CHARACTERIZATION OF FIELD STRAINS OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) USING MOLECULAR TECHNIQUES

by

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ABSTRACT

This study was aimed to apply different molecular techniques in the genotyping of field strains of infectious bursal disease virus (IBDV) currently present in the United States and in some other countries. The different techniques included the reverse transcription-polymerase chain reaction / restriction fragment length polymorphism (RT-PCR/RFLP), heteroduplex mobility assay (HMA), nucleotide and amino acid sequence analysis, and riboprobe *in situ* hybridization (ISH). From 150 samples analyzed from the United States, 80% exhibited RFLP identical to the variant Delaware E strain, other strains detected included Sal-1, D-78, Lukert, PBG-98, Delaware A, GLS IBDV standard challenge strain –like (STC-like). The analysis of the deduced amino acid sequence of the VP2 hypervariable region from six strains classified as Delaware variant E, revealed some amino acid substitutions that make them somewhat different from the original variant E strain isolated in the mid 1980s. The isolate 9109 was classified as a standard strain, but, it exhibited a unique RFLP pattern characterized by the presence of the *Ssp* I restriction site characteristic of the very virulent IBDV (vvIBDV) strains. The pathogenic properties of this isolate were compared to those of isolate 9865 (variant strain) and the Edgar strain. All three strains induced subclinical disease, however by *in situ* hybridization some differences in the tissue tropism were observed. The viral replication of the variant isolate 9865 was more restricted to the bursa of Fabricius. Isolate 9109 and the Edgar strain were also observed in thymus, cecal tonsils, spleen, kidney and proventriculus. A variety of inactivated IBDV strains received from Latin America were detected by RT-PCR/RFLP. The more interesting findings include the presence in Mexico and Venezuela of IBDV strains with unknown RFLP patterns, and the observation of RFLP patterns indicative of vvIBDV strains in Brazil and Dominican Republic. Finally, the HMA was evaluated as a method for genotyping IBDV. The HMA was able to differentiate between standard, antigenic variants and very virulent strains. Minor differences between antigenic variants were also detected. The results obtained by HMA were similar to that obtained with RFLP and phylogenetic analysis.

INDEX WORDS: Infectious bursal disease, Field isolates, Molecular characterization, restriction fragment length polymorphism, Heteroduplex mobility assay, *In situ* hybridization

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2002

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August 2002

DEDICATION

To my family; my mom Lydia, my siblings Ana Lilia, Rafael, and Alfonso, and my little niece Liliana for the things we share...

ACKNOWLEDGEMENTS

I am deeply thankful to my major professor, Dr. Pedro Villegas, for giving me the opportunity of being a graduate student under his direction. I would like to find a better way to express my gratitude but, I could not find it, so… THANKS!.

I express also my appreciation to each professor that served on my advisory committee, Dr. Corrie C. Brown, Dr. Donald L. Dawe, Dr. Maricarmen Garcia, and Dr. Mark W. Jackwood. Thanks for your input for my research project and for your contributions to my professional development.

I want to thank my good friends and colleages Dr John El-Attrache, Dr. Carlos Estevez, and Dr. Ivan Alvarado. You made my time at Dr. Villegas' lab a quite experience. It was a real honor, I will miss all of you.

I wish to thank Dr. Miguel Ruano, Dr. James Stanton, and Dr. Erika Behling-Kelly for your technical assistance.

Finally I want to thank to the faculty, staff and students of the Department of Avian Medicine. I spent a wonderful time with you. Georgia and all of you will be always in my mind.

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CHAPTER I

INTRODUCTION

Purpose of the Study

Infectious bursal disease (IBD) is an acute and highly contagious disease of young birds. IBD is distributed worldwide, and the effects of the disease are very significant in the commercial poultry industry. The disease may be fatal when highly virulent strains are involved, however, when antigenic variants of IBDV are present, the disease is subclinical and leads to various degrees of immunosuppression. After the infection with variant strains, the birds may become extremely susceptible to other pathogenic agents, such as Newcastle disease virus, infectious bronchitis virus, infectious laryngotracheitis virus, *Salmonella* spp or to opportunistic agents such as *Escherichia coli.* Furthermore, immunosuppressed birds will not respond properly to vaccination and may suffer severe or persistent postvaccinal reactions.

The disease has a global distribution, and various forms of disease have been described. There is a high variation in the genetic properties between strains, these variations may play a role determining the antigenic and pathological characteristics of the viruses present in the field. Since IBDV is ubiquitous and extremely resistant to environmental conditions, most of the efforts to control the disease are focused on vaccination programs. Different criteria are used for establishing vaccination programs, and different vaccine strains are used indiscriminately, without a complete knowledge of

the characteristics of the IBDV strains prevalent in the field. Thus, the diagnosis and accurate identification or typing of the different strains play a key role in the proper development of vaccination programs. There are different diagnostic approaches for detecting and typing IBDV, however, the application of molecular techniques is increasing and becoming more popular.

The structural protein VP2 of IBDV has been determined to be the major antigen that elicits a neutralizing response. There is a hypervariable region of the VP2 gene that contains most of the differences are present between IBDV strains. Since this hypervariable region of the VP2 gene exhibits a number of changes depending on the IBDV strain, most of the molecular techniques for genotyping IBDV are focused on analyzing this region.

This study was aimed at applying different molecular techniques in the genotyping of field IBDV strains currently present in the United States and in some other countries. The different techniques applied include the reverse transcription-polymerase chain reaction / restriction fragment length polymorphism (RT-PCR/RFLP), heteroduplex mobility assay (HMA), nucleotide and amino acid sequence analysis, and riboprobe *in situ* hybridization (ISH). It is expected that the findings of this study will contribute to the knowledge of the current situation of IBDV strains and to enhance the diagnostic methods for genotyping IBDV.

Objectives and Originality

The use of molecular techniques in the diagnosis and subtyping of IBDV offers several advantages over the conventional methods. The molecular techniques are characterized by their sensitivity, specificity and rapidity. The molecular technique most widely used for subtyping IBDV is RT-PCR/RFLP. There are several reports describing the usefulness of this technique. This method readily distinguishes the different IBDV strains. The first objective of this study was to characterize the molecular properties of seven recent IBDV isolates from the state of Georgia, based on the differences of the hypervariable region of the VP2 gene detected by RT-PCR/ RFLP.

Results from RT-PCR/RFLP and nucleotide analysis indicated the prevalence of the Delaware variant E in commercial broiler flocks in Georgia. However, these viruses showed some peculiar changes that make them somewhat different from the original Delaware variant E strain initially isolated by Dr. Rosenberger (University of Delaware, Newark DE) in the mid 1980s. Another interesting finding was the presence of the restriction site for the enzyme *Ssp* I in field strains in the United States and some Latin American countries. The presence of that restriction site is characteristic of the highly virulent strains, and has been considered as a molecular marker for very virulent IBDV (vvIBDV) strains. To the best of our knowledge, this is the first report on *Ssp* I –positive strains in the United States.

The second objective was the molecular characterization by RT-PCR / RFLP of IBDVs from Latin American countries. Several genotypes were detected in the different countries in Latin America. The most relevant finding is the confirmation of the presence

of vvIBDV type viruses in Brazil and the Dominican Republic. Some IBDV isolates exhibiting unique RFLP patterns were detected in Mexico and Venezuela, these viruses were not completely typeable by the system.

The third objective of this study was the introduction of HMA as a method for subtyping field IBDV. HMA is a technique to detect mutations present in PCR amplicons. With the use of HMA, two different PCR amplicons can be compared. HMA has been useful for molecular epidemiology of viral diseases relevant in human health. The applications of HMA include: a) subtyping of viral species, b) measuring the diversity within viral genome populations (quasispecies studies), and c) screening for genome variants among a large number of samples.

The use of HMA has been focused on the analysis of pathogenic agents such as human immunodeficiency virus, hepatitis C virus, measles virus, GB virus C (hepatitis G), human influenza virus, poliovirus and others. This method has also been applied experimentally to the genotyping and classification of mutations related to some genetic diseases such as phenylketonuria and von Willebrand's disease. However, the application of HMA in avian medicine has been limited. There is one report of the use of HMA for analyzing the fusion protein cleavage site for subtyping Newcastle disease virus (NDV), the technique allowed for the distinction of vaccine-like viruses from other NDV isolates potentially virulent for chickens.

