3.2. Histopathology

No differences in the bursa were detected between non-vacc/non-chall and vacc/chall groups (Fig. 3.1C and C.1). Remnants of bursal follicles with epithelial cells, fibroplasias, and macrophage infiltration along with diffuse lymphoid depletion were visible with severe lymphocytolysis in the bursas from non-vacc/chall group at 7 and 14 d.p.c. Follicular regeneration was observed in the same group at 21 d.p.c. No pathological changes following challenge were observed in the vacc/non-chall and vacc/chall groups in comparison to the non-vacc/non-chall group.

3.3. Phenotypic characteristics of subpopulation A and B bursal B-lymphocytes

Bursal subpopulation A and B were phenotyped using B-lymphocyte surface expressed antigens and flow cytometric analysis. At 3 d.a. subpopulation A was close to 50% of the total analyzed bursal cells and decreased overtime to 25% at 125 d.a. (Fig. 3.3D). At 3 d.a.

subpopulation B was less then 20% and at 125 d.a. reached 54% from the total analyzed bursal cells. Subpopulation B had higher percent of cells than subpopulation A following 97 d.a.

The intensity of the fluorescent signals from MUI36 and 78 surface antigens were stronger and the percent positive cells in subpopulation B were higher then in A between 3-125 d.a. (Fig. 3.3C). The MUI36 percent positive cells in subpopulation A and B was approximately 50% and 90%, respectively at all time points. The percent $Bu1b^{+}$ cells in subpopulation A and B were approximately 100% and 58%, respectively (Fig. 3.3C) and did not change overtime.

The MUI78 percent positive cells at 3 d.a. from subpopulation A and B was 32 and 40% and at 125 d.a. was 35 and 78%, respectively (Fig. 3.3A). In the two subpopulations the percent MHCII positive cells was close to 95% and no differences were observed between both subpopulations between 3-125 d.a. (Fig. 3.3A). There was no detectable signal from the MUI79 antigen at all time points.

No differences in the percent $Lewis^{x}$ positive cells between subpopulation A and B were observed during 3-31 d.a. Interestingly from 38 d.a. to the end of the experiment the percent positive cells in both subpopulations decreased. In subpopulation B staining was no less than 76%, however, in subpopulation A only 40% of the cells were positive at the last time point at 125 d.a. (Fig. 3.3B). Between 104 and 125 d.a. fewer IgM positive cells was observed in subpopulation A than in subpopulation B.

3.4. ELISA

Specific IBDV seroconversion in the vacc/non-chall group was observed at 14 d.p.v., peaked at 28 d.p.v., and was undetectable at 34 d.p.v. The IBDV titer in vacc/chall group peaked

at 7 d.p.c., was higher than in vacc/non-chall and non-vacc/chall groups, and negative at 21 d.p.c. The IBDV titer in non-vacc/chall group peaked at 7 d. p.c. was higher than in the vacc/non-chall group through 21 d. p.c. (Fig. 3.4D). Total serum IgA was elevated in the vacc/chall group between 7-34 d.p.v. and in the non-vacc/chall group between 7-21 d.p.c. (Fig. 3.4A). Total serum IgG was decreased in the non-vacc/chall group at 7 d.p.c. and elevated between 14-21 d.p.c. (Fig. 3.4B). Total serum IgM was elevated only in vacc/non-chall group at 7 d.p.v. and in the non-vacc/chall group at 7 d.p.c. (Fig. 3.4C).

4. Discussion

This study examined the effect of IBDV vaccination and/or challenge on B-cell populations and serum immunoglobulins. B-cell kinetics and subpopulation phenotype were analyzed following IBDV vaccination and challenge using surface expressed antigens and flow cytometric analysis. In the bursa, we observed two B-cell subpopulations designated as A and B with different flow cytometry profiles and kinetics. In addition, specific IBDV seroconversion and total serum immunoglobulin levels were measured following IBDV infection.

It was previously suggested that the age of IBDV infection is a critical factor for the humoral immune response (Hudson et al., 1975; Ivanyi, 1975; Giambrone et al., 1977; Hirai et al., 1979, 1981; Ivan et al., 2001Cloud et al., 1992; Rodenberg et al., 1994; Corley and Gimbrone, 2002) and disease outcome (Ivanyi and Morris, 1976). The only reduction in percent B-cells following IBDV infection was observed subpopulation B bursal IgM^+ cells. This subpopulation was slightly reduced following vaccination and gradually depleted in non-vacc/chall group following challenge. The different level of cell reduction may be explained by

lower numbers of susceptible cells at 3 d.a. Bursal follicle restoration and an increase in subpopulation B IgM^+ cells at 21 days post challenge (d.p.c.) was observed. The rapid bursal follicle B-cell repopulation following challenge with the nonattenuated Edgar strain indicates the presence of IBDV nonsusceptible immature B-cells which are at an earlier developmental stage than subpopulation B. Subpopulations A and B IgM^+ cells in vacc/chall group following challenge were not affected by IBDV.

Subpopulation A and B were phenotyped by subpopulation cell number and B-cell surface expressed antigens during two time periods 3-45 d.a. and 90-125 d.a. Age-related structural and functional changes in the bursa have been previously studied. Although there are differences between genders and bird species, in general rapid growth of the bursal follicles occurs within the first 28 d.a., signs of functional involution are observed around 56 d.a. with complete bursal involution at approximately 200 d.a. (Bickword et al., 1985; Franchini and Ottaviani, 1999; Ciriaco et al., 2003). Interestingly, the reduction of subpopulation A and increase of subpopulation B occurs between 97-125 d.a., a period of initial bursal functional involution.

A close correlation between percent $Bu1b^+$ and $MUI36^+$ cells from both subpopulations between 3-125 d.a. was observed. IBDV resistant $Bu1b^+/Lewis^{x-}$ and $MUI36^+$ cells at the bursal cortico-medullary junction were previously reported (Ivan et al., 2001; Ramm et al., 1991). $Bu1b^+$ cells repopulate bursal follicles depleted by IBDV before the functionally active $Lewis^{x+}$ cells are detected (Ivan et al., 2001). The Bu1 is an early B-cell surface antigen which is expressed before Ig rearrangement (Rothwell et al., 1996) and has been used as a marker for B-cell clone differentiation (Pink et al., 1985 a.b.; Houssaint et al., 1987 a.b.). This antigen is not restricted to cell cycle, immunoglobulin, major histocompatibility complex (MHC), and B-cells.

It is also expressed on macrophages and monocytes (Pink and Rijnbeek, 1983; Veromaa et al., 1988 a.b.) but not on plasma cells (Houssaint et al., 1987 a.b.). The MUI36 antigen is a B-cell determinant and is not Ig, MHCII, or B-cell restricted. Initially $MUI36^{+}$ cells are distributed evenly in the bursal follicle and later restricted to plasma cells. While the percent $Lewis^{x}$ positive cells in both subpopulations did not differ during 3 and 31 d.a. there was a reduction in positive cells in subpopulation A starting from 38 d.a. The expression of $Lewis^{x}$ during the bursal phase of avian B-cell differentiation is a stage-dependent process. It was suggested that similar to sialyl $Lewis^{x}$ the terminal oligosaccharide $Lewis^{x}$ is important for cell adhesion and homing. The $Lewis^{x}$ initial expression coincides with the initial immunoglobulin gene conversion and subsequently is down-regulated with the bursal lymphocytes maturation (Masteller et al., 1995 a.b;.Lee et al., 1996). In addition, although the IBDV cell receptor is not identified it was suggested to contain N-glycosylated protein (Ogawa et al., 1998).

The major histocompatibility complex has been linked to immune response against IBDV and MHCII was suggested to restrict the T-cell dependent secretory antibody response against this virus (Juul-Madsen et. all, 2002; Hudson et al., 2002). Most of the cells in both subpopulations were MHCII positive between 3-125 d.a. MHCII is expressed on antigen presenting cells and is involved in presentation and recognition by T-cells (Vainio et al., 1988). MHCII expression coincides with IgM expression and increases during the bursal embryonic phase of B-cell differentiation. It is not B-cell restricted and is also expressed on Mø and monocytes (Ewert et al., 1984). Initially at 3 d.a. the number of $MUI78^{+}$ cells in subpopulation A and B was similar. However, between 90 and 125 d.a., the number of $MUI78^{+}$ cells in subpopulation B was higher than in subpopulation A. MUI78 is an MHCII restricted antigen also

expressed on Mø-like and dendritic cells. The MUI79, a Mø marker (Ramm et al., 1988, 1991), was not expressed in both populations.

Bursal regeneration is necessary following IBDV infection for normal antibody response (Edwards et al., 1982; Kim et al., 1999). The effect of IBDV on the bursa resembles a bursectomy, which performed at different age affects different immunoglobulin classes. B-cell commitment is irreversible and an age-dependent process in the order of IgM→IgG→IgA (Kincade and Cooper, 1971; Cooper et al., 1972; Ekino et al., 1995). IgM^+ cells leave bursa prior to IgG^+ and bursectomy prior the IgM→IgG switch inhibits IgG expression (Van Alten et al., 1968; Cooper et al., 1969). Bursectomy at early embryonic stage leads to complete agamaglobulinemia, at later stage to lack of IgG but above normal IgM synthesis (Cooper et al., 1969). IgA^+, B-cells seed the peripheral organs after IgG^+ cells and bursectomy prior embryonic day 18 will lead to lack of IgA synthesis (Kincade and Cooper, 1973; Leslie et al., 1976). Although there was significant reduction of subpopulation B in non-vacc/chall group following challenge the immunosuppression may be temporary. The non-bursal lymphoid sites help in restoration of the humoral function in B-cell depleted animals (Ratcliffe et al., 1986; Houssaint et al., 1989, 1991) as theses sites can partially replace the bursal microenvironment (Lerner et al., 1971; Bryant et al., 1973). The bursal lymphocyte number is dependent on B-cell proliferation, apoptosis, and migration of mature cells to the peripheral lymphoid organs (Lassila, 1989; Paramithiotis and Ratcliffe, 1994; Paramithiotis et al., 1995). Although IgM^+, B-cells in the bursa have undergone Ig V(D)J recombination and gene V conversion (Reynaud et al., 1987; McCormack et al, 1989) expends few weeks after hatching (Reynaud et al., 1986) the hyperconversion process continues until bursa involutes (Masteller et al., 1995). Infection with

IBDV hinders this process and most likely decreases the B-cell repertoire when chickens are infected at early stage but not after 21 d.a. (Fitzsimmons et al., 1973; Huang and Dreyer, 1978).

Subpopulation A IgG^+ cells in the non-vacc/chall group following challenge and IgA^+ cells in the vacc/non-chall and non-vacc/chall groups following vaccination and challenge, respectively were increased. In a previous study, a significant difference in IgA^+ cells between vacc/non-chall and non-vacc/non-chall groups was not observed using flow cytometry (Rodenberg et al., 1994). The differences between the two studies may be due to the strain variations or the infectious dose used in the experiments.

The percent positive B-cell cells in the spleen did not change between vacc/non-vacc and non-vacc/non-chall groups. In the non-vacc/chall group IgM^+ cells decrease post challenge. The same observation was made in a previous flow experiment (Cloud et al., 1992). However, in a different study IgG^+ and IgM^+ B-cells in the spleen decreased post-IBDV infection (Hirai et al., 1981). The differences may be due to the antibody reactivity used in the previous experiments. We have not differentiated the percent IgM^+ cells that are also IgG^+. It was reported that IgG^+ cells in the bursa may be double-positive IgG^+/IgM^+ (Kincade and Cooper, 1971; Ekino et al., 1995) but most of the IgG is exogenous, trapped as an IgG-antigen complex (Ekino et al., 1995). Some of the IgG can also originate from plasma cells surrounding the bursal follicles (Cooper et al., 1966, 1972).

The specific secretory IgA plays a significant role in the mucosal immunity (Tomasi and Bienenstock, 1968) and the total serum IgA was elevated in both vacc/non-chall and non-vacc/chall groups following vaccination and challenge, respectively. We did not observe an antibody class-switch the IgM→IgG in vacc/chall group as was previously suggested (Wu et al., 1998 Butter et al., 2003). The total serum IgM was elevated only for short period in the

vacc/non-chall and non-vacc/chall group following vaccination and challenge. The total serum IgG was reduced in the non-vacc/chall group following challenge in comparison to the other three groups but elevated in the next two time points. The specific IBDV seroconversion in the non-vacc/chall group was elevated faster, higher, and persisted longer than in the vacc/non-chall group. At 7 d.p.c. in the non-vacc/chall group when the specific IBDV titer peaked, the total serum IgG was at its lowest level. In the vacc/chall group titers decreased quickly following challenge when compared to the other two groups. The differences of the serum immunoglobulin levels may be due to IBDV strain variations. Neutralization assays demonstrated significant difference between antigenic and immunogenic variants of IBDV. More virulent strains induce higher anti-IBDV titers and the viral antigen persists for longer time (Rodriguez et al., 2002 a.b.c.; Rautenschlein et al., 2003). In our experiments, the total serum immunoglobulin concentrations were lower than in non-SPF chickens and we were unable to measure total serum IgA and IgM at 3 d.a. Previously, it was reported that in SPF chickens IgG^+ and IgA^+ plasma cells were detected as early as 14 d.a. and IgM^+ at 5 d.a. (Jeurissen et al., 1989). The germ-free environment hinders B-cell development in peripheral lymphoid organs (Kincade and Cooper 1971; Honjo et al., 1993) due to inadequate antigenic stimuli. Bursa isolated at nineteen day of embryonation from environmental antigen leads to decreased numbers of IgG^+, B-cells and reduces B-cells proliferation (Ekino et al., 1980, 1995; Sayegh and Ratcliffe, 2000). In SPF chickens the cytoplasmic IgM, μ chain peak is delayed and the surface μ chain has lower expression (Ramm et al., 1988) than in normal chickens.

The two subpopulations co-express the B-lymphoid surface antigens $Lewis^x$, IgM, Bu1b, MUI36, MUI78, and MHCII. With the exception of MHCII, all are expressed differentially. The percent positive cells for these antigens in subpopulation B were higher than in subpopulation A.

In addition, the cell number and the percent MUI78^{+} or Lewis^{x+} cells are age-dependent. After 90 d.a MUI78 is up-regulated in subpopulation B while Lewis^{x} is down-regulated in subpopulation A.

In conclusion, IBDV resistant and susceptible bursal IgM^{+}, B-cell subpopulations were identified. The IBDV resistant subpopulation A most likely consists of immature cells which act to repopulate the bursa following IBDV infection. The subpopulation B was decreased following IBDV vaccination and challenge. The subpopulation B depletion did not reduce the total serum IgA, IgG, and IgM immunoglobulin levels nor did it affect IgG^{+} and IgA^{+}, B-cells in spleen.

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Figure legend

Fig. 3.1. Representative dot plots of flow cytometry data showing two bursal IgM^+, B-cells subpopulations. Subpopulation A and B dual stained with IgM and Bu1b antibodies coupled to R-phycoerythrin (PE) and fluorescein isothiocyanate (FITC), respectively (A). Compellation of dot plots of flow cytometry data and bursa samples stained with hematoxylin and eosin from vacc/chall (B and C) and nonvacc/chall (B.1 and C.1) groups. The groups were vaccinated at 3 days of age and challenged at 24 days of age. Arrows indicate subpopulation A and B. Significant reduction of subpopulation B IgM^+, B-cells was observed only in the non-vacc/chall group.

Fig. 3.2. Kinetics of B lymphocyte in bursa measured with flow cytometry. A) IgA^+, B-cells, subpopulation A; B) IgG^+, B-cells, subpopulation B; C) IgM^+, B-cells, subpopulation A, and D) IgM^+, B-cells, subpopulation B. Arrows indicate IBDV vaccination and challenge.

Fig. 3.3. Phenotyping subpopulation A and B, B-lymphocytes in bursa with B lymphocyte surface antigens using flow cytometry: A) MHCII and MUI78; B) $Lewis^x$; C) Bu1b and MUI36, and D) Nonstained subpopulation A and B kinetics.

Fig. 3.4. Total serum immunoglobulin levels A) IgA, B) IgG, and C) IgM and D) anti-IBDV titers. Arrows indicate IBDV vaccination and challenge.

Table 3.1
Identification of susceptible and resistant B-cell subpopulations. Experimental design.

Group	Dose	Age of inoculation (days)	Days after inoculation							Birds
			0	7	14	0 21	7 28	14 35	21 42	
I Non-vacc / Non-chall	n.a.[a]	n.a.	+	+	+	+	+	+	+	21
II Vacc / Non-chall	$10^{4.4}$[b]	3		+	+	+	+	+	+	18
III Vacc / Chall	$10^{1.8}$	3, 24					+	+	+	9
IV Non-vacc / Chall	$10^{1.8}$	24					+	+	+	9

Total number of birds										57

[a]n.a. = not administered
[b]= infectious dose ID_{50}
+ = bursa, spleen, and serum samples were taken

Fig. 3.1.

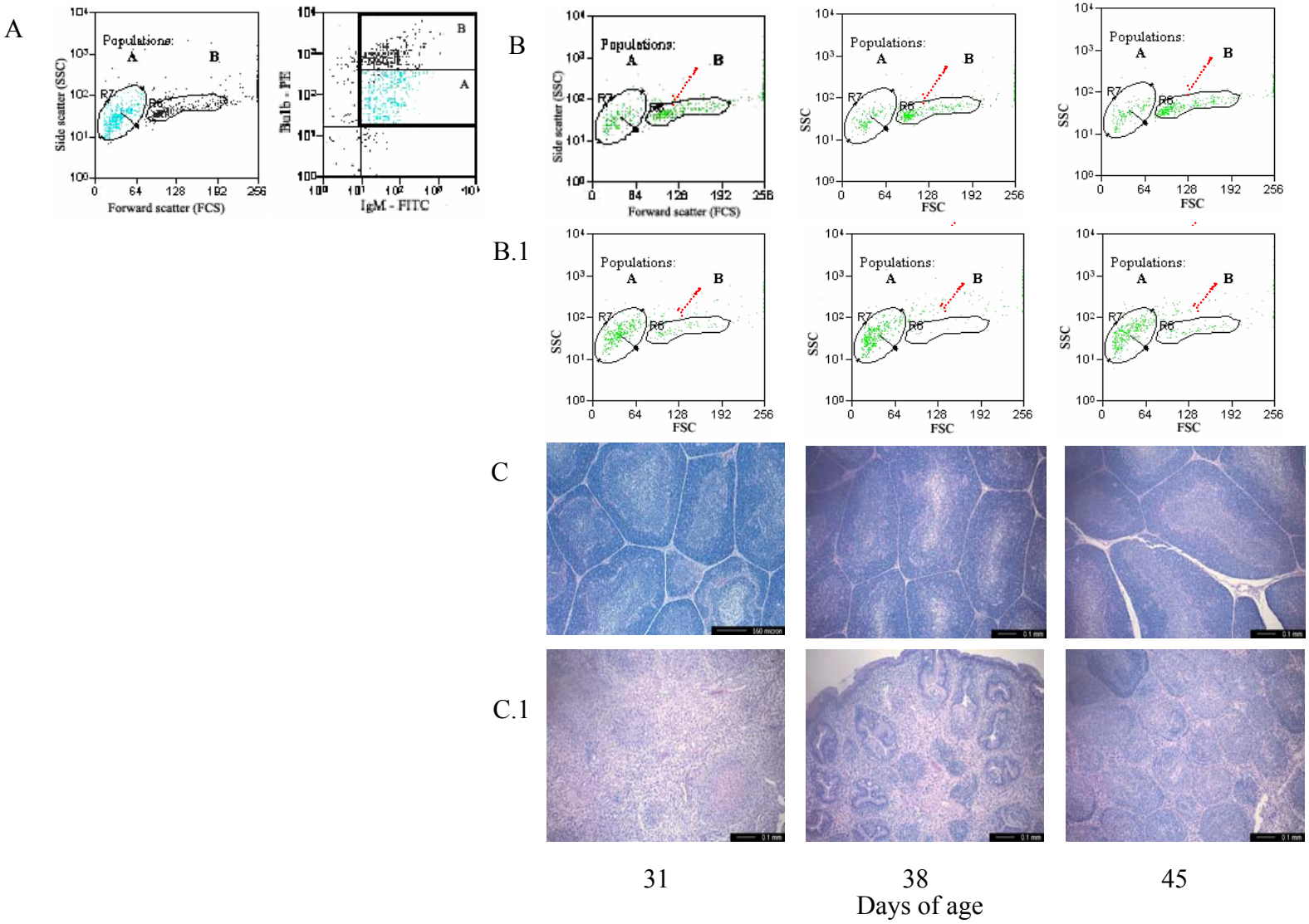

A
B
B.1
C
C.1
Populations:
A
B
R7
R6
Side scatter (SSC)
Forward scatter (FCS)
Bu1b - PE
IgM - FITC
SSC
FSC
31
38
45
Days of age

Fig. 3.2.