In this research, HMA was used to compare the hypervariable region of the VP2 gene of IBDV. The results indicate that the technique readily distinguishes between the different subtypes of IBDV: standard, antigenic variants and vvIBDV strains. The advantage of using HMA over RT-PCR/RFLP is that the complete sequence of the PCR

amplicons is analyzed; whereas with the use of RFLP only some regions of the nucleotide sequence are analyzed, make it difficult for IBDV typing. Furthermore, relationships between isolates and strains can be determined thus; evolutive and epidemiological information can be obtained. Finally, HMA may also be an auxiliary tool for selecting the vaccine strains that are more related to the field strains.

The fourth objective of this study was to determine the tissue tropism using riboprobe ISH of two recent field isolates of IBDV obtained from commercial broiler flocks. The use of riboprobe ISH is increasing and its usefulness in cellular biology and pathology is promising. This technique combines the specificity of the molecular techniques but also provides information about the location of nucleic acid sequences within the tissues.

CHAPTER II

LITERATURE REVIEW

Part 1: Infectious Bursal Disease Virus

a) History

Infectious bursal disease virus (IBDV) is the etiological agent of Gumboro disease or infectious bursal disease (IBD). IBD is a highly contagious viral disease of young chickens, characterized by destruction of the lymphocytic cells present in the bursa of Fabricius, producing a severe immunosuppression. The disease was first reported by Cosgrove in 1957. It was initially recognized as “avian nephrosis”, the syndrome became known as “Gumboro disease”. The clinical features of the syndrome included whitish or watery diarrhea, followed by anorexia, depression, trembling, severe prostration, and death. At necropsy, the birds exhibited dehydration, hemorrhages in the leg and thigh muscles, urate deposits in kidneys and enlargement of the bursa of Fabricius (33).

The early consensus was that avian nephrosis or Gumboro disease was caused by the Gray strain of infectious bronchitis virus (IBV) because of gross changes in the kidney and because IBDV and IBV were concurrent in many cases. This misconception also arose because the two infections were concurrent in many cases and the IBDV was difficult to isolate with the available methods (98). After subsequent studies, Winterfield

et al. (175), succeeded in isolating the causative agent in embryonating eggs, and later Hitchner proposed the term "infectious bursal disease" for the disease.

In 1984 and 1985, the Delmarva Peninsula broiler growing area experienced a significant increase in mortality. The clinical syndrome had significant variability, but often respiratory in nature. Lesions ranged from moderate to severe, with death usually being attributed to *E. coli* infection (34). Using vaccinated sentinel birds, Rosenberger *et al*. (140) isolated four isolates designated as A, D, G, and E. These isolates differed from standard strains in that they produced a very rapid bursal atrophy associated with minimal inflammatory response. The available killed standard vaccines did not afford complete protection against these new four Delaware isolates as with the challenge by the standard challenge virus (STC). The Delaware isolates, A, D, G and E were designated as antigenic variants and killed vaccines were developed, tested and proven effective against variants (142). Currently these types of variants are widely disseminated in the United States (151), (154).

Since 1987, acute IBDV cases with up to 30% to 60% mortality in broilers and pullets flocks respectively became commonly reported in Europe and the first reports were made by Chettle *et al*. 1989 (28), and van den Berg *et al* in 1991 (169). Surprisingly, some of these acute outbreaks occurred in broilers where all the hygienic and prophylactic measures had been taken. These findings indicated a dramatic change in the field situation. Although no antigenic drift was detected, these strains of increased virulence were identified as very virulent IBDV (vvIBDV) strains (166). The European situation has been dominated for a decade by the emergence of vvIBDV strains. These strains have now spread all over the word (44). However, in West Africa severe acute

IBD is hardly new. For ten years prior to the emergence of European vvIBDV strains, several reports had mentioned unusually high IBD-induced mortalities in local or imported chickens (55), (128). However, the unusual severity of these African cases was attributed as much to concurrent aggravating factors such as climatic stress or coinfection with parasites, bacteria and other viruses as to any increased virulence of the local viruses (44). In the Americas, acute IBD outbreaks due to vvIBDV strains have already been reported in Brazil (36), (69), and there are reports of vvIBD in Dominican Republic.

New Zealand was free of the disease during the mid 1980s, but IBDV was diagnosed in late 1993 and became endemic within months of the initial outbreak (84).

b) Economic significance of IBD

Uncomplicated IBD with the original type I results in up to 90% flock morbidity and 20% mortality in susceptible 3- 8 week-old hybrid Leghorn replacement pullets. In broilers aged 3 to 6 weeks, lower flock morbidity (50%) occurs following infection, and primary mortality from IBD seldom exceeds 3%. Until the emergence of very virulent strains of IBDV in 1986, the significance of IBD was confined to the immunosuppression. In broilers, immunosuppression is denoted by the high prevalence of viral respiratory infections and elevated mortality due to airsacculitis and coliseptisemia. In addition, the condemnation rate in processing plant may be increased tenfold from 0.5% to over 5% in affected flocks.

It was reported in broiler flocks with subclinical IBD a decrease of 14% in the financial return compared with unaffected flocks in Ireland. In other study, a reduction of 10% in profit was attributed to relative depression in body mass and feed conversion efficiency due to IBD (112). In studies on vvIBDV, it was shown that infection with

strain 849VB in 38-day-old hybrid Leghorn pullets, resulted in 60% losses, whereas, broiler chicks infected at the same age showed 17% mortality (166).

c) Etiology

IBDV is a member of the *Birnaviridae* family, (21) which has only one genus, *Birnavirus* (106). Among the most important features of this family are the bisegmented nature of the viral genome, as well as double-stranded RNA. The *Birnaviridae* family includes three genera: Genus *Aquabirnavirus* (type species: infectious pancreatic necrosis virus or IPNV), Genus *Avibirnavirus* (type species: infectious bursal disease virus or IBDV), and Genus *Entomobirnavirus* (type species: *Drosophilla* X virus or DXV). (37). Other birnaviruses have been isolated from bivalve mollusks such as Tellina virus (163), and Oyster Virus (39), (92), and Japanese eels (100). To date, no Birnavirus that cause disease in mammals including humans has been discovered.

IBDV particles are non-enveloped, single shelled with diameter of 60 to 70 nm (39), (56), (62), (125). Detailed analysis of images negatively stained and of shadowed preparations has suggested that the architecture of the capsid of IBDV is based on the geometry of a skew T=13 icosahedral lattice of the right-handed type (T=13d) (130). By cryomicroscopy, it was determined that the subunits forming the capsid are predominantly trimer clustered. Due to the conformation of the subunits, the capsid acquires a nonspherical shape (15).

d) Viral genome structure and organization

The genome of IBDV is formed by two segments of double-stranded RNA (dsRNA) with two segments detected by polyacrylamide gel electrophoresis (38), (81), (117). Molecular weights of the two double stranded segments are 2.2×10^6 and 1.9×10^6

Da, respectively (116). The length of both segments is 3.2 kb and 2.8 kb respectively (67).

Segment A contains two partially overlapping open reading frames. The first encodes a nonstructural polypeptide of 17 kDa known as VP5, which is dispensable for replication *in vitro* but important for virus-induced pathogenicity (120), (119). The second ORF encodes a 109-kDa polyprotein that is autoproteolytically cleaved into three polypeptides, VPX, VP3 and VP4. VPX is further processed to produce a polypeptide known as VP2 (4), (67), (118). VPX, VP2, and VP3 are the major structural proteins that form the virus capsid (15), while VP4 appears to be responsible for the proteolytic maturation of the polyprotein (83), (88), (101).

Segment B encodes VP1, a 95-kDa protein which is the RNA-dependent RNA polymerase (RdRp) responsible for the replication of the genome and synthesis of mRNAs (37), (155). VP1 shares a number of primary sequence features with RNA polymerases from diverse origins (24).

At the 5’ and 3’ ends in both genome segments of IBDV, there are direct terminal and inverted repeats that are likely to contain important signals for replication, transcription and packaging. It is not known whether virulence variations are due to mutations in these regions (122). The inverted adjacent repeats at the 3’ terminus on segments A and 5’ terminus on segment B have the potential to form stem and loop secondary structures (89), which are involved in the processes of RNA replication, translation and encapsidation like other RNA viruses such as poliovirus (149).

e) RNA synthesis

The mechanism of synthesis of both virus-specific ssRNA and dsRNA during infection with IBDV has not been clearly determined. Within the Birnavirus genus, an RNA-dependent RNA polymerase has been demonstrated in IBDV (155). Genome-linked proteins have been demonstrated in three different Birnavirus, (116), (131), (136), (155), indicating that they replicate their nucleic acid by a strand displacement (semiconservative) mechanism (12), (114), (155).

f) IBDV Proteins

The patterns of newly synthesized proteins do not differ between uninfected and IBDV infected cells analyzed by SDS-PAGE, indicating that IBDV does not shut off the synthesis of cellular proteins (7). Four mature viral structural proteins denominated VP1, VP2, VP3 and VP4 are detected in the infected cells. (7), (38), (39), (125). A non structural protein denominated VP5 has been identified, the function of this protein is still unknown, but it is not essential for viral replication (120), (119).

During the processing of the polyprotein precursor into pVP2, VP3 and VP4, it has been reported the existence of two sites, essential for the cleavage of the VPX-VP4 and VP4-VP3 precursors, respectively. These sequences are highly conserved among IBDV strains form serotypes 1 and 2 (143). However Rodríguez *et al*. (2002) postulated that VP4 allows multiple hit sites to increase its effectiveness (138).

VP2 is the most abundant viral protein, accounting for 51% of the serotype I IBDV. This is the major protein component of the viral capsid. A conformational dependent neutralizing epitope was detected in this protein. The transition from the

precursor of VP2 (pVP2) to VP2 involves the cleavage of pVP2 near its C terminus (4). VP2 has also been identified as an inducer of apoptosis (52).

VP3 is also a structural protein, and accounts for 40% of the virion proteins (87). VP3 is found only on the inner surfaces of virus-like particles (108). It is though that this protein plays role in the assembly of viral particles, and packaging of the viral genome (105), (108), (158). It is likely that the outer subunit in the viral capsid correspond to VP2, carrying the dominant neutralizing epitope, and that the inner trimers correspond to protein VP3, (15).

VP4 is the viral protease involved in the processing of the precursor polyprotein (4). It is a proteolytic enzyme-like protein, which uses a Ser-Lys catalytic dyad to act on specific substrates and cleavage sites (14). The integrity of VP4 was shown to be essential for the proteolytic processing of the polyprotein (41), (83). It has been suggested that VP4 itself, or through proteins under its control, plays a role in the activation of VP1 (14).

VP5 was the last IBDV protein identified (120). This protein is not essential for IBDV replication *in vitro* and *in vivo*, however, it plays an important role in viral pathogenesis (181). It has been demonstrated that have cytotoxic properties and it has been suggested that this protein plays also a role in the release of the IBDV progeny (104).

g) Host susceptibility

Domestic fowl are the natural host of the IBDV (59). All breeds are affected and it was observed that white Leghorns exhibit the most severe problems and have the highest mortality rate (106). Turkeys may be infected with serotypes 1 and 2 but do not

show clinical signs of the disease (82), (113). There is, however, considerable potential for immunosuppression or interaction with other diseases under field conditions (97). The serotype II was identified in clinically unaffected adults in Ireland (111). Ducks may develop IBD antibodies that can be detected by serum virus neutralization, but neither gross nor microscopic lesions are observed following infection. Antibodies were detected in five of 29 weavers (*Ploceus cucullatus*) and one of eight finches (*Uraeginthus bengalus*) (124). Surprisingly, serologic evidence was detected in Antarctic adelie penguins; the source of contact with IBDV has not been defined (53).

h) Transmission

The IBDV is highly contagious and the disease may be spread by direct contact between infected and susceptible flocks. Infected chickens shed IBDV one day after infection and can transmit the disease for at least 14 days (172). There are neither experimental data nor field observations to suggest that IBD can be transmitted vertically by the transovarian route (106).

Indirect transmission of virus most probably occurs on fomites (feed, clothing and litter) or through airborne dissemination of virus-laden feathers and poultry house dust (9). IBDV is very persistent in the environment of a poultry house. Houses from which infected birds were removed were still infective for other birds 54 and 122 days later (10). Virus can remain viable for up to 60 days in poultry house litter (172).

The lesser mealworms, *Alphitobius diaperinus* may be reservoir hosts (110), (150). IBDV has also been isolated from *Aedes vexans* mosquitoes, however, the virus was not pathogenic to susceptible Leghorn pullets (66).

i) Clinical aspects

Schematically, the global situation can be divided into three principal clinical forms:

The classical form, as described since the early 1960s, is caused by the classic moderate virulent strains of IBDV. The incubation period of IBD ranges from 2 to 4 days after exposure. One of the earliest signs of the classical infection in a flock is the tendency for some birds to pick at their own vents. The disease is characterized by acute onset of depression. Birds are reluctant to move, they exhibit ruffled feathers, whitish or watery diarrhea, pericloacal feathers are stained with urates, there is trembling, prostration. The feed intake is depressed but water consumption may be elevated. Severely affected birds become dehydrated and die (33), (50), (95).

The immunosuppressive form, principally described in the United States, is caused by low-pathogenicity strains of IBDV, as well as by variant strains, such as the Delaware variants or GLS strains, which partially resist neutralization by antibodies against the so-called “classic” or standard strains (151).

The acute and very virulent form, described initially in Europe, and then spread to Asia, Africa and some countries in Latin America, is caused by hypervirulent strains of IBDV, and it is characterized by an acute progressive clinical disease, leading to high mortality rates on affected farms. The initial outbreaks in Europe were characterized by high morbidity (80%) and significant mortality attaining 25% in broilers and 60% in pullets over a 7-day period (28), (126), (169).

j) Gross lesions

Chickens which die acutely of primary IBD infection show dehydration of the subcutaneous fascia and musculature of the thigh, inguinal and pectoral areas (33), (106). Hemorrhages may be observed in the mucosa of the proventriculus at the junction with the gizzard (95). Kidneys show enlargement and pallor with accumulation of crystalline urate in tubules (33). The renal lesions were prominent in early outbreaks in the United States, this may be attributed to the fact that cases of IBD were coinfected with nephropathogenic strains of avian infectious bronchitis (106).

The bursa of Fabricius is the main organ in which lesions develop following exposure to IBDV (30). Chickens that die or are sacrificed at early stages after the infection show a doubling in size of the bursa due to edema. The bursa is pale yellow in color and shows striations. By the 5^{th} day the bursa returns to normal weight, but it continues to atrophy, and from the 8^{th} day forward it is approximately one-third its original weight (106). Variant strains have been reported that do not induce an inflammatory response (139), (148). However, it was reported that one variant strain was able to induce inflammatory lesions (57).

Splenic enlargement has been documented, with small gray foci uniformly disperse through the parenchyma (106), (115). IBDV has been suggested to be part of an etiologic complex causing proventriculitis in broilers (68).

The vvIBDV strains are able to cause greater decrease in thymic weight index and more severe lesion in cecal tonsils, thymus, spleen, and bone marrow, but the bursal lesions are similar (106).

k) Histologic lesions

The IBDV is extremely lymphocidal, producing lesions in the bursa of Fabricius and other lymphoid tissues. Infection with standard and variant strains can result in death of bursal B lymphocytes. Necrosis of lymphocytes in the medulla of the organ can be detected within one day of infection. By the third day an inflammatory response with edema, heterophil infiltration, congestion and hemorrhage is present in infections by standard strains. At this time the follicles may be reduced to a necrotic center surrounded by heterophils. From the fourth day after infection the acute inflammatory reaction declines, and as necrotic debris is cleared by phagocytosis, cystic cavities are formed. Fibroplasia occurs in the surrounding connective tissue and the covering epithelium becomes infolded and irregular (30), (134). Sharma *et al*. (1989) observed that the infection with the variant A strain did not result in an acute inflammatory response, and follicular lymphoid necrosis was evident at three days after infection (148).

The development of lesions by IBDV in thymus depends on the pathotype of the virus (70), (161). IBDV induced cortical thymocyte depletion is caused by apoptosis (70). The highly pathogenic strains of IBDV from Europe and Japan are associated with severe thymocyte loss when compared to less pathogenic strains (161).

The spleen may suffer some hyperplasia of reticuloendothelial cells. The Harderian gland may be severely affected. Normally this gland is infiltrated and populated with plasma cells as the chicken ages. The infection with IBDV prevents this infiltration (157). In cecal tonsils, there may be acute heterophilic inflammation, destruction of lymphocytes, with regeneration on the fifth day after infection (59).