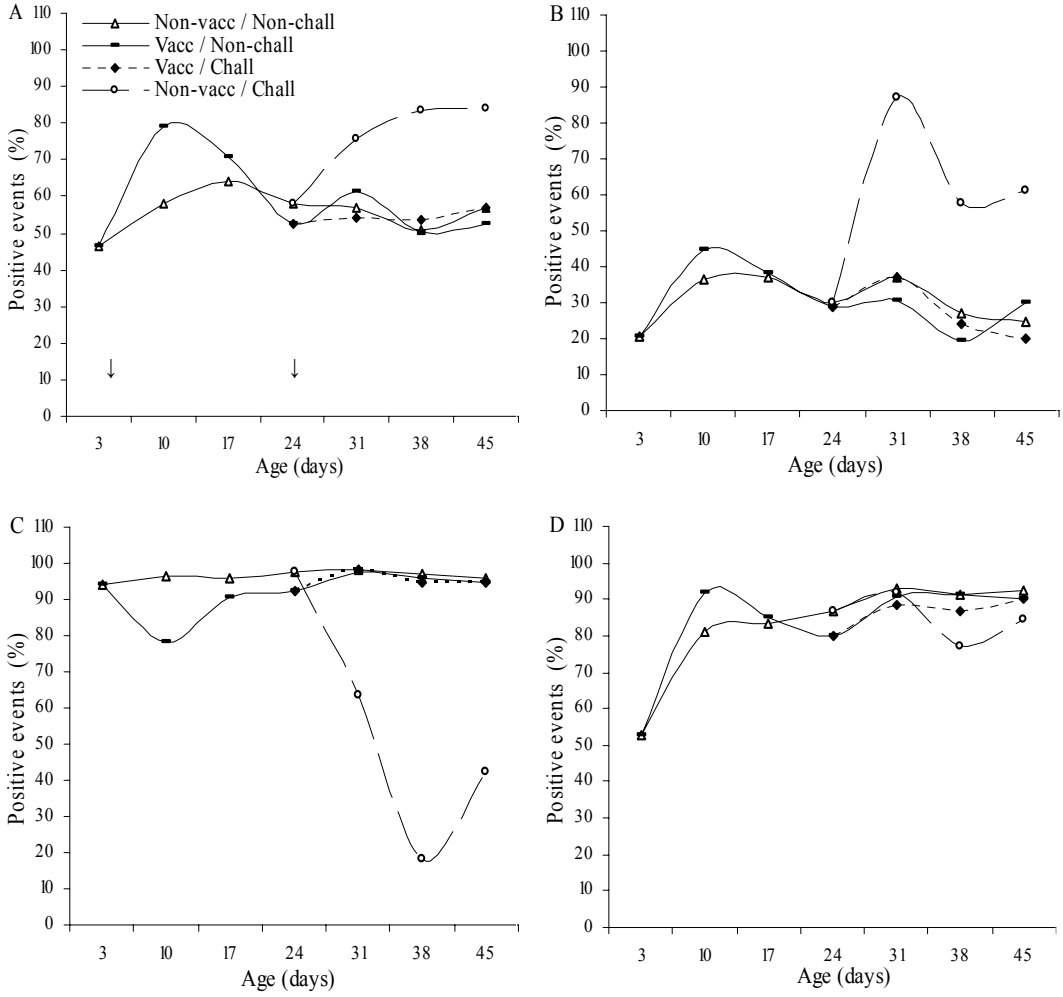

A
B
C
D
Non-vacc / Non-chall
Vacc / Non-chall
Vacc / Chall
Non-vacc / Chall
Positive events (%)
Age (days)
0
10
20
30
40
50
60
70
80
90
100
110
3
10
17
24
31
38
45

Fig. 3.3.

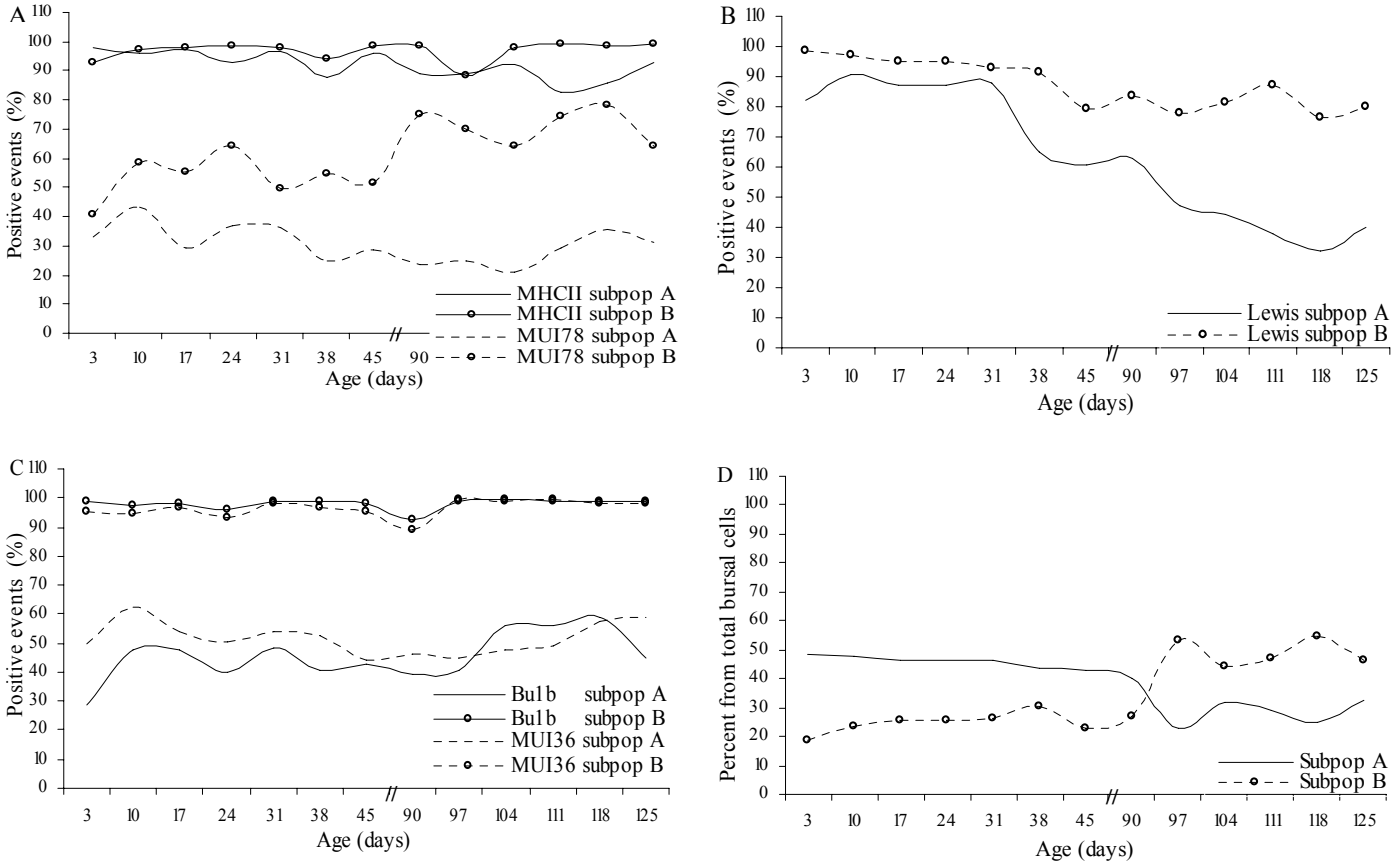
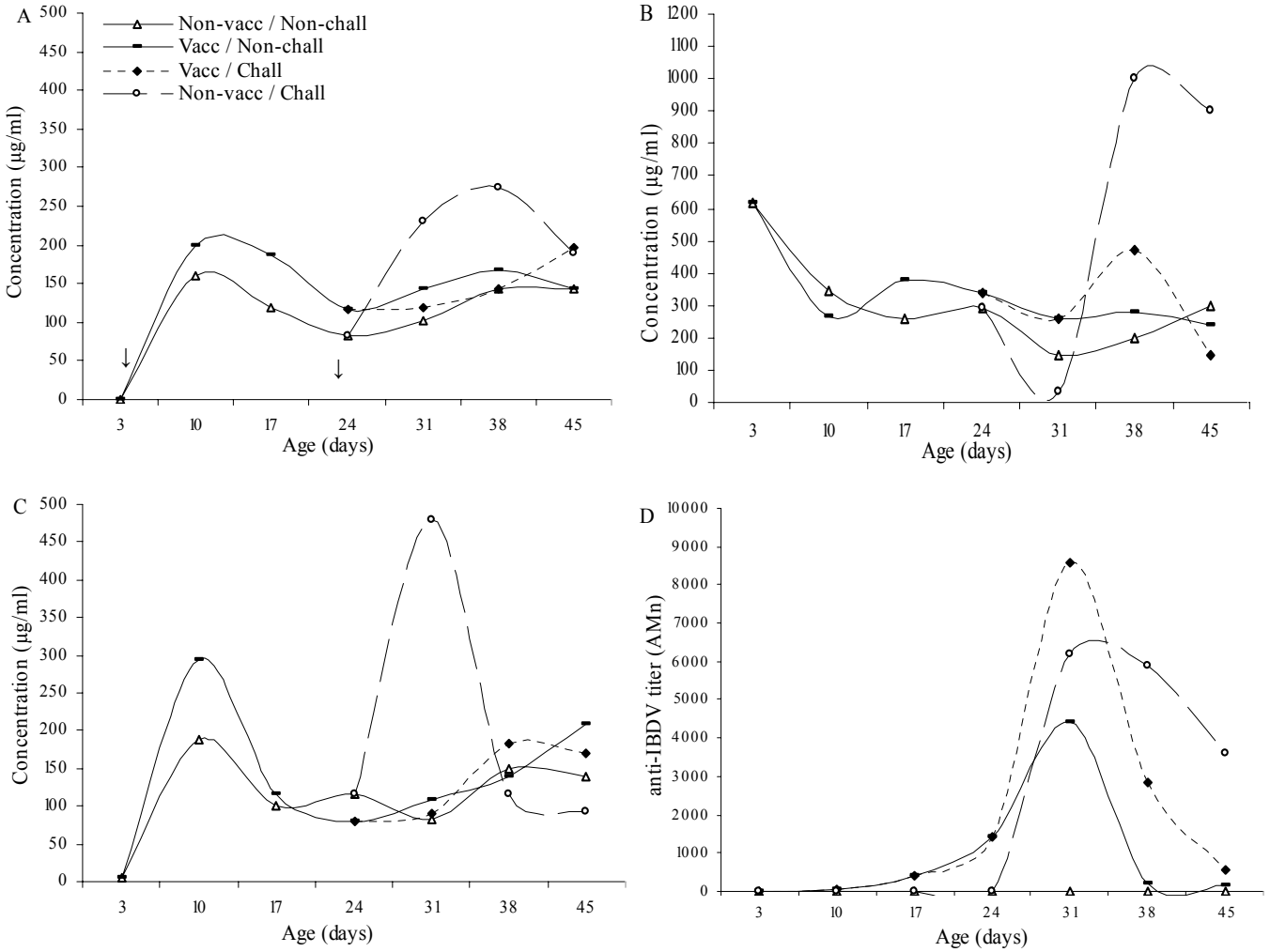

A
Positive events (%)
Age (days)
MHCII subpop A
MHCII subpop B
MUI78 subpop A
MUI78 subpop B
B
Positive events (%)
Age (days)
Lewis subpop A
Lewis subpop B
C
Positive events (%)
Age (days)
Bu1b subpop A
Bu1b subpop B
MUI36 subpop A
MUI36 subpop B
D
Percent from total bursal cells
Age (days)
Subpop A
Subpop B

Fig. 3.4.


A
Non-vacc / Non-chall
Vacc / Non-chall
Vacc / Chall
Non-vacc / Chall
Concentration (µg/ml)
Age (days)
B
Concentration (µg/ml)
Age (days)
C
Concentration (µg/ml)
Age (days)
D
anti-IBDV titer (AMn)
Age (days)

CHAPTER IV

MOLECULAR CHARACTERIZATION OF FOUR IBDV STRAINS WITH DIFFERENT PATHOGENICITIES USING FULL-LENGTH SEQUENCE ANALYSIS[1]

[1]Daniel I. Petkov, Erich G. Linnemann, Darrell R. Kapczynski, and Holly S. Sellers. To be submitted to *Virus Genes*.

Abstract. Characterization of field isolate 9109, Lukert, Edgar cell culture-adapted (CCA), and Edgar chicken embryo-adapted (CEA) serotype 1 IBDV strains using full-length genomic sequences is reported. IBDV genomic segments A and B were sequenced and the nucleotide and deduced amino acid (aa) sequences were compared with previously reported full-length sequenced IBDV strains. We found that the viral protein VPX and amino acid sequences between aa 202-451 and 210-473 but not the VP2 protein are the best representatives of the entire IBDV genome. The greatest variability was found in the VP2 and 5' noncoding region of segment B among IBDV strains. The deduced amino acid sequences of the VP1 protein varies in length among the strains analyzed. The RNA-dependent, RNA-polymerase motifs within VP1 and the VP5 protein were highly conserved among isolates.

Although within the VP2 processing site amino acid sequence of Lukert was similar to the classical while the Edgar CCA and CEA were more similar to the very virulent strains, it was determined that these strains have sequence characteristics of the classical strains. In addition, close relatedness between Lukert, Edgar CCA and CEA was observed. Although phylogenetic analysis of the VP1, VP3, and VP4 proteins indicated that 9109 is a classical type virus, this isolate shares unique amino acid changes with very virulent strains within the same proteins. Phylogenetic analysis of the 3' and 5' noncoding regions of segment A revealed that 9109 is more similar to the very virulent strains compared to the classical strains. In the VP2 protein several amino acids were conserved between variant E and 9109 strains. Thus it appears that 9109 isolate has characteristics of classical, very virulent, and variant strains.

Our analysis indicates that although VPX amino acid comparison may be initially useful for molecular typing, full length genomic sequence analysis is essential for complete molecular characterization.

Key words: IBDV, sequencing, phylogenetic analysis

Introduction

Infectious bursal disease virus (IBDV) causes lymphoid depletion in the bursa of Fabricius, a primary lymphoid organ of chickens. Infected chickens are immunosuppressed, can be predisposed to secondary infections, and respond poorly to immunization (1, 2, 3, 4, 5). Infectious bursal disease has a great economic impact on the poultry industry worldwide (6).

Two serotypes of IBDV have been identified (7), however, only serotype 1 viruses cause disease in chickens. Strains within serotype 1 differ in their pathogenicity and antigenicity, and are generally classified as very virulent (8, 9), classical (10), or antigenic variants (11, 12, 13).

IBDV is a non-enveloped, bi-segmented (14), double stranded RNA virus belonging to the *Birnaviridae* family (15). Segment A is approximately 3,260 nucleotides (nts) and contains two open reading frames (ORFs). The ORF1 (438 nts) partially overlaps ORF2 (3,039 nts) (16,17, 18) and encodes the viral VP5 protein (19). The monocistronic ORF2 encodes a precursor polyprotein VPX-VP4-VP3 and is auto-catalytically cleaved at amino acids (aa) A^{512}-A^{513} for VPX-VP4 and A^{755}-A^{756} for VP4-VP3 (20,21). The VPX protein, aa 1-512, is processed to VP2 which encodes for 441 aa protein (22). Most of the research has been focused on the VP2 protein, determinant for cell tropism (23,24) and inducing neutralizing antibodies (25). It has been demonstrated that changes within VP2 antibody binding amino acid region at position 206-350 (26) can lead to antigenic variants (11,27, 28). The group-specific VP3 protein (29, 30) induces non-neutralizing antibodies (31) and may be involved in replication and genome packaging (32). VP4 is a *cis*-acting viral protease that processes the polyprotein (1, 26, 33).

Segment B is approximately 2827 nts and encodes for VP1, the RNA-dependent, RNA-polymerase (RdRp) (34, 18, 35). The protein plays a role in viral encapsidation (36) and primes

the viral RNA synthesis (35). In addition, VP1 initiates protein synthesis by resolving 5' unique terminal repeats and recruits the 40S ribosomal subunit (37). The RdRp activity is 3' non-coding region (NCR) dependent on segment A and B. The 3' NCR has a signal recognition function for replication, transcription, and translation (38). The replication efficiency and virulence factors have been linked to segment B (39, 40). Although the VP1 protein of the very virulent (vv)IBDV strains form a distinct cluster separate from the classical strains (41), chimeric IBDV viruses containing segment A and B from classical and very virulent strains, respectively do not show increase of the viral virulence or pathogenicity (42,43).

The level of attenuation and the system used for viral propagation affects the pathogenicity of related viruses. The Lukert strain is a further attenuated Edgar cell culture adapted strain (40). The Edgar, bursa-derived strain is a pathogenic field isolate (44) and compared with both Edgar CEA and Lukert strains is the most invasive and pathogenic (45,46).

Most of the current molecular techniques used for IBDV typing utilize amplification of representative genomic sequence (47) or VP2 fragment by reverse transcriptase (RT) polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis (48,49). The most widely used typing method is the amplification of VP2 gene, 743 nucleotides (nts) fragment between 701-1444 nts of ORF2 using RT-PCR followed by digestion with restriction enzymes. Different strains can be grouped into genotypes based on restriction fragment length polymorphism (RFLP) patterns (16, 50, 51). It has been previously suggested that VP2 is not the only virulent determinant (43, 52) and that viral pathogenicity can be determined by genomic regions beyond this protein (53).

Characterization of IBDV strains with different pathogenicities: field isolate 9109, Lukert, Edgar CCA, and Edgar CEA strains is reported. We have sequenced the full-length

genomes of these strains. Analysis of the nucleotide and deduced amino acid sequences of segments A and B were compared with previously reported full-length sequences from serotype 1 very virulent, classical, antigenic variants, and serotype 2 IBDV strains. Our analysis revealed that although amino acid sequence of VPX may be useful, complete genomic sequence information for strain characterization is essential for complete molecular typing.