Histologic lesions of the kidney are nonspecific and probably occur because of severe dehydration of affected chickens. Lesions observed consisted of large casts of homogeneous material infiltrated with heterophils (59).

l) Immunosuppressive properties of IBDV

Clinical and subclinical infection with IBDV may cause immunosuppression. Both humoral and cellular immune responses are compromised (147). The first indication of damage in the immune system was reported by Helmboldt and Gardner in 1967 (59). In 1970, Cho demonstrated that white leghorn chickens exposed to IBDV at one day of age were consistently more susceptible to develop visceral tumors and nerve enlargement by Marek's disease virus. In 1972 Allan *et al*. reported that IBDV infections at early age were immunosuppressive, severely depressed the antibody response to Newcastle disease (2).

Virus replication in bursa leads to extensive lymphoid cell destruction in the medulary and cortical region of the follicles (159). The acute lytic phase of the virus is associated with a reduction in circulating IgM+ cells (63), (137). IBDV-exposed chickens produce suboptimal levels of antibodies against a number of infectious and non-infectious antigens (90), (31), (51), (177).

Only the primary antibody response is impaired, the secondary responses remain intact (54), (137), (148), and this humoral deficiency may be reversible. Although destruction of Ig-producing B cells may be one of the principal causes of humoral deficiency, other possible mechanisms may include the adverse effect of IBDV on antigen-presenting and helper T cell functions (147).

T-cells are resistant to infection with IBDV (61), and there is no evidence that the virus actually replicates in thymic cells (160), (148). However, there is evidence that *in vitro* mitogenic proliferation of T cells of IBDV exposed birds is severely compromised, this mitogenic inhibition is mediated most probably by macrophages, the way how IBDV induces macrophages to exhibit this suppressor effect is not clear (147).

Sharma *et al*. (2000) (147) detected a dramatic infiltration of T-cells in the bursa during the acute phase, accompanied by the precipitous drop in the number of IgM+ cells. The infiltrating cells were predominantly CD8+ from the seventh day of infection. It was suggested that T-cells modulate the infection, limiting the viral replication in the bursa in the early phase of the disease, and they also promote bursal tissue damage and delay recovery, possibly through the release of cytokines and cytotoxic effects (135). Cytotoxic T cells may exasperate virus-induced cellular destruction by lysing cells expressing viral antigens. T cells may also promote the production of inflammatory factors increasing the tissue destruction such as nitric oxide (147).

The effect of IBDV on innate immunity is centered in the modulatory effect of IBDV on macrophage functions. There is evidence that the *in vitro* phagocytic activity of these cells is compromised (147).

m) Diagnosis of IBDV

Several diagnostic procedures can be applied in the diagnosis of IBD.

Clinical and differential diagnosis. The clinical diagnosis of the clinical forms of IBD is based on the signs and the lesions of the bursa of Fabricius. The differential diagnosis should include the velogenic viscerotropic Newcastle disease, chicken infectious anemia, mycotoxicosis and nephropathogenic forms of infectious bronchitis. In

subclinical and immunosuppressive forms of IBD, Marek's disease and chicken anemia should be considered (97), (106).

Histological diagnosis. Since the lesion caused by IBDV are well characterized (30), (134), the histopathological diagnosis is frequently used. This approach has the advantage of giving valuable information about the virulence of the IBDV strain involved and the possible time when the infection occurred.

Serological diagnosis. Current serological tests include serum-virus (141) neutralization and ELISA (107). ELISA is widely used because is a sensitive and rapid method. With ELISA is easy to handle high amounts of samples. With the serological techniques is possible to detect the immunologic response in an outbreak or evaluate vaccination programs (127).

Viral isolation. The virus can be isolated in embryonated eggs, cell cultures or by inoculation of susceptible birds. Inoculation in birds is the best method, because the other methods may modify the original characteristics of the IBDV field strains (141).

Detection of viral antigens. Viral antigens may be detected by direct or indirect fluorescent antibodies techniques, immunohistochemistry, agar gel immunodiffusion and antigen-capture ELISA (AC-ELISA). The use of monoclonal antibodies in the capture detection allows for more precise antigenic characterization (154).

Molecular techniques. The reverse transcription-polymerase chain reaction (RT-PCR) allows for the detection of viral RNA from infected clinical samples (79), (99), (176). Differentiation of the strains is possible if the RT-PCR products are further analyzed using restriction enzymes (75), (74), (76). Other molecular techniques include the use of DNA probes (73), (78).

n) Antigenic differences

The capsid protein, VP2, is the major host protective immunogen. Immunization of susceptible chickens with purified VP2 elicits neutralizing antibodies and confers protection against virulent virus challenge (8), (49). Monoclonal antibodies raised against VP2 have the ability to neutralize the virus (4), (8), (152), (154). By using one neutralizing monoclonal antibody, it was recognized a specific antigenic region of VP2 between amino acids 206 and 350, since this epitope was denaturated by SDS, it was determined that is a conformational-dependent epitope (4).

The presence antigenic epitopes on VP3 protein has been reported but these antibodies are not totally neutralizing (4), (48).

Antigenic diversity between IBDV serotypes has been recognized since 1980, when serotypes 1 and 2 were defined on the basis of their lack of *in vitro* cross neutralization (111). By the use of monoclonal antibodies it has been stated that IBDV strains belonging to serotypes 1 and 2 do not share major neutralizing epitopes (8), (145). Some researchers have developed polyvalent neutralizing antiserotype 1 monoclonal antibodies such as monoclonal antibodies 1, 6, 7 8 and 9 (45), monoclonal antibody 8 (154), and monoclonal antibodies 6F6 and 7C9 (168).

Antigenic differences have been demonstrated within serotype 1, and the study of different strains has led to dividing serotype 1 into six subtypes, differentiated by cross neutralization assays using polyclonal sera (77).

Studies with monoclonal antibodies demonstrated the presence of a number of modified neutralizing epitopes in the antigenic variant strains detected in the United States (154). It has been suggested that the US isolates might have undergone an

antigenic shift (153). There appears to be a minimum of at least five neutralization epitopes on the standard IBDV strains defined by the monoclonal antibodies 8, 179, R63, B69 and 10. Delaware viruses have lost the B69 site, GLS viruses lack the B69 and R63 sites, and DS326 virus lacked the sites for monoclonal antibodies B69, R63 and 179 (154). Thus on the basis of the reactivities with various monoclonal antibodies, the IBDV viruses are antigenically grouped as classic or standard, GLS, DS326 and Delaware type variants (165).

After an epidemiological study using Snyder's monoclonal antibodies, it was determined that in isolated and less dense broiler growing areas (ME, NE, WA, OR, OK, CA, OH, and FL), the standard strains were predominant. The more dense Eastern areas (MS, AL, GA, VA, NC) specially the Delmarva area, the Delaware and GLS were predominant and the isolation of standard IBDV was a more rare event. Other areas such as AR, PA, TN MO, TX and IN appeared to be more in a transitional stage with respect to their IBDV populations (154).

In spite of their enhanced pathogenic properties, the vvIBDV were considered to be still closely antigenically related to the standard strains such as the Faragher 52/70 strain, on the basis of high cross-neutralization indices (47). Using neutralizing monoclonal antibodies developed by Snyder to characterize US IBDV variants, van der Marel studied twelve European isolates of IBDV, and detected no important differences between the standard strain 52/70 and vvIBDV (170). Similar data was produced by Öppling at al (129). However, Etterradossi *et al*. (1997) developed nine monoclonal antibodies and detected modified binding and neutralizing properties with French vvIBDV strains. All their monoclonal antibodies neutralized most mild or intermediate

vaccines, whereas two monoclonal antibodies did not neutralize a French vvIBDV strain, as well as US variants A, and the European strain Faragher 52/70. According to their results they suggested that a neutralizing epitope is possibly modified in the European vvIBDV strains, and the differentiation of vvIBDV strains may be achieved by the use of monoclonal antibodies (45), (46).

Part 2: Molecular Basis of IBDV Variability

After studies of amino acids alignments, the presence of a hypervariable region in the gene coding for VP2 has been detected. The amino acid changes between strains are not evenly distributed throughout the open reading frame but are clustered in certain regions. Most of the changes that occur in VP2 are located between amino acid 239 and 332 (6), (94), (165). This highly variable region falls entirely within those sequences of VP2 identified as the minimum region required for reaction with virus neutralization monoclonal antibody (4), (49), (178).