Materials and methods

Viruses

The field isolate 9109 and the classical attenuated Lukert strain were kindly provided by Dr. Pedro Villegas, University of Georgia, Athens, GA. The 9109 isolate was isolated at the Poultry Diagnostic and Research Center, University of Georgia from immunosuppressed broiler flocks showing subclinical signs of IBDV, bursal atrophy, and minimal mortality. In addition, 9109 displays unique RFLP pattern when compared to the reference strains used for genotyping at PDRC. Edgar cell culture-adapted (CCA) (120-ADV-2001) and Edgar chicken embryo-adapted (CEA) (124-ADV-9501) challenge strains were purchased from National Veterinary Services Laboratory (Ames, IA). The 9109 isolate was propagated in 21 day of age (d.a.) specific pathogenic free (SPF) chickens. At 72 hours post infection, bursas were aseptically collected, snap frozen in liquid nitrogen and stored at -80 C until future use. Lukert and Edgar CCA were propagated in primary chicken embryo fibroblasts (CEF) derived from 9-11 d.a. SPF embryos as previously described (54).

RNA extraction

Total RNA was extracted directly from the Edgar CEA strain from the purchased virus without propagation using the RNeasy mini kit (Qiagen, Valencia, CA). Total RNA was extracted from Lukert or Edgar CCA chicken embryonic fibroblasts infected cells using the same kit. The supernatant was removed at 48-72 hours post-infection, cells overlaid with 300 µl RLT buffer containing 0.143 M β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA) and scraped, as recommended by the manufacturer. Total RNA was extracted from homogenized bursas harvested from chickens infected with the 9109 isolate. All samples of extracted RNA were incubated with Proteinase K (Qiagen) at 55 C for 30 min prior to RNA column purification.

Reverse transcription-polymerase chain reaction (RT-PCR) of segment A and B

The first strand cDNA was synthesized using modified, previously reported segment specific primers: segACEF5 or segACEF3 for segment A, and segBCEF3 for segment B (42, 43). Additional primers for PCR and sequencing were designed as needed using PrimerSelect software (DNAstar, Lasergene, v. 5, Inc., Madison, WI) and previously published sequences obtained from GenBank (Table 4.1). Initially 4-5 µg total RNA and the gene-specific primer were denatured at 98 C for 5 min and cooled at 4 C for 10 min. The first-strand cDNA was synthesized at 50 C for 30 min in a 20 µl reaction containing 1x first-strand buffer, 0.01 M DTT, 0.5 µM gene-specific primer, 40 units RNase-out (Invitrogen, Life Tech., Carlsbad, CA), 200 units SuperScript II and 0.5 mM deoxynucleoside triphosphates mix (dNTP) (Invitrogen). The

RT mixture was then incubated at 70 C for 15 min. Two units of RNase H^- was added and incubated at 37 C for 20 min.

Segment A was amplified in two overlapping fragments with PCR primer pair set segAVP25/segACEF3 and segAVP23/segACEF5 with expected product size of 2691 and 1417 nts, respectively. Segment A positive RT-PCR controls were generated using the primer pair set segAVP25/segAVP23 with an expected size of 848 nts. The last 129 nts at the 5' end of segment A, Edgar CEA was amplified using the product from 5' RACE and primer pair set segAVP23/segACEF5. Segment B was amplified with a single PCR reaction using the primer pair set segBCEF3/segBCEF5 annealing to the extreme 3' and 5' ends, respectively and yielding an expected product of 2827 nts. The PCR reaction was synthesized in a 100 µl mixture containing 5 µl of the RT reaction, 1X PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, primers at 0.5 µM each and 2.5 units of recombinant or Platinum *Taq* DNA polymerase (Invitrogen). The PCR cycle parameters were as follows: 94 C for 3 min followed by 35 cycles at 94 C for 15 s, 55 C for 15 s, 72 C for 5 min and a final extension at 72 C for 30 min. The RT-PCR amplified products were electrophoresed on a 0.8% agarose gel and visualized with ethidium bromide as per manufacturer's recommendations.

5' RACE (rapid amplification of cDNA ends)

The sequences of the 5' termini of the viral genome were amplified using the 5' RACE system Version 2.0 (Invitrogen) as per the manufacturer's recommendations. Segment A and B specific primers were constructed with the PrimerSelect software (DNAstar, Lasergene, v. 5) using the previously reported nucleotide sequence of CEF94 (GenBank accession AF194428 and

AF194429, respectively) (Table 4.1). First strand cDNA for segment A was synthesized using 5 µl total RNA using SuperScript II reverse transcriptase, segment A specific reverse primer segA676. The reaction was carried out at 98 C for 10 min, 4 C for 5 min, 50 C for 50 min (reverse transcription), 70 C for 15 min, and a final incubation with an RNase mix at 37 C for 30 min. The first strand was S.N.A.P. column purified and an oligo-dC tail was added to the 3' end of the cDNA using terminal transferase and dCTP. The tailed cDNA was used as a template for polymerase chain reaction (PCR1) with abridged anchor (AAP) and segment A specific primer segA507. The second round of PCR (PCR2) was performed using 1 µl of PCR1 product with abridged universal amplification (AUAP) and segment A specific primer segA323. Similarly the first strand cDNA for segment B was synthesized using segment B specific reverse primer segB671. The oligo-dC tailed segment B cDNA was then used as a template for PCR1 with AAP and the segment B specific primer segB460. One microliter of the PCR1 product was used in PCR2 with AUAP and segment B specific primer segB344. All RACE-PCR reactions were carried out at 94 C for 3 min followed by 35 cycles at 94 C for 15 s, 55 C for 15 s, 72 C for 1 min and final extension at 72 C for 30 min. The 5' RACE products were electrophoresed on a 2.0 % agarose gel. Gel purification was performed with the QIAEXII-gel extraction kit (Qiagen).

Cloning and sequencing of RT-PCR products

The purified DNA was cloned into the pCR4-TOPO or pCRII-TOPO vectors according to the manufacturer's recommendations with maximum incubation times and transformed into competent *E. coli*, DH5α-T1R cells (Invitrogen). Transformants were selected on Luria-Bertani (LB) agar media (Q-BIOgene, CA, USA) containing 50 µg/ml kanamycin and screened by PCR

with segment specific primers. Six clones from each PCR reaction were stored and three used for sequencing. Clones were expanded overnight at 37 C in 10 ml LB media containing 50 µg/ml kanamycin, centrifuged at 4 C for 10 min at 800 g, resuspended in fresh LB media with 10% glycerol, and stored at -80 C until further use.

Plasmid DNA was purified using the Qiagen miniprep kit (Qiagen) according to manufacturer's recommendations. Double-stranded DNA was sequenced by the dideoxy chain termination procedure using the automated ABI Prism 310, Genetic Analyzer (Applied Biosystems, ABI, Foster City, CA). Plasmid specific (M13 reverse and forward), as well as, IBDV segment specific primers were used for sequencing with primer walking. Clones obtained from 5' RACE were sequenced with primers segA323 and segB344 for segment A and B, respectively. A consensus sequence was obtained using three clones which were sequenced at least three times with forward and reverse primers.

Multiple alignment and phylogenetic analysis

The nucleotide sequences obtained from the four IBDV strains in this report were deposited in GenBank with accession numbers for 9109 (AY462027, AY459321), Lukert (AY918948, AY918947), Edgar CCA (AY462026, AY459320) and Edgar CEA (AY918950, AY918949).

Previously published full-length nucleotide sequences of segment A and B used in our analysis have the following GenBank accession numbers: serotype 1 very virulent isolates D6948 (AF240686, AF240687), UPM97/61 (AF247006, AF527040), HK46 (AF092943, AF092944), UK661 (X92760, X92761), OKYM (D49706, D49707), serotype 1 classical isolates

CEF94 (AF194428, AF194429), CEF (AF493979), P2 (X84034, X84035), wild type Cu1(wt) (AF362747, AF362748), Cu1 (D00867, AF362775), serotype 1 Delaware, variant E isolate (AF133904, AF133905) and serotype 2 isolates OH (U30818, U20950) and 2382 (AF362773, AF362774).

The nucleotide and deduced amino acid sequences were aligned with the previously published full-length IBDV sequences using ClustalW multiple sequence alignment program and the pair distance calculated using Lasergene, v. 5 (DNAstar). Unrooted cladograms were generated using maximum parsimony analysis with Neighbor-Joining clustering and 1000 bootstrap replicates using the Phylogenetic Analysis Using Parsimony software (PAUP, v.10). The 5' and 3' NCR of segment A and B were analyzed using the Mfold software (v. 3) (55).

Results

There was a high nucleotide and amino acid similarity between IBDV strains used in this study. The amino acid sequences had higher similarities than nucleotide sequences. The similarity between both nucleotide and amino acid sequence of all strains analyzed for the VPX-VP4-VP3 polyprotein was representative for VP1, VPX, VP2, VP3, VP4, and VP5 genes (Table 4.3). The VPX-VP4-VP3 amino acid sequence similarity for the Edgar CCA and CEA was 96.6% and for Lukert and Edgar CEA and CCA was 96.4% and 96%, respectively. Lukert, 9109, and Edgar CEA were more similar to Cu1 strains 97.3%, 97.1%, and 96.5%, respectively than to Edgar CCA strain.

The similarity between amino acid and nucleotide sequences of Lukert and the classical strains Cu1, Cu1w, and P2 was higher within VP4, VP5, and the VP2 processing site at 100%

and 98.6%, respectively. The VP2 processing site in the 9109 isolate had 100% amino acid similarity to the classical strains Cu1, Cu1wt, CEF94, and P2. Within the same site Edgar CEA was more similar to Lukert and very virulent strains 90.4% compared to the other IBDV strains.

Segment B analysis

Segment B in Lukert, 9109, Edgar CCA and CEA strains was 2827 nucleotides (nts) in length. Edgar CCA has a coding region of 2646 nts (112-2757) and encodes for VP1 protein containing 881 amino acids (aa). The 9109 isolate and Edgar CEA have coding regions of 2640 nts (112-2751) and encodes 879 amino acids. Comparison between IBDV strains 9109 and Edgar CEA have a T→C transition at nucleotide position 2638 and an insertion of six nucleotides at position 2641-CCATGA-2646. The VP1 protein has several unique amino acid substitutions (Table 4.2 and 4.4). Specifically, Edgar CEA and CCA have one substitution G^{46} and T^{121}, respectively. Lukert has unique amino acid substitutions at G^{172}, V^{356}, P^{79}, S^{527}, and F^{720}. The 9109 isolate has one unique substitution at M^{809} and amino acids T^{13} and L^{546} are conserved in P2 and Cu1wt, Arg^{682} is conserved in Winterfield and variant E. In addition, at amino acid position 147 field isolate 9109 has D and very virulent strains have N compared to the other strains.

Segment A analysis

Segment A in all four strains was 3260 nts in length. ORF1, encoding the VP5 protein, contains 438 nts and encodes 145 amino acids in the four sequenced strains with the start codon

at nucleotide position 97 and stop codon at 543 nt. The amino acid substitution at position 16 D→A is conserved between Lukert, Edgar CCA, CEA and serotype 2 OH and 2382 strains. The second substitution in Edgar CEA is at aa 135 (H to R). VP5 in very virulent strains contains four additional amino acids at the start NH_3-MLSL-COOH compared to the rest of the strains analyzed (Table 4.2 and Fig. 4.3).

The open reading frame two, encoding for the polyprotein, contains 3038 nts and encodes 1012 amino acids. The polyprotein start codon is at nucleotide position 131 and stop codon at 3169 nt. Comparison of the polyprotein nucleotide sequences revealed that isolate 9109 has an insertion of C at position 2178 and deletion at 2209 nt. The VPX protein consists of 512 amino acids, VP4 consists of 243 amino acids, and VP3 consists of 257 amino acids in the four sequenced strains.

Multiple alignment of the predicted amino acid sequence of the VPX protein revealed several amino acid substitutions and potential antigenic regions (manuscript in preparation). Analysis of the VP3 protein revealed two unique substitutions I^{785} and P^{993} in Lukert, Edgar CCA and CEA (Table 4.2 and Fig. 4.1). The 9109 isolate has unique substitution A^{1005} also present in OKYM and vvUPM9761. Several other substitutions, Q^{922} in Lukert and F^{858}, Q^{922}, and A^{948} in Edgar CCA were also revealed.

Analysis of the VP4 protein revealed the following unique amino acid substitutions: 14 in 9109, 7 in Edgar CEA, and 1 in Edgar CCA (Table 4.2 and Fig. 4.2). The 9109 isolate has substitution at I^{541}, present in OKYM, variant E, and OH strains, D^{547} within motif I, Y^{680} present in vvUPM9761 and OKYM, and I^{686} present in Edgar CCA and variant E, and a unique extensive ^{726}SQSTRLGQA^{734} region. Edgar CEA has 6 substitutions between domains I and II at R^{576}, ^{578}RIRPF^{582}, and at amino acid position 584 (E to G) in domain II. No unique substitutions were

found within motif III, aa 644-661, substrate-binding domain IV, aa 697-705 (33,20), catalytic triad H^{546}, D^{589}, S^{652} (60), and catalytic dyad S^{652}, K^{692} (33).

The 5' and 3' noncoding regions (NCR) sequences

The nucleotide sequences of the 5' and 3' NCR of the four sequenced IBDV strains were compared to the published NCR sequences of vvD6948, CEF94, P2, segment A and vvD6948, CEF94, P2, Cu1, and CEF, segment B IBDV strains. The 5' NCR of segment A in all strains is 97 and 131 nts for ORF1 and ORF2, respectively, while segment B has 112 nts. The 3' NCR of segment A has 91 nts in all strains, however the length of segment B differs: 78 nts in P2, 75 nts in vvD6948, 9109, Edgar CEA, and CEF94, and 70 nts in Edgar CCA and Lukert. Segment A in Lukert has mutation at position 75 (U→C) and Edgar CEA at nucleotide position 3257 (C→A). The 18S rRNA binding motif in segment A (19,61) and the 5' NCR of segment A ORF2 promoter region -131 to -100 nts (62) were highly conserved among all strains analyzed.

The unrooted phylogenic analysis

Unrooted phylogenetic analysis using the Neighbor-Joining method with 1000 bootstrap replicates was performed with the aligned segment A and B deduced amino acid sequences. Unrooted analysis allows identification of related descendants without explicitly designating a common ancestor. The analysis of the less divergent trees from VP1 (Fig. 4.4A) and RdRp motif (data not shown), VP4 (Fig. 4.5C), I-IV motifs (data not shown), and VP3 (Fig. 4.5D) demonstrated formation of two branches represented by very virulent and classical strains. In the

same trees 9109 clustered with the classical strains. The phylogenetic analysis of the VP1, VP3, and VP4 identified a closer relationship between Lukert, Edgar CCA and CEA and classical strains with high bootstrap probability. vvD6948 in VP3 clustered with classical strains (Fig. 4.2D). In the VP2 processing site, Lukert was similar to the classical strains and Edgar CCA and CEA were more similar to the very virulent strains (manuscript in preparation). In VP2, Edgar CCA and Lukert clustered together, Edgar CEA formed its own cluster, and 9109 clustered with variant E (Fig. 4.4C). The 5' and 3' NCR of segment A 9109 isolate was found to be similar to vvD6948 (data not presented).

In the cladograms obtained from multiple alignments of the VPX-VP4-VP3, VP5 (Fig. 4.5A, B) and VP2, VP5-VPX-VP4-VP3-VP1, VPX the very virulent and serotype 2 strains (Fig. 4.4B, C) were represented in two distinct branches. In the same trees, 9109 and variant E clustered together with very high probability as predicted by high bootstrap confidence level. In the same cladograms, Lukert, Edgar CCA, and CEA, and classical strains formed individual clusters. Edgar CEA and CCA were more similar to Lukert in VPX, VPX-VP4-VP3, VP5, and VP3 (Fig. 4.4B and Fig. 4.5A, B, D). Clustering pattern obtained from the VPX and amino acid sequences between aa 202-451 and 210-473 cladograms represented the VP5-VPX-VP4-VP3-VP1 and VPX-VP4-VP3 (Fig. 4.4B and Fig. 4.5A).

Discussion

We describe the full-length sequence characterization of four IBDV strains with different pathogenicities. The nucleotide and deduced amino acid sequences of these strains were compared with previously reported full-length sequenced serotypes 1 and 2 IBDV strains.

The four sequenced strains were determined by the phylogenetic analysis of VP1 protein to be classical strains. The segment B was highly conserved among analyzed strains. The VP1 protein in Lukert and Edgar CCA contains two more amino acids compared to 9109 and Edgar CEA strains. Similar variations have been previously described for P2, Cu1, Cu1M, and CEF94 strains (41) but the significance is still unknown. Edgar CEA has a substitution at amino acid position 46 (S to G) within the Ser-phosphorylation region (59). At amino acid position 147 only 9109 and virulent strains have substitutions. Amino acids previously suggested to play a role in cell-specific replication (63), virulence (45), phosphorylation, glycosylation, NTP-binding motifs (56), and the RNA-dependent, RNA-polymerase (RdRp) motifs (56, 57, 58, 59) were highly conserved between all strains analyzed.

Substitutions at amino acid position 785 (L to I) and 993 (Q to P) in Lukert, Edgar CCA, and CEA are in the VP3- and double strand (ds)RNA-binding domains, respectively (65, 66, 67). The 9109 isolate has Q^{981} in the dsRNA- and A^{1005} in VP1-binding domain (65, 66, 67). In addition, it was determined that A^{1005} is a vvIBDV characteristic and may be significant for the pathogenicity (68, 69). Previously, the VP3 carboxyl terminus, aa 857-1012, was found to be important for vvIBDV pathogenicity (70) and formation of VP3-VP2 complex, which has role IBDV replication and genome packaging (32, 36).

Mutations in the VP4 protease may affect the polyprotein cleavage and may have a role in viral adaptation and pathogenicity. Substitutions in the VP4 protein at amino acid positions 541 (V to I) and 547 (N to D) in 9109 are within motif I and several substitutions in Edgar CEA are between serine-protease motifs I and II. The amino acid Y^{680} observed in 9109 was previously proposed as very virulent marker (68, 69).

Edgar CCA, CEA, and Lukert strains have substitutions within the VP5 protein at amino acid position 16 (D to A) indicating that this amino acid may not be important for viral pathogenicity as serotype 2 strains have the same substitution. In addition Edgar CEA contains a second substitution at amino acid position 135 (H→R). The cytotoxic protein is important for the viral release (71, 72), induction of apoptosis (73), and pore formation in the cells (74). Although previous analysis of VP5 did not reveal amino acids unique to the very virulent pathotype (75), amino acid substitutions could affect VP5 function. A IBDV VP5 knock-out mutant has been shown to replicate in the bursa without bursal lesions (52) and does not induce apoptosis (73) as does the wild type.

The stem-loop structure, not the sequence of the 3' NCR, is an important functional determinant (76) and plays a role in the protein-primed RNA synthesis (77) and RNA packaging (78). The mutation at nucleotide position 3257, segment A in Edgar CEA is within the inverted terminal repeat 3255-3260 nts at the 3' NCR and part of the stem-loop structure with energy level -3.1 *vs.* -11.7 kcal/mole in the other sequenced strains. The mutation at position 75 (U→C) in Lukert within the 5' NCR of segment A is in the putative 18S rRNA binding domain (61) and may affect the transcription and translation efficiency.

The viral protein VPX and amino acid sequences between aa 202-451 and 210-473 but not VP2 protein are the best representatives of the entire IBDV genome. The greatest variability was found in the VP2 and 5' noncoding region of segment B among IBDV strains. The RNA-dependent, RNA-polymerase motifs within VP1 and the VP5 protein were highly conserved among isolates.