Hydrophilicity profiles of this region show that there are two hydrophilic peaks at either end of this region, the larger peak being from amino acids 212 to 224 and the other from 314 to 324. These hydrophilic regions have been shown to be important in binding of neutralizing antibodies and, hence, are presumed to be a main part of the neutralizing domain (58), (145). It is interesting that the majority of amino acid variation in this region falls within these two peaks (6),(94).

Variations in IBDV antigenicity have been shown to depend on changes in hydrophilic peaks. The serotype 2 strain 23/82 (145), the North-American antigenic

variants A, E, GLS and DS326 (58), (94), (165), and neutralization resistant escape mutants (145), (168) all exhibit amino acid changes in these hydrophilic peaks, whereas only changes in the hydrophobic domain are found in typical serotype 1 strains (165).

A nucleotide sequence comparison suggested that four amino acid mutations located in the VP2 protein of the Delaware E strain were crucial for the ability of this variant to escape neutralizing antibodies. These amino acids were located at positions 213, 222, 318, and 323 (58). By restriction enzyme and amino acid sequence analysis, point mutations have been detected at residues 222, 254 and 323. However, the amino acid residues 222 and 254 are consistently mutated in the variant strains (40), (80). All the variants, except for the Mississippi strain, underwent a shift in amino acid number 222 from proline to threonine, and to glutamine in the Delaware variant A, other standard strains exhibit serine at this position (40), (80), (165). Glycine is present in the standard strains, as amino acid residue number 254, whereas the variants exhibit serine at this position (40), (80).

Most of the variant strains contain lysine and serine at positions 249 and 254 respectively, the only exception is the Mississippi isolate, which exhibits glutamine at position 249. Not all the variant strains contain these two residues, but if these residues are present in one strain, it is very likely that this strain corresponds to an antigenic variant. Hence, the amino acids lysine 249 and serine 254 can be considered as the signature for the variant strains (25), (40).

The ability of IBDV to mutate extensively within the variable domain and still replicate suggests that the changes in this region do not disrupt the viral life cycle. In the case of the Delaware variant A, there are six amino acid changes that are located at

positions 222, 249, 254, 270, 286 and 318, this indicates that minor changes are sufficient to alter a major neutralization epitope (94).

Vakharia *et al*. (1994) (165) used monoclonal antibodies to correlate antigenic variations with amino acid sequence substitutions in the hypervariable region of VP2. They found that the amino acid residue glutamine at position 249 might be involved in the binding of neutralizing antibody B69 that recognizes epitopes in standard strains. All the variant viruses have lysine instead of glutamine at this position, and they escape binding with the antibody B69.

Using a baculovirus expression system to synthesize all the structural proteins of present in the segment A of IBDV, Vakharia *et al*. (1997), produced virus like particles. They mapped the antigenic sites by producing chimeric cDNA clones of IBDV using the variant GLS plasmid as a backbone and inserting fragments from D78 and Delaware strain. At least two antigenic sites present on the surface of IBDV, one resides between amino acid residues 222 and 249, and the other between 269 and 323. Amino acid substitutions at residues 222, 249 and 254 in the GLS VP2 protein generated the B69 epitope, which was not present in the variant virus. Similarly, substitutions at residues 269, 284, 286, 318, 321 and 323 in the GLS VP2 protein generated all the epitopes specific for the Delaware E strain recognized by the monoclonal antibodies B67 and R63, but deleted the GLS-specific epitopes recognized by the antibody 57 (164).

The origins of vvIBDV are still unclear, according to the analysis of the proteins VP1, VP3 and VP4, the vvIBDV strain UK661 is the most divergent of the serotype I viruses. It was assumed that the source of vvIBDV was likely one unrecognized reservoir. Since it was determined that VP1 nucleotide sequences do not correlate with

serotype, it was hypothesized that segment B is able to reassort with segment A. These findings indicate that not a genetic recombination but genetic reassortment may play a role in the emergence of vvIBDV (22), (179).

The role of VP1 in the virulence of IBDV has not yet established. It is likely that the viral polymerase would influence the replication rate and, thus the pathogenic potential of a virus. The VP1 sequences of very virulent IBDV strains are genetically distinct from that of classical virulent or attenuated strains thus, VP1 of vvIBDV constitutes a genetic lineage distinct from that of classical virulent or attenuated strains and serotype 2 strains as well (71).

In highly virulent strains three specific amino acid residues have been reported at position 222 (Ala), 256 (Ile), 294 (Ile) and 299 serine which differ from classical strains (23). These substitutions are also present in other strains isolated from other countries such as Germany (182), Bangladesh (72) China (25), Israel (132), Japan (102), (179), Taiwan (103), Malaysia (32), (65), Nigeria (182), Vietnam (162), and Brazil (36), (69).

Australian IBDV strains belong to a separate and distinct genetic group of strains which are separate from all other classical, variant and vvIBDV strains. In Australia there is the coexistence of standard and variant IBDV strains, but neither the standard nor the variant strains found in this area are closely related to those prevalent in the USA, since amino acid substitutions within both hydrophilic regions are significantly different between the US strains and the Australian strains. From phylogenetic analysis it appears that the Australian variants have not arisen directly from the classic strains but may have been introduced into Australian flocks as a separate event and co-existed with the standard strains ever since (144).

Phylogenetic analysis of two IBDV isolates from New Zealand, showed that these isolates were closely related to the attenuated PBG98 and Cu1 strains and distant from the standard, very virulent and antigenic variants (26).

A motif defined by the amino acid sequence SWSASGS which is located immediately downstream from the second hydrophilic peak has been found both in pathogenic and vaccine strains. This heptapeptide although tentatively was proposed as a marker for virulence (58), it may also be found in egg or cell-propagated attenuated IBDV strains (43), (167), (180).

Positions 222 – 223 and 318 – 324 may be critical for the vvIBDV typical and atypical antigenicity respectively (43). These positions have been identified as "hot spots" for mutations in several escape mutants resistant to selected neutralizing monoclonal antibodies (145), (168).

Part 3: Heteroduplex Mobility Assay

DNA sequencing is the "gold standard" for detection of mutations, however, it is still too labor-intensive and cumbersome for routine procedures. In order to meet the need for rapid detection of mutations, a variety of mutation scanning techniques that are less costly and technically simpler than DNA sequencing have been developed. These methods include single-strand conformation polymorphism (SSCP), heteroduplex mobility assay (HMA), temperature or denaturing gradient gel electrophoresis (TGGE or DGGE), protein truncation test (PTT) and others (60).

Heteroduplex mobility assay (HMA) is a mutation scanning technique that detects mutations by virtue of the altered electrophoretic mobility of a DNA fragment which contain one or more mismatched bases (a heteroduplex) versus a fragment of two DNA strands which are perfectly complementary (a homoduplex) (60).

Two different PCR amplicons may be compared by HMA. The essence of HMA is that both amplicons are mixed together in approximately equal amounts and heated to denature them into individual single strands. On cooling this mixture in an appropriate way, the single strands will reanneal to form the original homoduplexes and also heteroduplexes composed of complementary single strands from the two different starting amplicons. The heteroduplexes will contain "bubbles" due to mismatches or "bulges" due to insertions or deletions. Because of the presence of bubbles or bulges, the heteroduplexes have an altered electrophoretic mobility and they will migrate slowly through a non-denaturing polyacrylamide gel, in comparison with the homoduplexes. Following gel electrophoresis both homoduplex and heteroduplex bands will be visualized (5), (60), (123).

There are two types of heteroduplexes. The first is formed when the sequence difference between the two DNA fragments is one or more single-base mutations; the resulting heteroduplex is termed a "bubble type" heteroduplex. The second type is formed when the difference between the two fragments is a small insertion or deletion; the resulting heteroduplex is termed a "bulge' type heteroduplex. "Bulges" result in a large structural perturbation with a significant bending of the DNA at the bulge; these heteroduplexes are readily resolvable from homoduplexes on polyacrylamide gels. The bulged sequences exhibit the slowest migration through the gel. In contrast, the multiple

mismatches ('bubbles') cause very much less bending of the DNA fragment and uniform patterns of chemical reactivity along the length of the mismatched sequences, suggesting a less well defined, and possibly flexible, structure (13).

Originally the HMA was described as a PCR artifact, Nagamine *et al*., (121) coamplified two homologous mouse genes (zinc finger Y genes, *Zfy-1 and Zfy-2*) with a single set of PCR primers, the expected pattern of two bands, due to a 18 bp deletion in *Zfy-2* was observed after electrophoresis on agarose gel. However, after polyacrylamide gel electrophoresis, two additional products of lower electrophoretic mobility were resolved. The authors demonstrated that these additional bands were heteroduplexes species.