Close nucleotide and amino acid relatedness between Lukert, Edgar CCA and CEA was established. Although Lukert, Edgar CCA and CEA strains have as expected sequence

characteristics of the classical strains when using the VP2 processing site Edgar CCA and CEA were classified as a very virulent strains.

Phylogenetic and sequence analysis of field isolate 9109 revealed unique features. The isolate has characteristics of classical, variant, and very virulent strain depending on the region analyzed. The 9109 isolate is more similar to the classical strains in the VP2 processing site, VP3, and VP4 proteins. Although we did not find the unique *SspI* restriction site within the VP2 of 9109 isolate (80, manuscript in preparation) previously suggested as a very virulent marker (79), we have identified two amino acid substitutions Y^{680} in VP4 and A^{1005} in VP3 which were also reported as vvIBDV markers. In addition, the amino acid at position 147 in the VP1 protein was substituted only in 9109 and very virulent strains. Phylogenetic analysis of the 5' and 3' NCR of segment A revealed similarity between 9109 and the vvD6948 strain. There are also several similarities between variant E and 9109 in VPX, VP2, and VPX-VP4-VP3 polyprotein. The predicted antigenic regions for 9109 isolate demonstrated similarities between 9109 and variant E (manuscript in preparation).

Our analysis revealed that although VPX amino acid comparison may be initially useful, complete genomic sequence information for strain molecular characterization is essential as partial sequences may falsely designate a particular strain as very virulent, classical, or variant.

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Figure legends

Fig. 4.1. Multiple alignment of the predicted amino acid sequence of VP3 protein of IBDV Lukert, 9109, Edgar CCA and CEA strains with corresponding sequences of published IBDV serotype one very virulent (vv) UPM9761 and OKYM, classical P2, Cu1, Cu1wt, variant E, and serotype two, OH strain. The start site of the VP3 is at amino acid (aa) 756 from ORF2 of segment A. The self- (756-853 aa), double stranded RNA (977-1003 aa), and VP1- (1003-1012 aa) domains are indicated as boxes. The amino acids sequence identical to the 9109 field isolate are indicated with dots and non-identical amino acids are presented with letters. The amino acid positions are based upon the 9109 isolate.

Fig. 4.2. Multiple alignment of the predicted amino acid sequence of VP4 protein of IBDV Lukert, 9109, Edgar CCA and CEA strains with corresponding sequences of published IBDV serotype one very virulent (vv) UPM9761 and OKYM, classical P2, Cu1, Cu1wt, variant E, and serotype two, OH strain. The start site of the VP4 is at amino acid (aa) 513 from ORF2 of segment A. VP4 motifs I, 539-550 aa, II, 583-597 aa, III, 644-661 aa, and IV, 697-705 aa are indicated with boxes. The amino acids sequence identical to the 9109 field isolate are indicated with dots and non-identical amino acids are presented with letters. The amino acid positions are based upon the 9109 isolate.

Fig. 4.3. Multiple alignment of the predicted amino acid sequence of VP5 protein of IBDV Lukert, 9109, Edgar CCA and CEA strains with corresponding sequences of published IBDV serotype one very virulent (vv) UPM9761 and OKYM, classical P2, Cu1, Cu1wt, variant E, and serotype two, OH strain. The start site of the VP5 is at amino acid 1 from ORF1 of segment A

and 145 is the last amino acid. The amino acids sequence identical to the 9109 field isolate are indicated with dots and non-identical amino acids are presented with letters. The dashes indicate amino acid deletion. The amino acid positions are based upon the 9109 isolate.

Fig. 4.4. Phylogenetic relationship based on the amino acid (aa) sequences of the (A) VP1, (B) VP2 aa 202-451, VP2 aa 210-473, VPX aa 1-512, VP5-VPX-VP3-VP4-VP1, and (C) VP2 for Edgar chicken embryo adapted (CEA), Edgar cell culture adapted (CCA), Lukert, and 9109 (shaded boxes) with other IBDV strains. The cladograms were generated using maximum parsimony analysis with Neighbor-Joining clustering and 1000 bootstrap replicates (confidence levels listed in parentheses) using the Phylogenetic Analysis Using Parsimony software (PAUP, v.10).

Fig. 4.5. Phylogenetic relationship based on the amino acid sequences of the (A) VPX-VP4-VP3, (B) VP5, (C) VP4, I-IV motifs, and (D) VP3 proteins for Edgar chicken embryo adapted (CEA), Edgar cell culture adapted (CCA), Lukert, and 9109 (shaded boxes) with other IBDV strains. The cladograms were generated using maximum parsimony analysis with Neighbor-Joining clustering and 1000 bootstrap replicates (confidence levels listed in parentheses) using the Phylogenetic Analysis Using Parsimony software (PAUP, v.10).

Table 4.1. Primers used for the full-length sequencing in this study.

Primer[a]	5' → 3'	Position[b]	Primer	5' → 3'	Position
5' RACE[c]			**RT-PCR**		
AUAP	GGCCACGCGTCGACTAGTAC		segA1474 f	CCGGGCCATAAGGAGGATAGC	1474-1494
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG		segA1665	GCGAGAGTCAGCTGCCTTATGC	1665-1644
segA75f	TCCTCCTTCTACAACGCTATCATT	72-95	segA1875f	TCATTGAAGGCGTGCGAGAAGAC	1875-1897
segA203	GTCCGGTTGTTGGCATCAGAAGG	203-181	segA 2522f	GTGACTGACATGGCCAACTTCGC	2522-2544
segA323	CAGGGAATCCAGGGAAAAAGACAA	323-300	segA2770	GTGCCCATTGAGTGCTACCC	2770-2751
segA507	GCGTTTATGGTGCCGTTTAGTG	507-486	segA3048	GCCTGGGATTGCGATGCT	3065-3048
segA676	GTCACCAAGCCTCACATACCCAAGAT	676-651	segA3069	GTTTTGGCTTGGGCTTTGGTAGAG	3092-3069
segB38f	AACGTGGCTACTAGGGGAGATAAC	38-61	segA3231	TGTTGTAAGTCCGAATTGGTGTCC	3231-3208
segB198	TTCCACGTCTTGTCCAGCAGTAGG	198-175	segACEF3	GGGGACCCGCGAACGG	3260-3245
segB344	ATTTGGTCGGTCTCATACTCCTCA	344-321	segBCEF5f	GGATACGATGGGTCTGACCCT	1-21
segB460	TAGGGCGATGTGTTGGGTAGTA	460-439	segB431f	TCCCAAAGTACTACCCAACACATC	431-454
segB671	TTTCTTCCAGTGGCGACCTCCTTCAT	671-646	segB804f	GATGTTGGTACTGACGGGAGACG	804-826
RT-PCR			segB1890f	ATTTTGTTCTGCTGCGTATCCCA	1890-1912
segACEF5f	GGATACGATCGGTCTGACCCCGG	1-23	segB2056	GAGCGGCGCCTGCGTTATTCT	2056-2036
segA320f	CCTGGCTCAATTGTGGGTGCTCA	320-342	segBCEF3	GGGGGCCCCCGCAGG	2827-2812
segAVP25f	CAACAGCCAACATCAACGAC	570-589	**TOPO vector**		
segA676	GTCACCAAGCCTCACATACCCAAGAT	676-651	T7	TAATACGACTCACTATAGGG	
segA1013f	GTGATTCCAACAAACGAGATAAC	1013-1035	T3	ATTAACCCTCACTAAAGGGA	
segA1254f	CAAATCCTGAACTAGCAAAGAACC	1254-1277	M13R	GTAAAACGACGGCCAG	
segAVP23	CACCTCCATGAAGTACTCAC	1417-1398	M13F	CAGGAAACAGCTATGAC	

[a] 'f' and 'r' indicate forward and reverse primer, respectively

[b]Primer position corresponds to the nucleotide positions at segment A and B of isolate CEF94 with GenBank accession numbers AF194428 and AF194429, respectively.

[c]5' RACE - rapid amplification of cDNA ends

Table 4.2. Total amino acid substitutions in the four sequenced IBDV strains within segment A and B coding regions and comparison with the other serotype 1 and 2 strains used in this study.

Strain	segment A						segment B	
	ORF1	ORF2						
	VP5	VP2 (1-441)[a]	VP2 (442-512)	VP4 (513-755)	VP3 (756-1012)	Total	substitutions	additions
9109	0	10	0	14	2	26	5	-
Edgar CCA	1	16	15	1	5	38	1	2
Edgar CEA	2	16	8	7	2	35	1	-
Lukert	1	18	1	0	3	23	5	2

[a]The start site of the VP2 is at amino acid (aa) 1 from ORF2, VX-VP4-VP3 polyprotein of segment A. The VX-VP4 is at aa 512-513 and the VP2 processing site is at aa 442-512. The amino acid positions are based upon the 9109 cleavage site for isolate.

Table 4.3. VPX-VP4-VP3 polyprotein deduced amino acid and nucleotide sequence alignment pair distances presented as percent similarity.

		Strain	Percent Similarity											
			1	2	3	4	5	6	7	8	9	10	11	
%	1	9109	100	*94.4*	94.6	95.7	96.0	96.7	97.1	97.1	96.8	96.7	88.6	%
nucleotide	2	Edgar CCA	95.0	100	96.6[a]	96.4	94.9	95.4	95.9	96.1	95.7	94.5	87.6	amino acid
identity	3	Edgar CEA	95.3	97.9	100	96.0	95.5	95.9	96.2	96.5	96.5	94.7	87.8	identity
	4	Lukert	95.8	97.9	97.5	100	96.0	96.5	97.1	97.3	97.1	95.7	88.8	
	5	vvUPM9761	94.8	93.8	94.2	94.6	100	98.9	97.2	97.3	97.5	95.8	89.0	
	6	vvOKYM	95.1	94.0	94.5	94.8	98.4	100	97.7	97.8	98.0	96.4	89.6	
	7	P2	96.8	96.2	96.3	96.9	95.4	95.7	100	99.4	98.7	97.1	90.1	
	8	Cu1	96.7	96.1	96.3	96.9	95.3	95.6	99.7	100	98.6	97.1	90.1	
	9	Cu1wt	96.5	96.1	96.4	97.0	95.4	95.7	97.9	97.7	100	96.9	89.7	
	10	Variant E	97.5	95.3	95.5	96.2	95.0	95.3	97.0	96.9	96.8	100	88.4	
	11	OH	83.0	82.7	82.5	83.3	82.9	82.9	83.7	83.6	83.6	83.0	100	

[a]The numbers in the shaded boxes indicate the highest percent similarities between strains.

Table 4.4. Unique amino acid substitutions in VP1 protein.

Strain	Amino acid position[a]																							
	4	12	13	46	61	69	75	90	92	104	121	125	136	145	146	147	150	160	163	170	172	174	192	
9109	I	S	T	S	V	E	T	G	V	Q	P	P	Y	N	E	D	D	R	A	M	Q	T	P	
Edgar CCA	.	.	K	.	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	
Edgar CEA	.	.	K	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	
Lukert	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	E	.	.	
vvUPM9761	V	.	K	.	I	.	.	.	.	.	.	.	.	T	D	N	.	K	P	.	.	.	S	
vvOKYM	V	Q	K	.	I	D	.	.	.	.	.	S	.	T	D	N	.	.	.	.	.	.	.	
Winterfield	.	.	K	.	.	.	.	R	.	H	.	.	.	.	.	G	.	.	.	I	.	.	.	
P2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	N	.	
Cu1	.	T	.	.	.	.	.	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	
Cu1wt	.	R	K	.	.	.	A	.	.	.	.	.	F	.	.	G	E	.	.	.	.	.	.	
Variant E	.	.	K	.	.	.	.	R	.	H	.	.	.	.	.	G	.	.	.	I	.	.	.	
OH[B]	V	T	K	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	

	204	205	212	213	242	287	289	310	311	319	356	370	380	390	393	425	426	431	469	479	484	508
9109	Q	L	V	G	D	T	L	Y	W	F	M	P	G	L	E	T	R	A	S	T	S	R
Edgar CCA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Edgar CEA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lukert	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	P	.	.
vvUPM9761	.	.	.	.	E	A	.	.	.	.	.	.	.	M	D	.	.	.	.	.	.	K
vvOKYM	.	.	.	.	E	A	.	.	.	.	.	T	V	M	V	I	S	V	.	.	.	K
Winterfield	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.
P2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cu1	H	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cu1wt	H	V	.	.	.	.	.	F	G	C	.	.	.	.	.	.	.	.	.	.	.	.
Variant E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.
OH	.	.	G	S	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	T	.	.	K

[a]The amino acid positions are based upon the 9109 isolate. The amino acids sequence identical to the 9109 field isolate are indicated with dots and non-identical amino acids are presented with letters. The dashes indicate amino acid deletion.
[b]Not all amino acid substitutions between positions 100-135 in OH strain are included in the table.
[c]vvUPM9761 and vvOKYM represent all very virulent strains used in this analysis.

Table 4.4 (Continued)

	511	515	527	546	562	617	640	646	650	667	675	677	682	685	687	695	697	718	720	722	788	789
9109	R	E	N	L	S	A	A	G	R	S	A	E	R	V	S	K	V	N	L	T	K	V
Edgar CCA	.	.	.	P	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.
Edgar CEA	.	.	.	P	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.
Lukert	.	.	S	P	.	.	.	.	.	.	.	.	K	.	.	.	.	.	F	.	.	.
vvUPM9761	S	.	.	P	P	.	.	S	.	.	.	.	K	.	P	R	.	.	.	N	.	.
vvOKYM	S	.	.	P	P	.	.	S	.	.	S	V	K	.	P	R	A	.	.	.	.	.
Winterfield	.	D	.	P	.	.	G	.	.	.	.	.	.	I	.	.	.	S	.	.	.	.
P2	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.
Cu1	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.
Cu1wt	.	.	.	P	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.
Variant E	.	D	.	P	.	.	G	.	.	.	.	.	.	I	.	.	.	S	.	.	.	.
OH	S	.	.	P	.	G	.	S	-	A	.	.	K	.	.	.	.	.	.	.	N	-

	793	809	831	871	879	880	881
9109	A	M	H	R	Q	-	-
Edgar CCA	.	V	.	.	.	Q	P
Edgar CEA	.	V	.	.	.	-	-
Lukert	.	V	.	.	.	Q	P
vvUPM9761	.	V	.	.	.	-	-
vvOKYM	.	V	R	.	.	-	-
Winterfield	.	V	.	.	.	-	-
P2	.	V	.	.	-	-	-
Cu1	.	V	.	.	-	-	-
Cu1wt	.	V	.	.	-	-	-
Variant E	.	V	.	.	.	-	-
OH	G	V	.	A	.	-	-

Fig. 4.1.

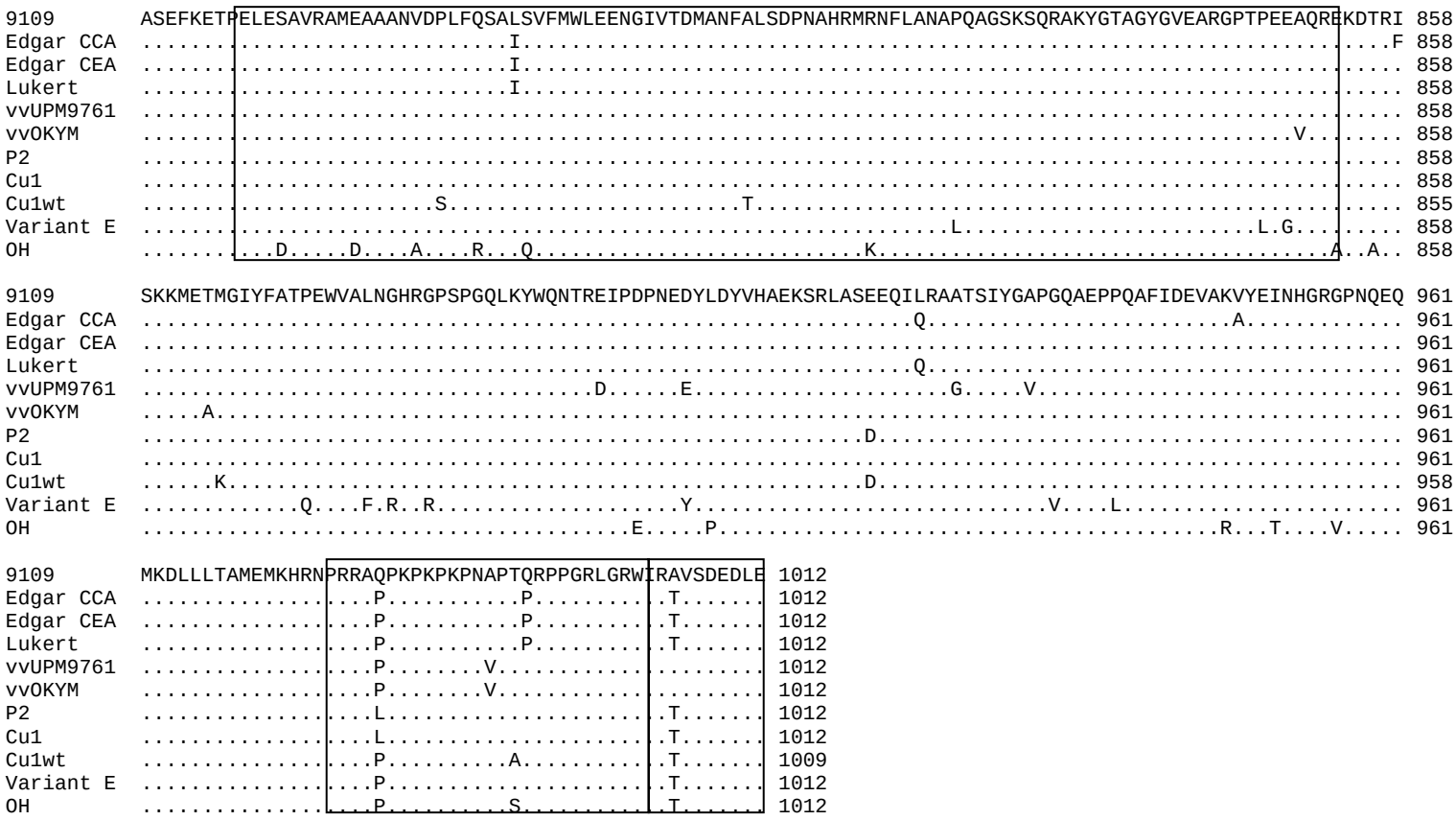

9109 ASEFKETPELESAVRAMEAAANVDPLFQSALSVFMWLEENGIVTDMANFALSDPNAHRMRNFLANAPQAGSKSQRAKYGTAGYGVEARGPTPEEAQREKDTRI 858
Edgar CCA .....I.....F 858
Edgar CEA .....I..... 858
Lukert .....I..... 858
vvUPM9761 858
vvOKYM .....V..... 858
P2 858
Cu1 858
Cu1wt .....S.....T..... 855
Variant E .....L.....L.G..... 858
OH .....D.....D....A....R...Q.....K.....A..A.. 858
9109 SKKMETMGIYFATPEWVALNGHRGPSPGQLKYWQNTREIPDPNEDYLDYVHAEKSRLASEEQILRAATSIYGAPGQAEPPQAFIDEVAKVYEINHGRGPNQEQ 961
Edgar CCA .....Q.....A..... 961
Edgar CEA 961
Lukert .....Q..... 961
vvUPM9761 .....D.....E.....G.....V..... 961
vvOKYM .....A..... 961
P2 .....D..... 961
Cu1 961
Cu1wt .....K.....D..... 958
Variant E .....Q....F.R..R.....Y.....V....L..... 961
OH .....E.....P.....R...T....V..... 961
9109 MKDLLLTAMEMKHRNPRRAQPKPKPKPNAPTQRPPGRLGRWIRAVSDEDLE 1012
Edgar CCA .....P.....P.....T..... 1012
Edgar CEA .....P.....P.....T..... 1012
Lukert .....P.....P.....T..... 1012
vvUPM9761 .....P.....V..... 1012
vvOKYM .....P.....V..... 1012
P2 .....L.....T..... 1012
Cu1 .....L.....T..... 1012
Cu1wt .....P.....A.....T..... 1009
Variant E .....P.....T..... 1012
OH .....P.....S.....T..... 1012

Fig. 4.2.