HMA has been adapted to rapid analysis of known, specific mutations. This modification of the technique involves the use of a synthetic construct that bears a small deletion (2 to 5 bp). This synthetic construct denominated heteroduplex generator (HG) enhances the bubble effect of a natural mutation resulting in greater separation. The HG is amplified by the use of the same set of primers as those used to amplify the DNA target under study. After the amplification, both the test amplicon and the amplicon prepared from the HG are mixed together. The mixture is heated, cooled and subjected to gel electrophoresis. If the mutation is present, the resulting heteroduplexes will have two types of mismatches: a bulge-type and a bubble-type (60).

The HMA has been used successfully for the molecular characterization of several viral agents such as genotyping hepatitis C viruses (HCV) (133), (173), HIV, (1), measles (93), human, avian, and swine influenza A viruses (42), Norwalk-like viruses (109), the GB virus C (hepatitis G) (174). Berinstein *et al*. (11) were able to distinguish

vaccine-like viruses from virulent isolates of Newcastle disease viruses by analyzing the F protein cleavage site coding sequences from several isolates using HMA.

Part 4: *In situ* hybridization

With the introduction of the *in situ* hybridization (ISH) techniques in the late 1960s, a new era in histology and cell biology was opened (29). ISH techniques allow specific nucleic acid sequences to be detected in morphologically preserved sections. In combination with immunohistochemistry, *in situ* hybridization can provide topological information about specific sequences or gene expression (3). ISH provides similar information to that provided by other molecular techniques, however, ISH has the added advantage of allowing for topologic assessment of nucleic acid localization which is not possible with traditional molecular biology techniques (17).

DNA and RNA probes are used for ISH procedures, however, the use of riboprobes is becoming increasingly popular. Riboprobes are made in an *in vitro* transcription reaction using a DNA template. These DNA templates are constructed by two methods a) by insertion of the desired sequence of DNA into a commercial vector containing specific RNA polymerase promoter sequences (eg. from phage T7 or the *Salmonella* phage Sp6), adjacent to the multiple cloning site into which cDNA is going to be inserted, and b) through construction by polymerase chain reaction incorporating polymerase binding sites into the primer sequences (27).

Antisense RNA (cRNA) probes have several advantages over cDNA probes (1) they are single stranded, thus avoiding the reannealing problem; (2) they hybridize with

greater stability to mRNA, enabling more stringent post hybridization washes; (3) unhybridized probe can be destroyed by post hybridization treatment with RNAse which spares the cRNA-mRNA hybrids; and (4) probes of uniform length can be obtained. It has been reported that antisense probes hybridize significantly better *in situ* than cDNA probes. Those are the reason why the use of riboprobes is increasing. The main disadvantage of the riboprobes is that they are sensitive to Rnases (27).

The nonradioactive probes have many advantages as compared to radioactive probes: practically, the absence of radioactivity takes away several inconveniences linked to the use of radioisotopes. The use of nonradioactive probes avoids the autoradiographic procedure, which is time consuming, expensive and difficult to organize on a routine basis. Nonradioactive probes have a long shelf life. They give a high cellular and subcellular resolution with a reaction limited to the cell compartment containing the nucleotide target (27).

At the moment, it can be considered that the nonradioactive probes present two major disadvantages: first, they are generally less sensitive than the corresponding radioactive riboprobes. However, improvements in sensitivity of nonradioactive probes are emerging. The second disadvantage is that they do not permit at the moment quantitative *in situ* hybridization because the standardization of the procedure and of the intensity of the reaction is difficult to obtain (27).

With the synthesis of biotin-labeled (d) UTP, the construction of biotinylated nucleic acids became possible (16) (96). However, biotin is an endogenous molecule of living cells associated with carboxylases and plays role in many reactions, especially in the liver and kidney (91), (171). In some cases this endogenous biotin can lead to false

positive results (29). In 1987, digoxigenin, a steroid isolated from digitalis plants (*Digitalis purpurea or Digitalis lanata*) was proposed as an alternative to biotin for labeling of hybridizing probes. Because the blossoms and leaves of these plants are the only natural sources of digoxigenin, no false positive reactions occur as occurred with biotin (29), (64). Detection of hybridized digoxigenin-labeled probes is mediated by high affinity anti-digoxigenin antibodies conjugated to either alkaline phosphatase, peroxidase, fluorescein and rhodamine (29), (86).

The sensitivity and efficiency of riboprobe ISH depend on several variables, for which optimal conditions must be determined: (a) the probe construction and hybridization conditions; (b) the type and efficiency of probe labeling; (c) the tissue preparation (fixation, embedding) which must allow the retention of the target of hybridization and/or the hybridized products; and (d) the method used for signal detection (29).

Riboprobe ISH is used for detection of cellular gene expression, to detect and roughly quantify specific cellular mRNA for the production of oncogenes, cytokines, hormones and cell surface receptors, and for studying viral infections, especially in acute infections (17). Riboprobe *in situ* hybridization has been used for detecting different viral agents of veterinary medicine relevance, such as African horse sickness virus (20), bluetongue virus (19), bovine virus diarrhea virus (35), rinderpest (18), and mouse adenovirus (85). For diseases important in avian medicine, the examples are infectious bursal disease (146), avian leucosis virus subgroup J (156) and chicken anemia virus[1].

[1] Banda, A. P. Villegas, J. El-Attrache, and C. Brown.- Clinical and molecular characterization of chicken anemia virus isolates obtained from commercial broilers. Unpublished results.

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CHAPTER III

MOLECULAR CHARACTERIZATION OF SEVEN FIELD ISOLATES OF INFECTIOUS BURSAL DISEASE VIRUS OBTAINED FROM COMMERCIAL BROILER CHICKENS[1]

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[1]Banda, A., P. Villegas, J. El-Attrache, and C. Estévez. 2001. Avian Diseases. 45:620-630.

SUMMARY.

Specific pathogen free (SPF) sentinel birds were used as an initial biological system to isolate infectious bursal disease virus (IBDV) field isolates from commercial broiler farms exhibiting recurrent respiratory problems and poor performance. Reverse-transcription and polymerase chain reaction (RT-PCR) was used to amplify a 248-bp product encompassing the hypervariable region of the IBDV VP2 gene. Restriction fragment length polymorphism (RFLP) analysis of the RT-PCR products were performed using the restriction endonucleases (RE) *Dra* I, *Sac* I, *Taq* I, *Sty* I, *Bst* NI, and *Ssp* I. Two isolates (619, 850) exhibited a RFLP pattern characteristic of Delaware variant E IBDV. Restriction enzyme digestion for four isolates (625, 849, 853, and 11153) revealed unmatched RFLP patterns when compared with reference IBDV strains. Nucleotide and deduced amino acid sequence analysis of the VP2 hypervariable region for these six isolates revealed identity (96.3 up to 98%) with Delaware E variant IBDV strain. However, serine at position 254, which is characteristic of Delaware variant strains, was substituted by asparagine in these six isolates. The seventh IBDV isolate (9109) also exhibited a unique RFLP pattern, which included the *Ssp* I restriction site, which is characteristic of very virulent IBDV (vvIBDV) strains. Nucleotide and amino acid sequence analysis of the hypervariable region for this isolate revealed identity (90%) with the standard challenge strain (STC). However, the leucine residue at position 294 was substituted by isoleucine. This substitution corresponds to one of the amino acids that are conserved in the vvIBDV strains. Antigenic index studies of the predicted amino acid sequence of the hypervariable region of VP2 from isolates 619, 625, 849, 850, 853, and

11153 exhibited a profile almost identical to variant E, whereas the isolate 9109 exhibited a profile characteristic of standard IBDV strains.

Key words: Variant-like IBDV field isolates, molecular characterization, commercial broilers.

Abbreviations: BF = Bursa of Fabricius; CEF = Chicken embryo fibroblasts; DEPC = Diethyl pyrocarbonate; DMSO = Dimethyl-sulfoxide;; IBD = Infectious bursal disease; IBDV = Infectious bursal disease virus; RE = Restriction endonuclease; RFLP = Restriction fragment length polymorphism; RNA = Ribonucleic acid; RT-PCR = Reverse transcriptase- polymerase chain reaction; SDS = Sodium duodecyl sulfate; SPF = Specific pathogen free; STC = Standard challenge strain; vvIBDV = very virulent infectious bursal disease virus.