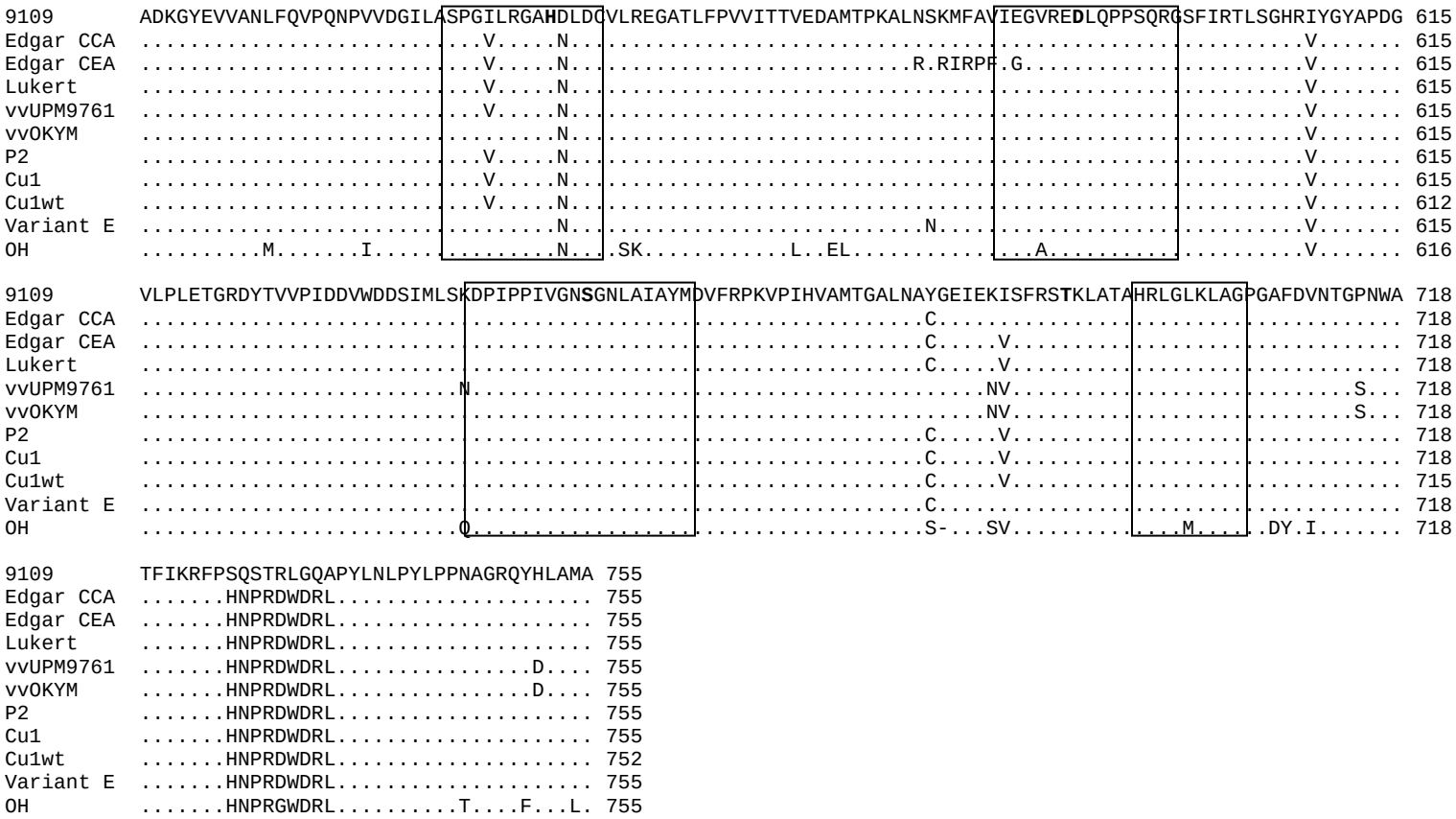

9109 ADKGYEVVANLFQVPQNPVVDGILASPGILRGAHDLDQVLREGATLFPVVITTVEDAMTPKALNSKMFAVIEGVREDLQPPSQRQSFIRTLSGHRIYGYAPDG 615
Edgar CCA ...V.....N.....V..... 615
Edgar CEA ...V.....N.....R.RIRPF.G.....V..... 615
Lukert ...V.....N.....V..... 615
vvUPM9761 ...V.....N.....V..... 615
vvOKYM .....N.....V..... 615
P2 ...V.....N.....V..... 615
Cu1 ...V.....N.....V..... 615
Cu1wt ...V.....N.....V..... 612
Variant E .....N.....N.....V..... 615
OH .....M.....I.....N...SK.....L..EL.....A.....V..... 616
9109 VLPLETGRDYTVVPIDDVWDDSIMLSKDPIPPIVGNSGNLAIAYMDVFRPKVPIHVAMTGALNAYGEIEKISFRSTKLATAHRLGLKLAGPGAFDVNTGPNWA 718
Edgar CCA .....C..... 718
Edgar CEA .....C.....V..... 718
Lukert .....C.....V..... 718
vvUPM9761 .....N.....NV.....S... 718
vvOKYM .....NV.....S... 718
P2 .....C.....V..... 718
Cu1 .....C.....V..... 718
Cu1wt .....C.....V..... 715
Variant E .....C..... 718
OH .....Q.....S-...SV.....M.....DY.I..... 718
9109 TFIKRFPSQSTRLGQAPYLNLPYLPPNAGRQYHLAMA 755
Edgar CCA .....HNPRDWDRL..... 755
Edgar CEA .....HNPRDWDRL..... 755
Lukert .....HNPRDWDRL..... 755
vvUPM9761 .....HNPRDWDRL.....D.... 755
vvOKYM .....HNPRDWDRL.....D.... 755
P2 .....HNPRDWDRL..... 755
Cu1 .....HNPRDWDRL..... 755
Cu1wt .....HNPRDWDRL..... 752
Variant E .....HNPRDWDRL..... 755
OH .....HNPRGWDRL.....T....F...L. 755

Fig. 4.3.

```
9109       ----MVSRDQTNDRSDDKPARSNPTDCSVHTEPSDANNRTGVHSGRHPGEAHSQVRDLDLQFDCGGHRVRANCLFPWIPWLNCGCSLHTAEQWELQVRSDAPDCPEPTGQ 106
Edgar CCA  ----.............D.................................................................................... 106
Edgar CEA  ----.............D.................................................................................... 106
Lukert    ----.............D.................................................................................... 106
vvUPM9761 MLSL.......G....E.............................R.............................F.............................. 110
vvOKYM    ----............E.............................R.............................F.............................. 106
P2         ----.................................................................................................... 106
Cu1        ----.................................................................................................... 106
Cu1wt     ----.................................................................................................... 106
Variant E  ----.................................................................................................... 106
OH         ----.............DG.H............D.....P........T...N....L..R.....T.........FS.R...........P..P....SA..ACE 106

9109       LQLLQASESESHSEVKHTPWWRLCTKRHHKRRDLPRKPE 145
Edgar CCA  ..................................... 145
Edgar CEA  ............................R.......... 145
Lukert     ..................................... 145
vvUPM9761  .........................W............ 149
vvOKYM     .........................W............ 145
P2         ..................S..................... 145
Cu1        ..................S..................... 145
Cu1wt      .....T............................... 145
Variant E  ............K........................ 145
OH         .....T..Q.PNRA..........S.WD...G....... 145
```

Fig. 4.4.

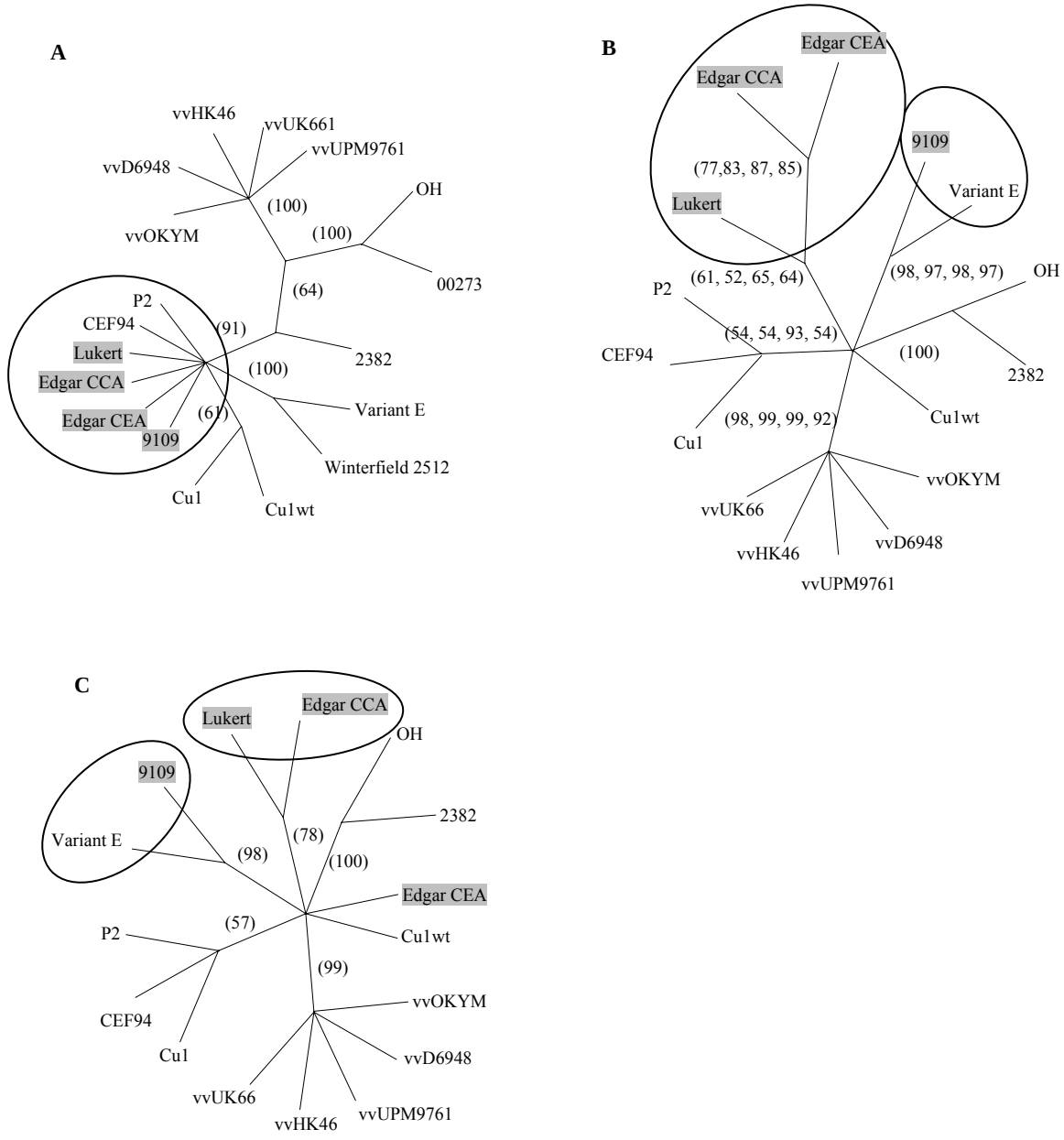
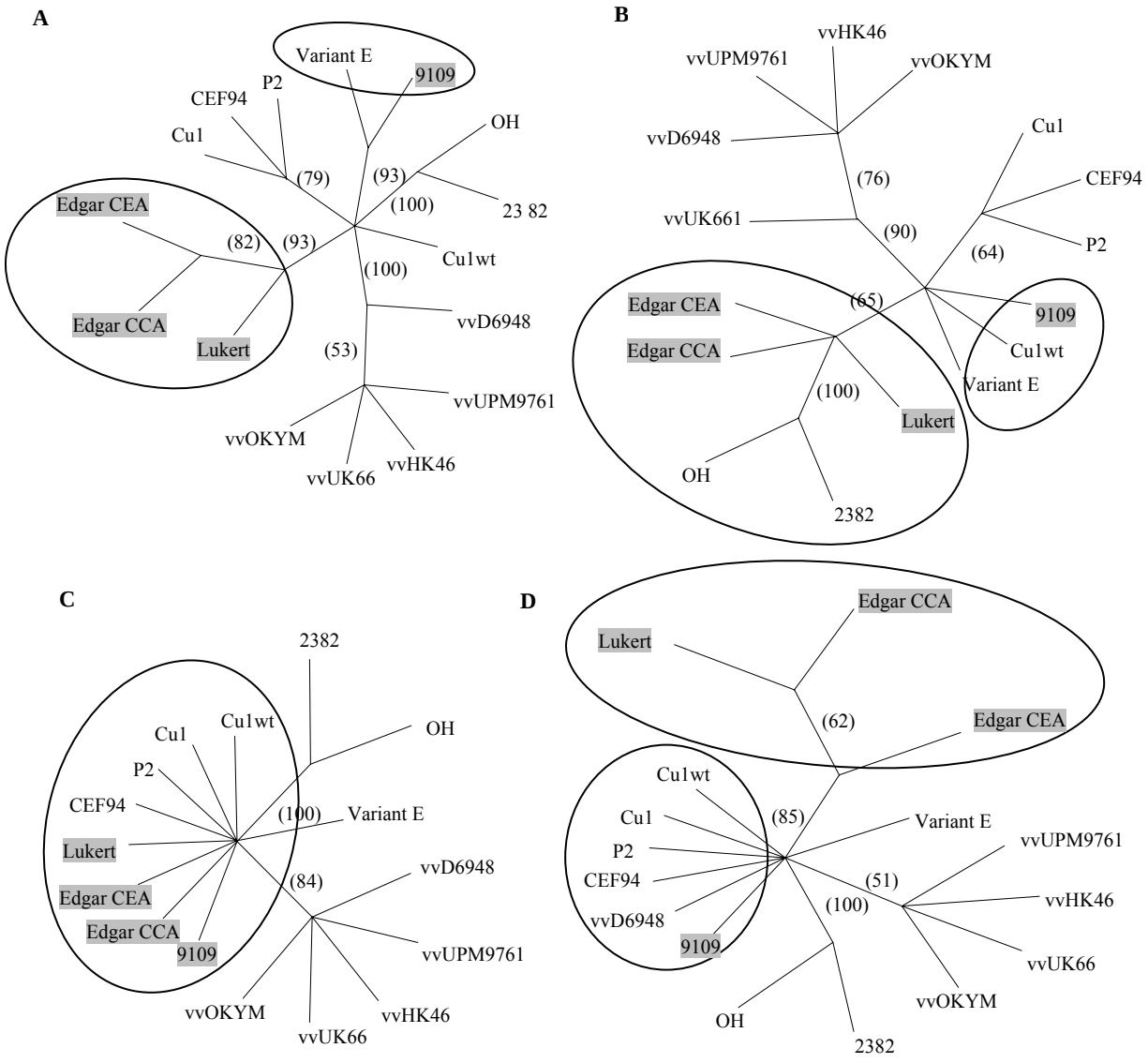

A
vvHK46
vvUK661
vvUPM9761
vvD6948
OH
(100)
vvOKYM
(100)
00273
(64)
P2
CEF94
(91)
Lukert
2382
(100)
Edgar CCA
Variant E
Edgar CEA
(61)
9109
Winterfield 2512
Cu1
Cu1wt
B
Edgar CEA
Edgar CCA
9109
(77,83, 87, 85)
Variant E
Lukert
(61, 52, 65, 64)
(98, 97, 98, 97)
OH
P2
(54, 54, 93, 54)
CEF94
(100)
2382
(98, 99, 99, 92)
Cu1wt
Cu1
vvOKYM
vvUK66
vvD6948
vvHK46
vvUPM9761
C
Lukert
Edgar CCA
OH
9109
2382
Variant E
(78)
(98)
(100)
Edgar CEA
(57)
P2
Cu1wt
(99)
vvOKYM
CEF94
vvD6948
Cu1
vvUK66
vvUPM9761
vvHK46

Fig. 4.5.


A
Variant E
9109
P2
CEF94
Cu1
OH
(79)
(93)
(100)
23 82
Edgar CEA
(82)
(93)
Cu1wt
(100)
Edgar CCA
vvD6948
Lukert
(53)
vvUPM9761
vvOKYM
vvUK66
vvHK46
B
vvHK46
vvUPM9761
vvOKYM
vvD6948
Cu1
(76)
CEF94
vvUK661
(90)
P2
(64)
Edgar CEA
(65)
9109
Edgar CCA
Cu1wt
Variant E
(100)
Lukert
OH
2382
C
2382
Cu1
Cu1wt
OH
P2
CEF94
(100)
Variant E
Lukert
vvD6948
Edgar CEA
(84)
Edgar CCA
9109
vvUPM9761
vvOKYM
vvHK46
vvUK66
D
Edgar CCA
Lukert
(62)
Edgar CEA
Cu1wt
Cu1
(85)
Variant E
vvUPM9761
P2
CEF94
(51)
vvHK46
vvD6948
(100)
9109
vvUK66
vvOKYM
OH
2382

CHAPTER V

COMPARISON OF VPX AMINO ACID SEQUENCES OF INFECTIOUS BURSAL DISEASE VIRUS STRAINS AND ANTIGENIC ANALYSIS[1]

[1]Daniel I. Petkov, Erich G. Linnemann, Darrell R. Kapczynski, and Holly S. Sellers. To be submitted to *Virus Research*.

Abstract

A portion of the polyprotein encoding a conformationally dependent, protective epitopes of infectious bursal disease virus (IBDV) was sequenced and analyzed. The deduced amino acid (aa) sequences of the VPX protein of four serotype 1 IBDV isolates with known different pathogenicities a field isolate (9109, Lukert, Edgar cell culture-adapted (CCA), and Edgar chicken embryo-adapted (CEA) strains) were compared with previously reported VPX sequences from very virulent, classical, and variant serotype 1, as well as, serotype 2 IBDV strains. The amino acid sequences 202-451 and 210-473 were representative for VPX and aa 210-440, 210-332, and 242-440 were representative for VP2 proteins.

The VP2 protein, aa 1-441 was analyzed for potential antigenic regions and unique amino acid substitutions using computer software programs. Newly predicted antigenic regions based on the antigenic index were observed within VP2. Our analysis revealed that of the previously reported hydrophilic peaks, only the major A and minor I regions were part of the newly predicted antigenic sites of VP2. The greatest amino acid variability within VP2 was revealed between aa 249-259. Amino acid determinants in VP2 for tissue-culture adaptation were conserved in Edgar CCA and Lukert. Extensive amino acid substitutions in an otherwise conserved VP2 processing site were present only in Edgar CCA and CEA strains.

The 9109 isolate shares several conserved amino acids with variant E in the VP2 protein and with the classical strains in the VP2 processing site. Lukert, Edgar CCA and CEA strains have amino acid sequence characteristics of the classical strains.

Key words: IBDV; VPX; VP2; sequencing; phylogenetic analysis

1. Introduction

Infectious bursal disease virus causes immunosuppression in chickens (Craft et al., 1990; Faragher et al., 1972; Giambrone et al., 1977; Kim et al., 1999; Sharma et al., 2000). The severity of the disease depends on the IBDV strain (Van den Berg et al., 2004), age of infection (Giambrone et al., 1976), and maternal antibodies (Lucio and Hitchner, 1979). Serotype 1 IBDV strains have been classified as very virulent (vv) (Chettle et al., 1989; Van den Berg et al., 1991), classical (Oppling et al., 1991; Rautenschlein et al., 2003), and antigenic variants (Heine et al., 1991; Snyder et al., 1988 a.b.).

IBDV is a non-enveloped, bi-segmented (Kibenge et al., 1988), double stranded RNA virus belonging to the *Birnaviridae* family (Dobos et al., 1979). Segment A contains two open reading frames (ORFs). The ORF1 encodes for a VP5 protein (Mundt and Muller, 1995) and partially overlaps ORF2 (Kibenge et al., 1990, 1991). The monocistronic ORF2 encodes for the precursor VPX-VP4-VP3 polyprotein cleaved at amino acids (aa) A^{512}-A^{513} for VPX-VP4 (Lejal et al., 2000; Sanchez and Rodriguez, 1999). The VPX protein, aa 1-512, is processed to VP2 protein, aa 1-441, responsible for inducing host protective neutralizing antibodies (Becht et al., 1988; Fahey et al., 1989; Hudson et al., 1986). The greatest amino acid variability of the IBDV genome is within VP2 protein. Changes within the minimally required VP2 antibody binding region, aa 206-350 (Azad et al., 1987; Bayliss et al., 1990), can lead to antigenic variants (Heine et al., 1991; Jackwood and Jackwood, 1994; Vakharia et al., 1994) with different pathogenicities and cell tropism (Boot et al., 2001; Mundt, 1999).

Within VP protein, aa 206-350 hydrophilic peaks designated as major A and B (Azad et al., 1987) and minor I, II, and III (Van den Berg and Martine, 1996) regions were identified. It

was suggested that virus neutralizing conformationally dependent VP2 epitopes exist beyond these region (Cui et al., 2003). Using isolates from a VP2 phage library it was suggested that the hydrophilic B region is not antigenic (Cui et al., 2003). Several amino acid substitutions located between the two major hydrophilic A and B regions were also related to virulence (Brown et al., 1994; Cao et al., 1998; Van den Berg and Martine, 1996; Yamaguchi et al., 1996). Classical strains have unique amino acid substitutions within the hydrophobic regions of VP2 protein (Vakharia et al., 1994) and only two of the seven amino acid substitutions at positions 222 and 251 previously determined as markers for very virulent strains are within the hydrophilic peaks (Van den Berg and Martine, 1996). Substitution at amino acid position 222 in the hydrophilic region A is not responsible for the failure of the monoclonal antibody (mAb) to recognize this site in the antigenic variants A, E, and GLS (Heine et al., 1991; Lana et al., 1992; Vakharia et al., 1994). Most of the mAb used for IBDV strain characterization bind outside of the previously reported hydrophilic regions including amino acids at position 76, 326, 253, 269, 311, 309, 269 (Vakharia et al., 1994).

The level of attenuation and the system used for viral propagation affects related viruses. Lukert is an attenuated Edgar strain adapted in primary avian cell cultures (Lukert et al., 1975). The bursa-derived Edgar strain is a pathogenic field isolate (Edgar and Cho, 1973) and compared to the chicken embryo adapted (CEA) and chicken culture adapted (CCA) strains is the most invasive and pathogenic and induces the highest neutralization antibody titers followed by Edgar CEA and Lukert (Rodriguez et al., 2002 a.b.).

Currently IBDV strain characterization is possible with the virus-neutralization assay (Jackwood and Saif, 1987; McFerran et al., 1980). This assay requires *in vitro* virus propagation and not all strains can be propagated *in vitro* (McFerran et al., 1980). Most of the current

molecular techniques used for IBDV typing utilize amplification of the VP2 fragment by reverse transcriptase (RT) polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis (Ikuta et al., 2001; Majo et al., 2002). The most widely used typing method is the amplification of VP2 gene, 743 nucleotide (nts) fragment between 701-1444 nts of ORF2 using RT-PCR followed by digestion with either *BstN I* or *Mbo I* restriction enzymes. Using this technique, serotype 1 strains can be grouped into six RFLP patterns (Jackwood and Sommer, 1997, 1999; Kibenge et al., 1990). However, some strains can not be clearly characterized by this procedure and regions outside of this VP2 fragment may also be important.

We report the nucleotide and amino acid sequences of the VPX protein of four IBDV strains of different pathogenicities: field isolate 9109, Lukert, Edgar cell-culture adapted and Edgar chicken-embryo adapted. We compared amino acid sequences with previously published VPX protein sequences of serotype 1 (very virulent, classical, antigenic variants) and serotype 2 IBDV strains. Short deduced amino acid sequences within VPX protein were analyzed for of VPX and VP2 representative sequences. In addition, the antigenic index of the complete VP2 protein was determined.

2. Materials and methods

2.1 Viruses

Field isolate 9109 was isolated from at the Poultry Diagnostic and Research Center (PDRC), University of Georgia and the classical attenuated Lukert strain (kindly provided by Dr.

Pedro Villegas, University of Georgia, Athens, GA). The field isolate 9109 was isolated from immunosuppressed broiler flocks showing subclinical signs of IBDV, bursal atrophy, and minimal mortality. In addition, 9109 has unique RFLP pattern when compared to the reference strains used for genotyping at PDRC. Edgar cell culture-adapted (CCA) (120-ADV-2001) and Edgar chicken embryo-adapted (CEA) (124-ADV-9501) challenge strains were obtained from National Veterinary Services Laboratory (Ames, IA). The 9109 isolate was propagated in 21 days of age (d.a.) specific pathogenic free (SPF) chickens. At 72 hours post-infection bursas were aseptically collected, snap frozen in liquid nitrogen and stored at -80 C. Lukert and Edgar CCA were propagated in primary chicken embryo fibroblasts (CEF) from SPF embryos, as previously described (Lukert and Davis, 1974).

2.2 RNA extraction

Total RNA was extracted from the Edgar CEA strain from the purchased virus without propagation using the RNeasy mini kit (Qiagen, Valencia, CA). Total RNA was extracted from Lukert or Edgar CCA infected chicken embryo fibroblast cells using the same kit. The supernatant was removed at 48-72 hours post-infection, cells overlaid with 300 µl RLT buffer containing 0.143 M β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA) and scraped with cell scraper as recommended by the manufacturer. Total RNA was extracted from homogenized bursas harvested from chicken infected with 9109 isolate. All samples of extracted RNA were incubated with Proteinase K (Qiagen) at 55 C for 30 min prior to RNA column purification.

2.3 Reverse transcription-polymerase chain reaction (RT-PCR) of segment A

The first strand cDNA was synthesized using modified previously reported segment specific primers: segACEF5 or segACEF3 for segment A (Boot et al., 2000). Additional primers were designed as needed using PrimerSelect software (DNAstar, Lasergene, v. 5, Inc., Madison, WI) and previously published sequences obtained from GenBank (Table 5.1). Initially 4-5 µg total RNA and the gene-specific primer were denatured at 98 C for 5 min and cooled at 4 C for 10 min. The first-strand cDNA was synthesized at 50 C for 30 min in a 20 µl reaction containing 1x first-strand buffer, 0.01 M DTT, 0.5 µM gene-specific primer, 40 units RNase-out (Invitrogen, Life Tech., Carlsbad, CA), 200 units SuperScript II and 0.5 mM deoxynucleoside triphosphates mix (dNTP) (Invitrogen). The RT mixture was then incubated at 70 C for 15 min. Two units of RNase H^- were added and incubated at 37 C for 20 min.

Segment A was amplified in two overlapping fragments with PCR primer pair set segAVP25/segACEF3 and segAVP23/segACEF5 with expected product size of 2691 and 1417 nts, respectively. Segment A positive RT-PCR controls were generated using the primer pair set segAVP25/segAVP23 with an expected size of 848 nts. The PCR reaction was synthesized in a 100 µl mixture containing 5 µl of the RT reaction, 1X PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, primers at 0.5 µM each and 2.5 units of recombinant or Platinum *Taq* DNA polymerase (Invitrogen). The PCR cycle parameters were as follows: 94 C for 3 min followed by 35 cycles at 94 C for 15 s, 55 C for 15 s, 72 C for 5 min and a final extension at 72 C for 30 min. The RT-PCR amplified products were electrophoresed on a 0.8% and visualized with ethidium bromide.

2.4 Cloning of segment A RT-PCR products and sequencing of VP2

The purified DNA was cloned into the pCR4-TOPO or pCRII-TOPO vectors according to the manufacturer's recommendations with maximum incubation times and transformed into competent *E. coli*, DH5α-T1R cells (Invitrogen). Transformants were selected on Luria-Bertani (LB) agar media (Q-BIOgene, CA, USA) containing 50 μg/ml kanamycin and screened by PCR with segment specific primers. Six clones from each PCR reaction were stored and three used for sequencing. Clones were expanded overnight at 37 C in 10 ml LB media containing 50 μg/ml kanamycin, centrifuged at 4 C for 10 min at 800 g, resuspended in fresh LB media with 10% glycerol, and stored at -80 C until further use.

Plasmid DNA was purified using the Qiagen miniprep kit (Qiagen) according to manufacturer's recommendations. Double-stranded DNA was sequenced by the dideoxy chain termination procedure using the automated ABI Prism 310, Genetic Analyzer (Applied Biosystems, ABI, Foster City, CA). Plasmid specific (M13 reverse and forward), as well as, IBDV segment specific primers were used for sequencing with primer walking. A consensus sequence was obtained using three clones which were sequenced at least three times with forward and reverse primers.

2.5 Multiple alignment and phylogenetic analysis of VP2 protein

The polyprotein sequences obtained from the four IBDV strains in this report were deposited in GenBank with accession numbers for 9109 (AAS10174.1), Lukert (AY918948), Edgar CCA (AAS10172.1) and Edgar CEA (AY918950).

Previously published polyprotein sequences used in our analysis have the following GenBank accession numbers: serotype 1 very virulent isolates D6948 (AAF85953.1), UPM97/61 (AAK72435.1), HK46 (AAD23373.1), UK661 (CAA63416.1), OKYM (BAA08555.1), serotype 1 classical isolates CEF94 (AAF16082.1), CEF (AAM11679.1), P2 (CAA58851.1), wild type Cu1(wt) (AAK51522.1), Cu1 (BAA00740.1), serotype 1 Delaware, variant E isolate (AAD32617.1) and serotype 2 isolates OH (AAC55351.1) and 2382 (AAK69713.1).

The deduced amino acid sequences were aligned with previously published IBDV VP2 sequences using ClustalW multiple sequence alignment program (DNAstar Lasergene, v. 5). Unrooted cladograms were generated using maximum parsimony analysis with Neighbor-Joining clustering and 1000 bootstrap replicates using the Phylogenetic Analysis Using Parsimony software (PAUP, v.10). Predicted antigenic regions within VP2 were obtained using the software program designed by Kolaskar (Kolaskar and Tongaonkar, 1990).

3. Results

3.1 VP2 protein analysis

The VPX protein consists of 512 amino acids (aa). Multiple alignment of the predicted amino acid sequences of different IBDV strains of the VPX protein revealed several amino acid substitutions (Fig. 5.1). Within the VP2 protein there are 18 amino acid substitutions in Lukert, 16 in Edgar CCA and CEA, and 10 in 9109 isolate. Lukert, Edgar CCA and CEA have unique substitution at aa 5 (Q→T) compared to the other strains used in this analysis. The VP2 processing site located between aa 442-512 (Da Costa et al., 2002; Lejal et al., 2000) is

conserved in 9109 isolate compared to the other strains but there are 15 aa substitutions in Edgar CCA, 8 in Edgar CEA, 1 in Lukert strain. In the VP2 processing site Edgar CCA and CEA have the same amino acid L^{451} as the very virulent strains.

Antigenic analysis of the VP2 revealed 18 to 20 potential antigenic regions for serotype 1 and 15 for serotype 2 strains. There are 20 antigenic regions in 9109, 19 in Lukert, and 18 in Edgar CCA and CEA as compared to consensus sequence of the analyzed strains. Among all serotype 1 strains used in this analysis fourteen amino acid regions shown as shaded in Figure 5.1 were highly conserved. There are a few exceptions within these antigenic regions including the single region aa 113-138 in Edgar CEA and the region aa 124-138 in Lukert is shifted to aa 132-143 and has 8 unique substitutions. In vvUPM9761 and OKYM strains region aa 291-298 is an exception within the conserved region aa 290-297 and has one unique amino acids. The greatest amino acid variability was revealed at aa 212-233, 236-265, 267-278, and 425-440 regions. The single antigenic region in Edgar CEA aa 212-233 is significantly shorter in 9109. This region in Lukert and Edgar CCA is divided into two regions comprised of aa 212-219 and aa 221-233. The antigenic region in Lukert and Edgar CCA aa 236-265 is divided into two aa 236-249 and aa 251-265 regions in Edgar CEA and 9109. The antigenic region aa 268-278 in Lukert, Edgar CEA and CCA has shifted to aa 267-275 in 9109 isolate. The greatest amino acid variability was revealed between aa 249-259. The most variable amino acid substitutions among strains were observed at positions 222, 242, 249, 253, 255, 270, 279, 284, and 286. The aa 222 in 9109 is T, in Edgar CEA is P, and in Edgar CCA and Lukert is S. The 9109 field isolate has unique substitutions at K^{249}, N^{251}, and N^{254}, Lukert at H^{249} and R^{251}, Edgar CEA at Q^{249}. Edgar CCA has substitutions at H^{249} and H^{253}, the last amino acid was conserved in P2 and Cu1. In Edgar CCA and Lukert the conserved V^{256} and G^{258} are substituted with A and D, respectively.

There are several other unique substitutions. Lukert, Edgar CCA and CEA have T^{5}, T^{270}, and S^{278} and 9109 isolate has substitutions at N^{213}, V^{242}, N^{279}, and N^{318}. At amino acid position 284 Edgar CEA and 9109 have A and Edgar CCA and Lukert have T. At position 286 Lukert, Edgar CCA and 9109 have I while Edgar CEA has T. Lukert has unique substitution at A^{314} and Edgar CEA at A^{252} and H^{354}. No substitutions were revealed in the serine rich motif ^{326}SWSASGS^{332} stipulated to have role in IBDV pathogenicity (Heine et al., 1991; Lin et al., 1993; Vakharia et al., 1994). The last VP2 predicted antigenic region was not conserved among strains. In Edgar CCA this region is between aa 379-386, in Edgar CEA is aa 430-438, in Lukert is aa 425-440, and in 9109 is divided into two regions composed of aa 425-432 and 434-440. Edgar CEA has 6 amino acid substitutions and 9109 isolate has one substitution R^{435}.

3.2 The unrooted phylogenic analysis

Unrooted phylogenetic analysis using the Neighbor-Joining method with 1000 bootstrap replicates was performed with alignments of deduced amino acid sequences of the VPX, VP2, the VP2 processing site, and amino acid fragments 202-451, 210-332, 210-440, 210-473, and 242-440. The unrooted analysis allows identification of related descendants without explicitly designating a common ancestor. The analysis of the VP2 processing site demonstrated formation of two branches (Fig. 5.2C): 1) serotype 2 and classical strains and 2) very virulent strains with Edgar CEA and CCA strains. The 9109 and variant E clustered with the classical strains. In the VP2 and VPX cladograms very virulent strains formed two distinct branches: 1) very virulent and 2) serotype 2 strains. In the same cladograms, 9109 and variant E clustered together with very high probability as determined by bootstrap. The amino acid sequences between 202-451

and 210-473 were representative of the VPX (Fig. 5.2A) cladogram and the sequences between aa 210-332, 210-440, and 242-440 were representative of the VP2 protein (Fig. 5.2B). As expected phylogenic analysis of the VP2 processing site identified a close relationship between Edgar CCA and CEA. These viruses were more similar to the very virulent than to the classical strains (Fig. 5.2C). While in the VPX analysis Edgar CEA and CCA were more similar to Lukert (Fig. 5.2A), in VP2, Edgar CCA and Lukert clustered together and Edgar CEA clustered with Cu1wt (Fig. 5.2B).

4. Discussion

The VPX protein of four IBDV strains with different pathogenicities was analyzed. The deduced amino acid sequences of these strains were compared with previously reported VPX protein sequences of serotypes 1 and 2 IBDV strains. This analysis identified unique amino acid residues and newly determined antigenic regions.

The newly predicted antigenic regions were based on the amino acid flexibility, hydrophilicity, and accessibility. Previously reported VP2 antigenic regions were based on hydrophilic peaks identified as the major A, aa 212-224, and B, aa 314-324 (Azad et al., 1987), and minor I, aa 248-252, II, aa 279-290, and III, aa 299-305 (Van den Berg and Martine, 1996). Our analysis revealed that only previously reported major A and minor I regions were part of the antigenic sites of VP2. The most variable amino acids are at positions 222 and 249. The greatest amino acid variability was observed between aa 249-259 which is part of the previously reported minor I region. The antigenic region A is part of region aa 212-233 in Edgar CEA, and aa 212-219 and 221-233 regions in Lukert and Edgar CCA. In 9109 and variant E the region between aa

212-233 was not identified as antigenic and antigenic region aa 267-275 is identical in both strains.

The amino acid substitutions at positions 222 (P→T, Q or S), at 249 (Q→K), and S^{254} are part of monoclonal antibody (mAb) epitopes and are considered US variant determinants (Cao et al., 1998; Hoque et al., 2001; Snyder et al., 1988 a.b.; Vakharia et al., 1994). The 9109 field isolate has a unique *SspI* restriction site in the VP2 gene (Banda et al., 2001) found in most vvIBDV strains (Lin et al., 1993). In this report T^{222} and K^{249} were observed within variant E and 9109. At the otherwise conserved G^{254}, variant E has S^{254} while 9109 has N^{254}. In addition, variant E and 9109 have other conserved amino acid substitutions at positions 213, 242, 249, 279, 284, 286, and 318. Therefore both isolates may share similar antigenicities. It has also been demonstrated that variants that do not react with a particular mAb may have amino acid substitutions (Eterradossi et al., 1998; Heine et al., 1991; Vakharia et al., 1994) or the lack of antigenic site.

Previously, it was shown that Edgar CEA and Lukert have different mAb binding patterns (Snyder et al, 1988 a.b. 1990, 1994; Van der Marel et al., 1990; Vakharia et al., 1994) which likely results from unique amino acid substitutions within the mAb binding epitopes. Lukert, 9109, Edgar CCA and CEA have substitutions at position 222 alone with substitutions in other antigenic regions. Edgar CEA has P^{222}, Q^{249}, and G^{254}. Edgar CCA and Lukert have S^{222}, H^{249}, and G^{254}. This contradicts the previous observations that only US variants have additional substitutions in the second hydrophilic domain (Brown and Skinner, 1996).

As shown by the VP2 crystal structure amino acids at position 222, 223, 251, 253, and 313 are surface accessible (Coulibaly, et al, 2005) and they likely within the antigenic regions. The amino acids important for cell culture adaptation and virulence at position 279 and 284 (Lim

et al., 1999; Mundt, 1999; Van Loon et al., 2002) and for neutralization-escape variants at position 322 (Schnitzler et al., 1993) were also found in the outmost loops of VP2 (Coulibaly et al., 2005) but do not participate in the antigenic regions determined in this study.