INTRODUCTION

Infectious bursal disease (IBD) or Gumboro disease is an acute, highly contagious viral disease of young birds characterized mainly by severe lesions in the bursa of Fabricius followed by immunosuppression. (3, 24, 32). Infectious bursal disease virus (IBDV) is a member of the *Birnaviridae* family with a genome consisting of two segments of double stranded (ds) RNA. IBDV is a nonenveloped icosahedral virus with a diameter of about 55- 60 nm (13, 28). Two serotypes of IBDV have been recognized and designated as I and II, but only the serotype I causes naturally occurring disease in chickens (13, 18, 26).

The effects of IBDV in chickens have been extensively reviewed (24, 29, 36). The severity of these effects varies with the virulence of the field virus, age of the birds, and level of maternally derived antibodies (22, 23). There are reports in Europe and Asia of very severe clinical outbreaks with high mortality rates (90% to 100%) caused by very virulent IBDV (vvIBDV) (6, 27, 36, 37). The subclinical and immunosuppressive form of the disease is prevalent in the United States (23). Significant problems are rarely observed, but reduction of body weight and lack of uniformity in the flock, are recognized in many mild outbreaks of IBDV. Differences in the severity of lesions in bursas of chicken can be observed following infection with IBDV (24, 25).

During the 1980's, serotype I variants were isolated by Rosenberger from the Delmarva area in the United States (30, 31). These variants were found to be antigenically different from previously characterized IBDV standard strains. Such isolates are referred to as antigenic variants of serotype I (24). These variants are not only antigenically different, but they are also pathogenic variants. In contrast to standard and vvIBDV strains, many variant IBDVs do not usually cause clinical disease or high mortality in 4-6 week-old chickens. Some isolates have exhibited great ability to rapidly induce bursal atrophy, thus severe immunossupression (23, 31).

Immunosuppression caused by variant strains of IBDV is a major problem primarily in heavily concentrated broiler-producing areas. The increase of immunosuppressive subclinical IBD and associated problems have a negative impact on the poultry industry because of the increase in mortality, poor performance, and condemnations in many commercial broiler companies (9). The current technologies for typing IBDV include molecular methods that amplify specific segments of the IBDV

genome by the reverse transcriptase – polymerase chain reaction (RT-PCR) followed by restriction fragment length polymorphism (RFLP) (7, 14, 16, 34). However, in the field there are numerous strains of IBDV that cannot be accurately characterized by this procedure. The objective of this study was to characterize the molecular properties of seven IBDV field isolates from the state of Georgia based upon differences in the hypervariable region of VP2 gene by restriction analysis of a 248 bp RT-PCR fragment and nucleotide sequence of a 698 bp RT-PCR fragment.

MATERIALS AND METHODS

IBDV field isolates and reference strains. Three-week-old sentinel birds (SPAFAS, Inc., Norwich, CT.) were raised for one week in direct contact with the commercial broilers from three different farms where three different vaccination programs were being used. These farms recurrently suffered from respiratory problems and poor performance. Bursal samples were obtained from sentinel birds. Five isolates (619, 625, 849, 850, and 853) were isolated using a sentinel SPF bird approach and they were previously identified and classified as "new type" of IBDV by RT-PCR/RFLP analysis using a published protocol (14, 16). Two aditional IBDV isolates, identified as 9109 and 11153, were isolated by the inoculation of SPF chickens with homogenized bursal samples obtained from birds from field. These two viruses exhibited unmatched RFLP patterns when compared with reference strains with the system currently used for genotyping IBD viruses at the University of Georgia (12). The Edgar strain was obtained from the National Veterinary Services Laboratory (NVSL) (Ames, IA). Delaware variant A and E were obtained from Merial-Select, Inc. (Gainesville, GA).

RNA extraction method. RNA from frozen bursas was extracted using an acid-guanidium-phenol-chloroform RNA extraction method (4). Briefly, bursal samples were minced and mixed with 1 ml of solution D (4M guanidium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). One portion of the mixture (0.1ml) was transferred to an Eppendorf tube where 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of phenol (water saturated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate. The final suspension was shaken and cooled on ice for 15 minutes. The samples were centrifuged at 10,000 g for 20 min at 4° C. After centrifugation, RNA present in the aqueous phase was transferred to a new tube and mixed with 1 ml of isopropanol and then placed at -20° C for 1 h to precipitate the RNA. After centrifugation at 10,000 g for 20 min, the resulting pellet was resuspended in 75% ethanol, sedimented, dried, and dissolved in 50 µl of 90% dimethyl-sulfoxide (DMSO) in diethyl pyrocarbonate (DEPC) water.

Primers. Two 23-nucleotide primers (IBDVP3 and IBDVP4) were developed by Simbios Biotecnologia (Sao Paulo, Brazil) (12). The OLIGO TM primer analysis software (National Biosciences Inc., Plymouth, MN) was used to design the primers. These primers were specific for the common sequences located on both sides of the VP2 gene hypervariable region and amplify a fragment of 248 bp at positions 804 to number1051, according to the numbering system of Bayliss *et al*. (1). For nucleotide sequence analysis, 698 bp RT-PCR products were obtained using the following set of primers 5-TCTGCAACAGCCAACATCAACG-3, as forward primer, and 5-TCAGGATTTGGGATCAGCTCGA-3, as reverse primer. This set was designed to amplify a product of 698 bp from nucleotide position 567 to 1264 of the VP2 gene.

RT-PCR method. RNA from bursal samples was reverse transcribed to cDNA and amplified by a one step RT-PCR (12) in 50 µl of the reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2 mM of each deoxyribonucleotide triphosphate (Sigma Chemical Co., St. Louis, MO), 8.8 U of RNase inhibitor (Promega Corp. Madison, WI), 24 U of M-LV reverse transcriptase (Gibco BRL, Life Technologies, Grand Island, NY), and 1 µM of each primer and 2 U of *Taq* DNA polymerase (Sigma Chemical Co., St. Louis, MO). Reverse transcription was performed at 37º C for 1 hour. Amplification was conducted for 40 cycles of 30 seconds at 94º C, 30 seconds at 50º C, and 1 minute at 72º C. A final extension step was performed at 72º C for 5 minutes. After amplification, samples were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide.

Molecular IBDV identification by RFLP. The 248 bp products obtained from the RT-PCR procedure were digested with restriction endonucleases (RE) *Dra* I, *Sac* I, *Taq* I, *Sty* I, *Bst* NI, and *Ssp* I (New England Biolabs Inc., Beverly, MA) according to the manufacturer's instructions. Digested fragments were subjected to electrophoresis on a 12.5% polyacrylamide gel and visualized by rapid silver staining. Electrophoretic patterns were compared with reference IBDV RFLP patterns as well as undigested controls.

Nucleotide and deduced amino acid sequence analysis. The 698 bp RT-PCR products were purified by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Purified PCR products were sequenced by the dideoxy-mediated chain-termination method (33). Sequence data were assembled using Seqman TM (Dnastar Inc., Madison, WI). Nucleotide sequence analysis was performed by Clustal method using the package

Megalin TM (Dnastar Inc., Madison, WI), and the predicted amino acid sequence was analyzed by Protean TM software. (Dnastar Inc., Madison, WI).

Nonsynonymous to synonymous ratios of nucleotide changes in the hypervariable region of VP2 gene were calculated by dividing the number of nonsynonymous nucleotide substitutions by the number of synonymous nucleotide substitutions.

Phylogenetic analysis of the segment of VP2 gene (from nucleotide position 567 to 1264) was performed by parsimony method with heuristic search and 1000 bootstrap replicates using the package PAUP 4b2 TM (Sinauer Associates Inc. Sunderland, MA). The tree was rooted to an outgroup sequence of the segment A of the Infectious pancreatic necrosis virus (IPNV) (accession NC 001915) which is the type virus of the A*quabirnavirus* genus.

Antigenic Index. Based on formulations described by Jameson and Wolf (19), potential antigenic sites of the deduced amino acid sequence were calculated considering surface probability, regional backbone flexibility, and probable secondary structure of the predicted amino acid sequence using the Protean TM software (Dnastar Inc., Madison, WI).

GenBank sequence accessions of reference strains used for comparisons included the variant A (M64285), variant E (X54858), the standard strains STC (D00499), and 52-70, (D00869), as well as the vvIBDV strains UK661 (X92760) and Ehime 91 (AB024076). The nucleotide sequence data of the seven field IBDV isolates has been deposited with GenBank Data Libraries. The assigned accession numbers are AY012677 (isolate 619), AY012678 (isolate 625), AY012679 (isolate 849), AY012680 (isolate 850), AY012681 (isolate 853), AY012682 (isolate 11153), and AY012683 (isolate 9109).