Amino acids Q^{253}, D^{279}, and A^{284} have been identified as markers for the classical strains and determinants for B-cell binding (Boot et al., 2001; Brandt et al., 2001). Substitutions of amino acids at position 253 (H→Q) or 284 (A→T) was determined as sufficient to adapt Edgar bursa-derived strain to gown in tissue-culture (Mundt, 1999) and to reduce the virulence *in vivo* (Van Loon et al., 2002). These mutations may eliminate an alpha-helix and result in a VP2 conformational change. In this study we found that Edgar CCA, P2, and Cu1 have H^{253} while the other three sequenced strains have Q which may affect virulence and cell tropism (Mundt, 1999). Variant E and 9109 have at 279 N and Lukert, Edgar CCA, and CEA have D. While at amino acid position 284 very virulent strains, as well as, Cu1wt, Edgar CEA, 9109, and variant E have (A) the highly attenuated cell-culture adapted Edgar CCA and Lukert strains have (T). Unique substitutions identified in very virulent strains are at aa 222, 242, 255, 286, 294, and 299. Very virulent strains have antigenic regions aa 213-233 and 236-265 identical to Edgar CEA and CCA, respectively.

The last predicted antigenic region is within VP2 carboxyl terminal and is not conserved among the four sequenced strains. It has been shown that VP2 carboxyl domain, aa 50-60 may play a role in the VP2-VP2 and VP2-VP3 interaction and capsid formation (Caston et al., 2001; Tacken et al., 2002, 2003). Amino acid substitutions or deletions within aa 442-512 may be lethal for the virus (Da Costa et al., 2002). In addition, Edgar CCA and CEA strains have numerous amino acid substitutions within the VP2 processing site and when compared to the

other IBDV strains at this site are similar to the very virulent. In the VPX and VP2 comparison Edgar CCA and CEA are similar to the classical strains.

Lukert, Edgar CCA and CEA strains have as expected sequence characteristics of the classical strains. The 9109 field isolate have several similarities with variant E within VP2 and VPX and classical strains in the VP2 processing site.

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Figure legends

Fig. 5.1. Multiple alignment of the predicted amino acid sequences of the VPX protein for Edgar chicken embryo adapted (CEA), Edgar cell culture adapted (CCA), 9109, and Lukert strains with previously sequenced other IBDV strains. The box with long dashes indicates the VP2 processing site. Previously reported VP2 variable domains are indicated with arrows and boxes (A, 1, B, 2, and 3). The important amino acids for tissue-culture adaptation are with bold letters. The shaded regions represent predicted antigenic regions. The amino acids sequence identical to the 9109 field isolate are indicated with dots and non-identical amino acids are presented with letters. The dashes indicate amino acid deletion. The amino acid positions are based upon the 9109 isolate.

Fig. 5.2. Phylogenetic relationship based on the amino acid (aa) sequences of the (A) VP2 aa 202-451, VP2 aa 210-473, VPX aa 1-512, (B) VP2 aa 210-440, aa 210-332, aa 242-440, VP2 aa 1-441, and (C) VP2 processing site for Edgar chicken embryo adapted (CEA), Edgar cell culture adapted (CCA), Lukert, and 9109 (shaded boxes) with other IBDV strains. The cladograms were generated using maximum parsimony analysis with Neighbor-Joining clustering and 1000 bootstrap replicates (confidence levels listed in parentheses) using the Phylogenetic Analysis Using Parsimony software (PAUP, v.10).

Table 5.1
Primers used for the VPX amplification and sequencing in this study.

Primer[a]	5' → 3'	Position[b]
RT-PCR		
segACEF5f	GGATACGATCGGTCTGACCCCGG	1-23
segA320f	CCTGGCTCAATTGTGGGTGCTCA	320-342
segAVP25f	CAACAGCCAACATCAACGAC	570-589
segA676	GTCACCAAGCCTCACATACCCAAGAT	676-651
segA1013f	GTGATTCCAACAAACGAGATAAC	1013-1035
segA1254f	CAAATCCTGAACTAGCAAAGAACC	1254-1277
segAVP23	CACCTCCATGAAGTACTCAC	1417-1398
segA1665	GCGAGAGTCAGCTGCCTTATGC	1665-1644
segACEF3	GGGGACCCGCGAACGG	3260-3245
TOPO vector		
T7	TAATACGACTCACTATAGGG	
T3	ATTAACCCTCACTAAAGGGA	
M13R	GTAAAACGACGGCCAG	
M13F	CAGGAAACAGCTATGAC	

[a] 'f' indicate forward primer.
[b]Primer position corresponds to the nucleotide positions at segment A of isolate CEF94 with GenBank AF194428 accession number.

Fig. 5.1.

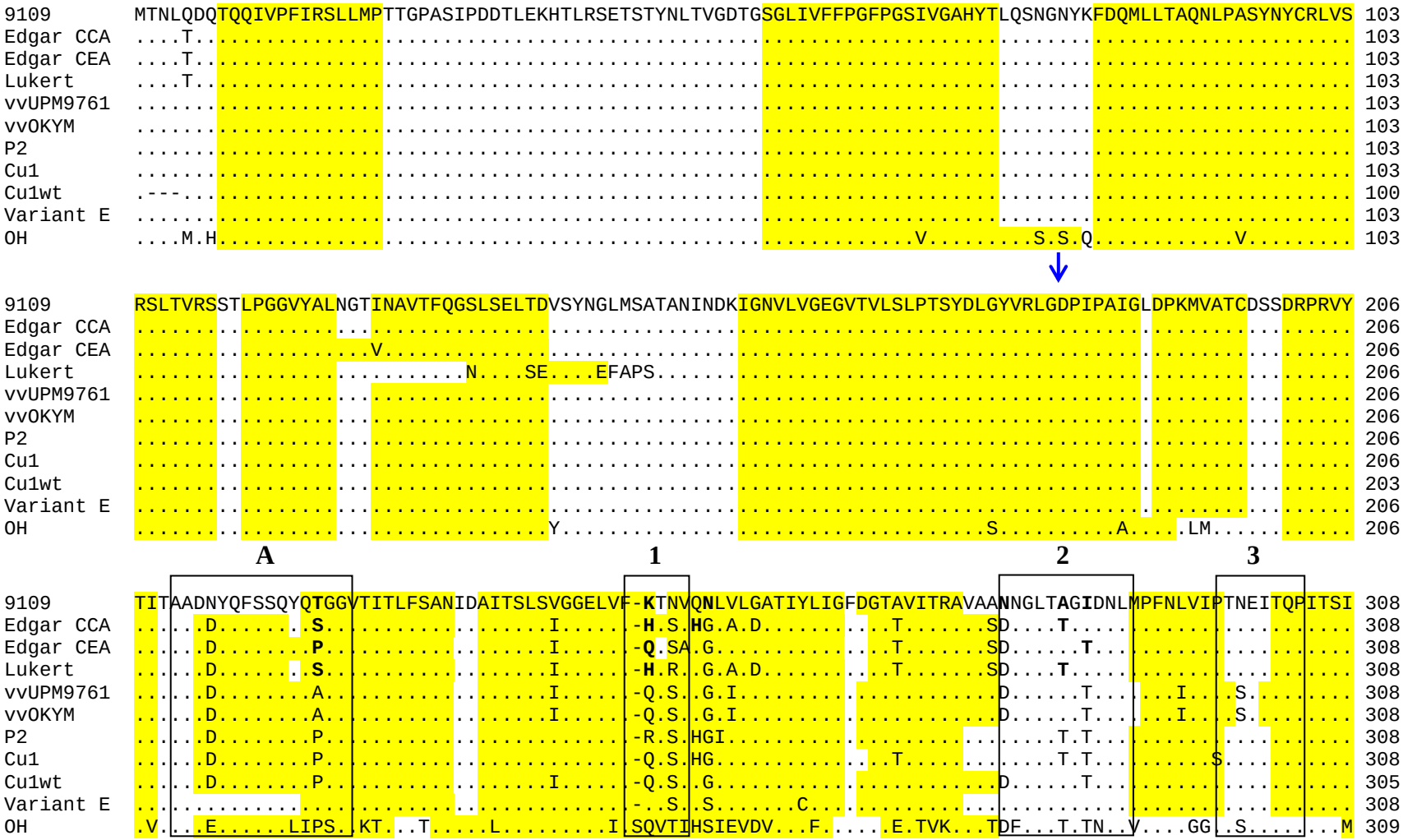

9109 MTNLQDQTQQIVPFIRSLLMPTTGPASIPDDTLEKHTLRSETSTYNLTVGDTGSGLIVFFPGFPGSIVGAHYTLQSNGNYKFDQMLLTAQNLPASYNYCRLVS 103
Edgar CCA ....T..... 103
Edgar CEA ....T..... 103
Lukert ....T..... 103
vvUPM9761 103
vvOKYM 103
P2 103
Cu1 103
Cu1wt .---..... 100
Variant E 103
OH ....M.H.....V.....S.S.Q.....V..... 103
9109 RSLTVRSSTLPGGVYALNGTINAVTFQGSLSELTDVSYNGLMSATANINDKIGNVLVGEGVTVLSLPTSYDLGYVRLGDPIPAIGLDPKMVATCDSSDRPRVY 206
Edgar CCA 206
Edgar CEA .....V..... 206
Lukert .....N....SE....EFAPS..... 206
vvUPM9761 206
vvOKYM 206
P2 206
Cu1 206
Cu1wt 203
Variant E 206
OH .....Y.....S.....A....LM..... 206
A
1
2
3
9109 TITAADNYQFSSQYQTGGVTITLFSANIDAITSLSVGGELVF-KTNVQNLVLGATIYLIGFDGTAVITRAVAANNGLTAGIDNLMPFNLVIPTNEITQPITSI 308
Edgar CCA .....D.....S.....I.....-H.S.HG.A.D.....T.....SD....T..... 308
Edgar CEA .....D.....P.....I.....-Q.SA.G.....T.....SD.....T..... 308
Lukert .....D.....S.....I.....-H.R..G.A.D.....T.....SD....T..... 308
vvUPM9761 .....D.....A.....I.....-Q.S..G.I.....D.....T.....I...S..... 308
vvOKYM .....D.....A.....I.....-Q.S..G.I.....D.....T.....I...S..... 308
P2 .....D.....P.....-R.S.HGI.....T.T..... 308
Cu1 .....D.....P.....-Q.S.HG.....T.....T.T.....S..... 308
Cu1wt .....D.....P.....I.....-Q.S..G.....D.....T..... 305
Variant E .....-..S..S.....C..... 308
OH .V...E.....LIPS..KT..T.....L.....I.SQVTIHSIEVDV...F.....E.TVK...TDF...T.TN..V....GG..S.....M 309

Fig. 5.1. (Continued)

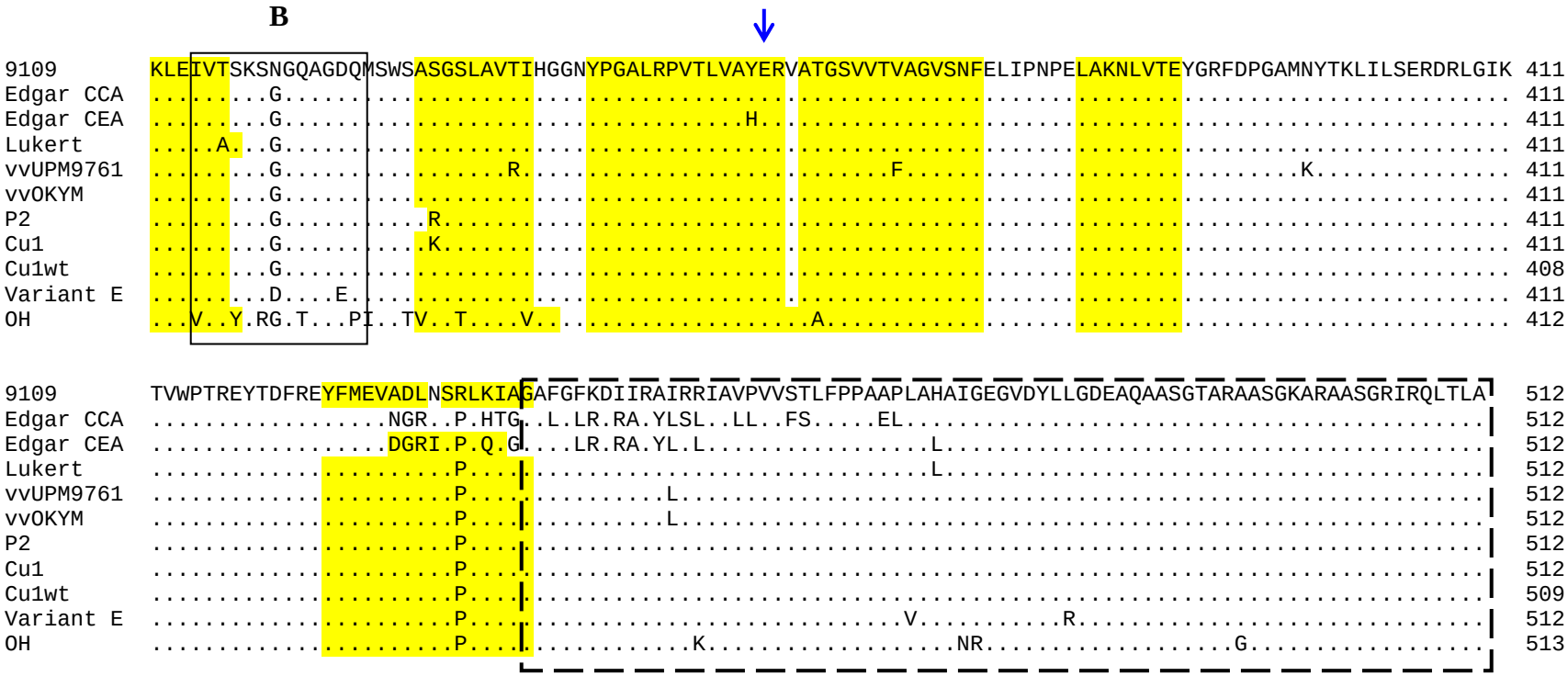
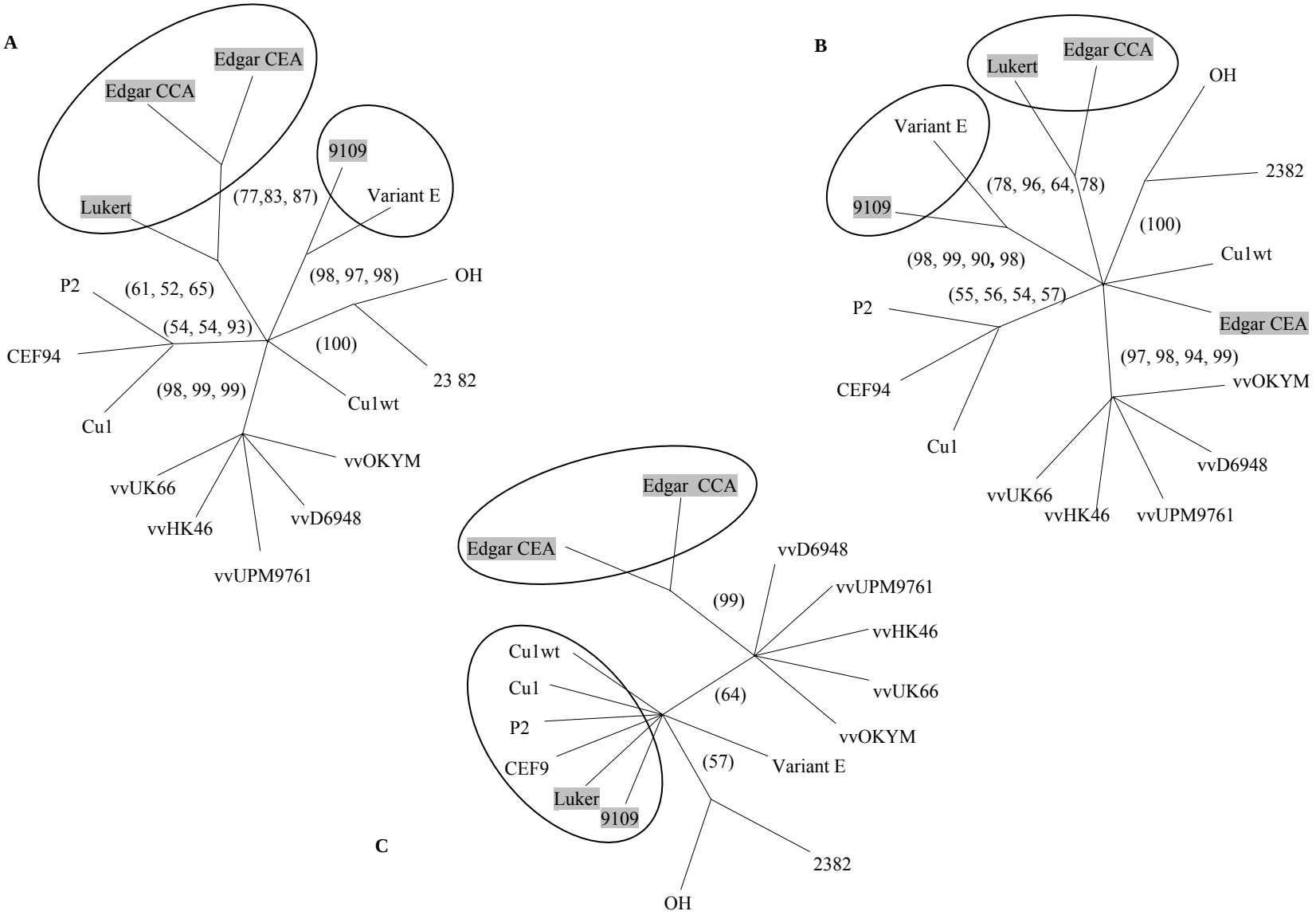


Fig. 5.2.


A
Edgar CEA
Edgar CCA
9109
Lukert
(77,83, 87)
Variant E
(98, 97, 98)
OH
P2
(61, 52, 65)
(54, 54, 93)
CEF94
(100)
23 82
(98, 99, 99)
Cu1wt
Cu1
vvOKYM
vvUK66
vvD6948
vvHK46
vvUPM9761
B
Lukert
Edgar CCA
OH
Variant E
2382
(78, 96, 64, 78)
9109
(100)
(98, 99, 90, 98)
Cu1wt
(55, 56, 54, 57)
P2
Edgar CEA
(97, 98, 94, 99)
CEF94
vvOKYM
Cu1
vvD6948
vvUK66
vvHK46
vvUPM9761
C
Edgar CCA
Edgar CEA
vvD6948
(99)
vvUPM9761
vvHK46
Cu1wt
Cu1
(64)
vvUK66
P2
vvOKYM
CEF9
(57)
Variant E
Luker
9109
2382
OH

CHAPTER VI

DISCUSSION AND CONCLUSIONS

Characterization of the chicken antibody response against infectious bursal disease virus (IBDV) and IDV impact on B-cells

The bursa of Fabricius, a primary lymphoid organ in chickens is a major site for B-cell division, gene conversion, and clonal differentiation and contains more than 85% IgM^+, B-cells. In chickens, cell progenitor commitment to the B-lymphocyte lineage occurs during a short period of time and immunoglobulin gene rearrangement is not a permanent process. Bursectomized and irradiated chickens at one day of age (d.a.) do not produce circulating antibodies. The lymphocytes in each of the bursal lymphoid follicles originate from few stem cells with a specific VDJ rearrangement. Diversification of the immunoglobulin classes is an irreversible, age-dependent process in the order of IgM→IgG→IgA. In the bursa, some of the IgM^+ cells become IgM^+/IgG^+ double positive cells. In addition, the bursal B-cell subpopulations have different abilities to populate bursal compartments and peripheral tissues.

Infectious bursal disease virus (IBDV) causes humoral and cellular immunodeficiency in chickens. In the bursa, IBDV infection results in a temporary reduction of proliferating immature IgM^+, B-cells and thus reduces the B-cell repertoire. Although following IBDV infection, the bursa remains the functional lymphoid organ wherein bursal follicles are restored, the B-cells have a reduced proliferating response. Earlier histopathological evaluation of the bursa suggests the existence of bursal IBDV resistant B-cells expressing surface determinants Bu1b or MUI36.

The precise IBDV cell target has not been identified *in vivo.* Earlier histopathological evaluation of the bursa suggests the existence of bursal IBDV resistant B-lymphocytes expressing surface determinants Bu1b or MUI36. In addition, there is no clear understanding of IBDV affect on the total serum immunoglobulin levels.

Since IBDV causes a reduction of immature IgM^+, B-cells, IgA^+, IgG^+, and IgM^+, B-cell subpopulations should be affected. Our objectives were to identify and phenotype the B-cell subpopulations susceptible to IBDV and those responsible for bursal regeneration using flow cytometric analysis. Bursa and spleen were collected between 3-45 d.a. from chickens vaccinated at 3 d.a. and challenged at 25 d.a. In addition, total serum IgG, IgA, and IgM immunoglobulins and specific IBDV antibody levels were also measured.

In the bursa, two B-cell subpopulations designated as A and B were identified based on cell size and granularity. The subpopulation B IgM^+, B-cells was slightly reduced following vaccination and gradually depleted in the non-vacc/chall group following challenge. Bursal follicle restoration and an increase of subpopulation B IgM^+ cells at 21 days post challenge (d.p.c.) was observed. A rapid bursal follicle B-cell repopulation following challenge with nonattenuated Edgar strain indicates the presence of IBDV nonsusceptible immature B-cells at an earlier developmental stage than subpopulation B. Subpopulations A and B IgM^+ cells in the vacc/chall group were not affected by IBDV challenge. Subpopulation A IgG^+ cells in the non-vacc/chall group following challenge and IgA^+ cells in the vacc/non-chall and non-vacc/chall groups following vaccination and challenge, respectively were increased.

Although there was significant reduction of subpopulation B in the non-vacc/chall group following challenge, the immunosuppression may be temporary. The percent positive B-cells in the spleen did not change between the vacc/non-vacc and non-vacc/non-chall groups. In the non-

vacc/chall group, IgM^+ cells decrease post challenge. The effect of IBDV on the bursa resembles a bursectomy, which performed at different ages affects different immunoglobulin classes. The IBDV infection hinders B-cell maturation and likely decreases the B-cell repertoire. Bursal regeneration is necessary following IBDV infection for normal antibody response. Previously, it was suggested that the non-bursal lymphoid sites help in restoration of the humoral function in B-cell depleted animals as theses sites can partially replace the bursal microenvironment. In addition, it was previously demonstrated that in the bursa the process of hyperconversion continues until the bursa involutes.

The two bursal B-cell subpopulations were phenotyped using B-cell surface expressed antigens and flow cytometric analysis. Bursas were collected from chickens during two time periods, 3-45 d.a. and 90-125 d.a. The first period coincides with rapid bursal B-cell replication and the second period coincides with the initial sings of functional bursal involution.

A close correlation between percent $Bu1b^+$ and $MUI36^+$ cells from both subpopulations between 3-125 d.a. was observed. Bursal IBDV resistant $Bu1b^+/Lewis^{x-}$ and $MUI36^+$ cells were previously reported. $Bu1b^+$ cells repopulate bursal follicles depleted by IBDV before the functionally active $Lewis^{x+}$ cells are detected. The Bu1 antigen is expressed in immature cells, before immunoglobulin rearrangement and is not expressed on plasma cells. Although the MUI36 is initially expressed on immature B-cells, at a later developmental stage this antigen is restricted to plasma cells. While the percent $Lewis^x$ positive cells in both subpopulations did not differ during 3 and 31 d.a. there was a reduction in positive cells in subpopulation A starting from 38 d.a. The expression of $Lewis^x$ may be important for cell adhesion and homing is down-regulated as bursal B-cells mature. The major histocompatibility complex II (MHCII) was suggested to restrict the T-cell dependent secretory antibody response against IBDV. MHCII

expression coincides with IgM expression and increases during the bursal embryonic phase of B-cell differentiation. Most of the cells in both subpopulations between 3-125 d.a. were MHCII positive. Initially, at 3 d.a. there was almost equal percent of the MHCII restricted antigen MUI78^{+} cells in subpopulation A and B and between 90-125 d.a. the number of MUI78^{+} cells in subpopulation B was higher than in subpopulation A.

Secretory IgA plays a significant role in the mucosal immunity and the total serum IgA was elevated in both vacc/non-chall and non-vacc/chall groups following vaccination and challenge, respectively. We did not observe an antibody class-switch the IgM→IgG in the vacc/chall group as was previously suggested. The total serum IgM was elevated only for short period in the vacc/non-chall and non-vacc/chall group following vaccination and challenge. The total serum IgG was reduced in the non-vacc/chall group following challenge in comparison to the other three groups but elevated in the next two time points. The specific IBDV seroconversion in the non-vacc/chall group was elevated faster, higher, and persisted longer than in the vacc/non-chall group. At 7 d.p.c. in the non-vacc/chall group when the specific IBDV titer peaked, the total serum IgG was at its lowest level. In the vacc/chall group titers decreased quickly following challenge when compared to the other two groups. The differences of the serum immunoglobulin levels may be due to IBDV strain variations. Neutralization assays demonstrated that more virulent strains induce higher anti-IBDV titers and the viral antigen persists for longer time.

In our experiments, the total serum immunoglobulin concentrations were lower than in non-specific pathogen free (SPF) chickens and we were unable to measure total serum IgA and IgM at 3 d.a. Previously, it was reported that in SPF chickens IgG^{+} and IgA^{+} plasma cells were detected as early as 14 d.a. and IgM^{+} at 5 d.a. The germ-free environment hinders B-cell

development in peripheral lymphoid organs due to inadequate antigenic stimuli that leads to decreased numbers of IgG^+, B-cells and reduces B-cells proliferation.

The subpopulation A and B demonstrated age-related changes such as a decrease of the proportion of subpopulation A and an increase of subpopulation B when compared with the total analyzed bursal cells. Interestingly, the reduction of subpopulation A and increase of subpopulation B occurs between 97-125 d.a., a period of initial bursal functional involution. The two subpopulations co-express the B-lymphoid surface antigens $Lewis^x$, IgM, Bu1b, MUI36, MUI78, and MHCII. With the exception of MHCII, all are expressed differentially. The percent positive cells for these antigens in subpopulation B were higher than in subpopulation A. In addition, the expression of $MUI78^+$ and $Lewis^{x+}$ was age-dependent. After 90 d.a MUI78 was up-regulated in subpopulation B while $Lewis^x$ was down-regulated in subpopulation A.

In conclusion, IBDV resistant and susceptible bursal IgM^+, B-cell subpopulations were identified. The IBDV resistant subpopulation A most likely consists of immature cells which act to repopulate the bursa following IBDV infection. The subpopulation B was decreased following IBDV vaccination and challenge. The subpopulation B depletion did not reduce the total serum IgA, IgG, and IgM immunoglobulin levels nor did it affect IgG^+ and IgA^+, B-cells in spleen.

Molecular characterization of four IBDV strains with different pathogenicities using full-length sequence analysis

Two serotypes of IBDV have been identified, however, only serotype 1 viruses cause disease in chickens. Strains within serotype 1 differ in their pathogenicity and antigenicity, and are generally classified as very virulent (vvIBDV), classical, or antigenic variants.

IBDV is a bi-segmented A and B, double stranded RNA virus belonging to the *Birnaviridae* family. Segment B encodes for VP1, the RNA-dependent, RNA-polymerase (RdRp). The replication efficiency and virulence factors have been linked to segment B. The protein plays a role in viral encapsidation, primes the viral RNA synthesis, and initiates protein synthesis. The RdRp activity is 3' non-coding region (NCR) dependent on segment A and B. The 3' NCR has a signal recognition function for replication, transcription, and translation. Although the VP1 protein of the vvIBDV strains form a distinct cluster separate from the classical strains, chimeric IBDV viruses containing segment A and B from classical and very virulent strains, respectively do not show increase of the viral virulence or pathogenicity. Segment A has two open reading frames (ORFs). The ORF1 encodes for the VP5 protein. The monocistronic ORF2 encodes for precursor polyprotein that is auto-catalytically cleaved to VPX, VP4, and VP3. The group-specific VP3 protein induces non-neutralizing antibodies and may be involved in replication and genome packaging. VP4 is a *cis*-acting viral protease that processes the polyprotein.

The VPX protein, amino acids (aa) 1-512, is processed to the VP2 protein, aa 1-441. The VP2 protein induces host protective neutralizing antibodies. The greatest amino acid variability in the IBDV genome is within VP2 protein. Changes within the minimally required VP2 antibody binding region, aa 206-350, can lead to antigenic variants with different pathogenicities and cell tropism. It has been previously suggested that VP2 is not the only virulent determinant and that viral pathogenicity can be determined by genomic regions beyond this protein. Within VP2, aa 206-350 hydrophilic peaks designated as major A and B and minor I, II, and III regions were identified. It was suggested that virus neutralization conformational dependent epitopes exist beyond these regions. Several amino acid substitutions located between the two major

hydrophilic A and B regions were also related to virulence. Classical strains have unique amino acid substitutions within the hydrophobic regions of VP2 protein and only two of the seven amino acid substitutions at positions 222 and 251 previously determined as markers for very virulent strains are within the hydrophilic regions. Most of the monoclonal antibodies (mAb) used for IBDV strain characterization bind outside of the previously reported hydrophilic regions.

Most of the current molecular techniques used for IBDV typing utilize amplification of a representative genomic sequence within VP2 by reverse transcriptase (RT) polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis. Different strains can be grouped into genotypes based on RFLP patterns. However, some strains can not be characterized by this procedure and regions outside of this VP2 fragment may also be important.

The level of viral attenuation and/or propagation affects pathogenicity of related viruses. The classical Lukert strain is a further attenuated Edgar cell culture adapted strain. The Edgar bursa-derived strain compared with both Edgar CEA and Lukert strains is the most invasive and pathogenic. Field isolate 9109 was obtained from immunosuppressed broiler flocks showing subclinical signs of IBDV, bursal atrophy, and minimal mortality. In addition, isolate 9109 has a unique *SspI* restriction site in the VP2 protein and displays unique RFLP pattern when compared to the reference strains used for genotyping at Poultry Diagnostic and Research Center, University of Georgia. The *SspI* restriction endonuclease site is also unique to vvIBDV strains.

The fields isolate 9109, Lukert, Edgar CCA, and Edgar CEA strains were sequenced and characterized. The full-length nucleotide and deduced amino acid sequences of these strains were compared with previously reported full-length sequences of serotype 1 very virulent, classical,

antigenic variants, and serotype 2 IBDV strains. Short deduced amino acid sequences within VPX protein were analyzed for representative sequences of IBDV genomes. In addition, antigenic index analysis of the complete VP2 protein was performed.

Although segment B was highly conserved among analyzed strains a few exceptions were identified. The four sequenced strains were determined by the phylogenetic analysis of VP1 protein to be classical strains. The sequence of the 5' NCR of segment B along with the total number of deduced amino acids of the VP1 protein varies. The VP1 protein in Lukert and Edgar CCA contains two additional amino acids compared to 9109 and Edgar CEA strains. Similar variations have been previously described for classical P2, Cu1, Cu1M, and CEF94 strains but the significance is still unknown. Edgar CEA has a substitution at aa 46 (S to G) within the Ser-phosphorylation region. Amino acids previously suggested to play a role in cell-specific replication, virulence, phosphorylation, glycosylation, NTP-binding motifs, and the RNA-dependent, RNA-polymerase (RdRp) motifs were highly conserved between all strains analyzed.

The VP5 protein was highly conserved. Lukert, Edgar CCA and CEA strains have substitutions within the VP5 protein at aa 16 (D to A), indicating that this amino acid may not be important for viral pathogenicity as serotype 2 strains have the same substitution. In addition Edgar CEA contains a second substitution at aa 135 (H to R). The cytotoxic protein is important for the viral release, induction of apoptosis, and pore formation in the cells. Although previous analysis of VP5 did not reveal amino acids unique to the very virulent pathotype, amino acid substitutions could affect VP5 function. A IBDV VP5 knock-out mutant has been shown to replicate in the bursa without bursal lesions and does not induce apoptosis as does the wild type.

Mutations in the VP4 protease may affect the polyprotein cleavage and may have a role in viral adaptation and pathogenicity. Substitutions in the VP4 protein at aa 541 (V to I) and aa

547 (N to D) in 9109 are within motif I and several substitutions in Edgar CEA are between serine-protease motifs I and II. The amino acid Y^{680} observed in 9109 was previously proposed as very virulent marker.

Substitutions at aa 785 (L to I) and 993 (Q to P) in Lukert, Edgar CCA, and CEA are in the VP3- and double strand (ds)RNA-binding domains, respectively. The 9109 isolate has Q^{981} in the dsRNA- and A^{1005} in VP1-binding domain. In addition, it was determined that A^{1005} is a vvIBDV characteristic and may be significant for the pathogenicity. Previously, the VP3 carboxyl terminus, aa 857-1012, was found to be important for vvIBDV pathogenicity and formation of VP3-VP2 complex, which has role IBDV replication and genome packaging.

The stem-loop structure of the 3' NCR is an important functional determinant and plays a role in the protein-primed RNA synthesis and RNA packaging. The mutation at nucleotide (nt) position 3257 of segment A in Edgar CEA is within the inverted terminal repeat (3255-3260 nts) at the 3' NCR and changes the energy level of the stem-loop, -3.1 *vs.* -11.7 kcal/mole in the other sequenced strains. The mutation at nt 75 (U to C) in Lukert within the 5' NCR of segment A is in the putative 18S rRNA binding domain and may affect the transcription and translation efficiency.

The viral protein VPX and amino acid sequences between aa 202-451 and 210-473 but not VP2 protein are the best representatives of the entire IBDV genome. In addition, aa 210-440, 210-332, and 242-440 were representative for VP2 proteins.

The newly predicted antigenic regions were based on the amino acid flexibility, hydrophilicity, and accessibility. Previously reported VP2 antigenic regions were based on hydrophilic peaks identified as the major A, aa 212-224, and B, aa 314-324 (Azad et al., 1987), and minor I, aa 248-252, II, aa 279-290, and III, aa 299-305. Our analysis revealed that only

major A and minor I regions were part of the antigenic sites of VP2. The most variable amino acids are at positions 222 and 249. The greatest amino acid variability was observed between aa 249-259 which is part of the previously reported minor I region. The antigenic region A is part of region aa 212-233 in Edgar CEA, and aa 212-219 and 221-233 regions in Lukert and Edgar CCA. In 9109 and variant E the region between aa 212-233 was not identified as antigenic and antigenic region aa 267-275 is identical in both strains. Edgar CEA and CCA strains have antigenic regions aa 213-233 and 236-265 that are identical to the very virulent strains. The last predicted antigenic region is within VP2 carboxyl terminal and is not conserved among the four sequenced strains. It has been shown that VP2 carboxyl domain, aa 50-60 may play a role in the VP2-VP2 and VP2-VP3 interaction and capsid formation. Amino acid substitutions or deletions within aa 442-512 may be lethal for the virus. In the VPX and VP2 proteins Edgar CCA and CEA are similar to the classical strains. Both Edgar CCA and CEA have numerous amino acid substitutions within the VP2 processing site and when compared to the other IBDV strains at this site are similar to the very virulent.

The amino acid substitutions at positions 222 (P to T, Q or S), at 249 (Q to K), and S^{254} are part of mAbs epitopes and are considered US variant determinants. In this report we found that variant E and 9109 strains have T^{222} and K^{249} and at the conserved G^{254} variant E has S^{254} while 9109 has N^{254}. In addition, variant E and 9109 have other conserved amino acid substitutions at positions 213, 242, 279, 284, 286, and 318. Therefore both isolates may share similar antigenicities. Amino acid determinants in VP2 for tissue-culture adaptation were conserved in Edgar CCA and Lukert. Previously, it was shown that Edgar CEA and Lukert have different mAb binding patterns which likely results from unique amino acid substitutions within the mAb binding epitopes. Lukert, 9109, Edgar CCA and CEA have substitutions at position 222

alone with substitutions in other antigenic regions. Edgar CEA has P^{222}, Q^{249}, and G^{254}. Edgar CCA and Lukert have S^{222}, H^{249}, and G^{254}. This contradicts the previous observations that only US variants have additional substitutions in the second hydrophilic domain.

Amino acids Q^{253}, D^{279}, and A^{284} have been identified as markers for the classical strains and determinants for B-cell binding. Substitutions of amino acids at position 253 (H to Q) or 284 (A to T) was determined as sufficient to adapt Edgar bursa-derived strain to gown in tissue-culture and to reduce the virulence *in vivo*. These mutations may eliminate an alpha-helix and result in a VP2 conformational change. In this study we found that Edgar CCA has at 253 H while the other three sequenced strains have Q which may affect virulence and cell tropism. Variant E and 9109 have at aa 279 N and Lukert, Edgar CCA, and CEA have D. While at aa 284 very virulent strains, as well as Edgar CEA, 9109, and variant E have A, the highly attenuated cell-culture adapted Edgar CCA and Lukert strains have T.

As shown by the recently published VP2 crystal structure, aa 222, 223, 251, 253, and 313 are surface accessible and they are likely within the antigenic regions. The amino acids important for cell culture adaptation and virulence at position 279 and 284 were also found in the outmost loops of VP2 but do not participate in the antigenic regions determined by this study.

Phylogenetic and sequence analysis of the field isolate 9109 revealed unique features. The isolate has characteristics of classical, variant, and very virulent strain depending on the region analyzed. The 9109 isolate is more similar to the classical strains in the VP2 processing site, VP3, and VP4 proteins. Although we did not find the unique *SspI* restriction site within the VP2 of 9109 isolate previously suggested as a very virulent marker, we have identified two amino acid substitutions Y^{680} in VP4 and A^{1005} in VP3 which were also reported as vvIBDV determinants. In addition, aa 147 in the VP1 protein was substituted only in 9109 and very

virulent strains. Phylogenetic analysis of the 5' and 3' NCR of segment A revealed similarity between 9109 and the vvD6948 strain. There are also several similarities between variant E and 9109 in VPX, VP2, and VPX-VP4-VP3 polyprotein. The predicted antigenic regions for 9109 isolate demonstrated similarities between this isolate and variant E.

Different nucleotide or deduced amino acid sequences used for virus characterization may influence the analysis of a particular strain or isolates. Close nucleotide and amino acid relatedness between Lukert, Edgar CCA and CEA was established. Although Lukert, Edgar CCA and CEA strains have as expected sequence characteristics of the classical strains when using the VP2 processing site Edgar CCA and CEA were classified as a very virulent strains.

Our analysis revealed that although VPX amino acid comparison may be initially useful, complete genomic sequence information for strain molecular characterization is essential as partial sequences may falsely designate a particular strain as very virulent, classical, or variant.