RESULTS

Molecular IBDV identification by RFLP. Results from the RE digestion of the amplified 248-bp VP2 product obtained from the seven field isolates are shown in Table 3.1. *Sac* I and *Taq* I cleaved isolates 619, 849, and 850; however, isolate 849 presented an additional RE site for *Taq* I; thus 3 fragments of 125, 60 and 63 bp were obtained. Isolates 625 and 853 were digested only by *Taq* I. Isolate 9109 was digested by *Sac* I, *Bst* NI, and *Ssp* I, and finally isolate 11153 was digested by *Sac* I, *Dra* I and *Taq* I. The Edgar strain exhibited restriction sites for *Sac* I and *Sty* I, but, variant A for *Dra* I and *Taq* I, and variant E for *Sac* I and *Taq* I. The exact position of the RE sites and the length of the fragments were confirmed by sequence analysis. The RE site positions along the hypervariable region of the VP2 gene are shown in Table 3.1.

Nucleotide and amino acid sequence analysis. The nucleotide sequence identity and divergence calculated by Clustal method of the amplified 698 bp VP2 products are shown in Table 3.2. Isolates 619, 625, 849, 850, 853, and 11153 were similar to Delaware variant E IBDV strain. A nucleotide sequence similarity of 94.5% was observed between isolates 619 and 625, whereas a 98.8% similarity was observed between isolates 625 and 853. The similarity with Delaware variant E strain ranged from 96.3 (isolate 619) to 98.0% (isolate 850). The number of nucleotide substitutions in comparison with variant E ranged from 9 for isolate 850, up to 19 in isolate 619. The nonsynonymous to synonymous ratio of nucleotide changes ranged from 0.1333 for isolate 853, up to 0.5833 for isolate 619, when these strains were compared with the variant E strain.

Isolate 9109 exhibited a 98% nucleotide similarity with the STC strain, whereas a 94% nucleotide similarity was shared with the vvIBDV UK661. The number of nucleotide substitutions of isolate 9109 in comparison with STC was 12, whereas 37 substitutions were observed when compared with UK661. The nonsynonymous to synonymous ratio when isolate 9109 was compared with the STC strain was 0.7142, and when it was compared with the UK661 the ratio was 0.2333. A nonsynonymous substitution in isolate 9109, occurring at nucleotide position 1011 where cytosine was substituted by adenine yielded the formation of the restriction site for *Ssp* I from nucleotide position 1008 to 1013.

Phylogenetic analysis of the amplified 698 bp product for the seven isolates and the IBDV variant E, STC, 52-70, ehime91, and UK661 strain is shown in Fig. 3.1. Three distinct branches were observed after generation of a phylogenetic tree using parsimony with a heuristic search and 1000 bootstrap replicates. Isolates 619, 625, 849, 850, 853, and 11153 were branched with variant E, isolate 9109 was branched with the classic strains STC and 52-70, while the vvIBDV strains UK661 and Ehime 91 distinctly were separated from both the classic and variant strains of IBDV.

The deduced amino acid sequences of the seven field isolates, variant E, 52/70, STC, Ehime 91 and UK 661 strains are shown in Fig. 3.2. The region analyzed included 140 amino acid residues from 200 to 340 of the segment A polyprotein according to the numbering system reported by Bayliss et al. (1). When compared with the Delaware variant E IBDV strain, the following amino acid changes occurred within the hypervariable region of VP2. Serine is substituted by asparagine at residue 251 for isolate 11153. Isolate 849 substituted valine for isoleucine at amino acid residue 252. Isolates

619, 625, 849, 850, 853, and 11153 substituted the amino acid residue serine for asparagine at position 254. At amino acid residue 299, serine substituted asparagine in isolates 619 and 850. For isolate 625 and 11153, aspartate is substituted by asparagine at residue 318, and glutamate substituted glycine at residue 322. Finally, aspartate substituted for glutamate in isolates 625, 849, 853, and 11153 at residue 323. When compared with the classic IBDV STC strain, amino acid substitutions for isolate 9109 included serine for leucine at residue 217, leucine for phenylalanine at residue 263, isoleucine for leucine at residue 294, and isoleucine for valine at residue 312.

Antigenic Index. The potential antigenic sites within the predicted amino acid sequence of the region of VP2 analyzed in isolates 619, 625, 849, and 9109, as well as the strains 52/70, Delaware variant E and UK 661 are shown and compared with classic and variant IBDV strains in Fig. 3.3. Four different discrepancies are observed between the Delaware variant E IBDV strain and the classic 52-70 strain. Delaware variant E exhibited two smaller peaks from the positions 209 to 216 and from 218 to 222. Variant E also was missing two peaks from positions 276 to 282 and 276 to 282. The antigenic profiles of isolates 619, 625, and 849 were almost identical to variant E. The potential antigenic profile for isolate 9109 was very similar to that observed for classic strain 52/70 and for vvIBDV strains Ehime 91 and UK661.

DISCUSSION

The IBDV structural protein VP2 has been identified as the major antigen that induces neutralizing antibody response in the host. The VP2 sequence is conserved with exception of the central area between amino acid residues 206 to 305, which has been

designated as the variable domain or variable region, where the majority of amino acid differences among IBDV strains appear (5, 10, 20). Consequently, RT-PCR and RFLP analysis of the VP2 gene has been used to differentiate serotypes and subtypes of IBDV (12, 14-16).

In the present study, RT-PCR was used to amplify a 248-bp fragment of the IBDV-VP2 gene from bursal samples. The fragment encompassed the variable region of VP2 from nucleotide position 804 to 1051. This typing system was based on the use of the RE *Dra* I, *Sac* I, *Taq* I, *Sty* I, *Bst* NI, and *Ssp* I. The main restriction endonuclease sites have been identified for the 248-bp amplified fragment of the VP2 region of the IBDV genome. Restriction enzymes *Sac* I, *Dra* I, *Sty* I, *Bst* NI, and *Ssp* I sites occurred only once and only in some sequences at positions 864, 873, 887, 949, and 1008, respectively. The *Taq* I site occurs at different positions 832, 875, or 988, depending on the sequence analyzed (12).

Using the RFLP system described above, variant A normally is cleaved by *Dra* I, and *Taq* I and variant E is cleaved by endonucleases *Sac* I and *Taq* I (12). Isolates 619, 625, 849, 850 and 853 were originally classified as “new type” by a previously published protocol (16). However, the PCR-RFLP method that is currently used in our lab, genetically identified 619 and 850 as variant E IBDV strains, because of the RFLP patterns produced by *Sac* I and *Taq* I. Isolate 849 exhibited a similar pattern to variant E but with an additional restriction site for *Taq* I. Isolates 625 and 853 were digested only by *Taq* I. Although these patterns did not exactly match any defined RFLP profile, their patterns are similar to those observed in field isolates of variant E IBDV strains. This similarity was confirmed by nucleotide sequence analysis and phylogenetic analysis.

Isolate 11153 exhibited the restriction endonuclease sites for *Dra* I, *Sac* I, and *Taq* I, which was also a unique RFLP pattern, but the similarity with variant E (97.2%) was also determined by nucleotide sequence analysis.

The range of nonsynonymous to synonymous ratios of nucleotide changes (0.1333 to 0.5833) for isolates 619, 625, 849 850, 853, and 11153, indicated that most of the nucleotide substitutions were silent, because few substitutions resulted in amino acid changes when compared with the variant E strain. In contrast, when the ratio of isolate 9109 was compared with the STC strain, this ratio was higher (0.7142). This ratio might be indicative of possible antigenic changes.

Dormitorio *et al.* (5) reported that the variant IBD viruses Ark, Ga, Del-E, GLS, DS326 and Del-A all contained lysine and serine as residues 249 and 254, respectively; whereas most of the classic IBDV strains contain glutamine and glycine at those same positions. One exception is the Mississippi strain in which serine appears at residue 254 (5). The amino acids lysine 249 and serine 254 are conserved in Delaware variant viruses and are considered identifiable signatures for these IBDV strains. Amino acid sequence analysis of the VP2 region (between amino acid residues 146 to 376) of isolates 619, 625, 849, 850, 853, and 11153 demonstrated that lysine appeared at position 249. However, at position 254 the serine residue was substituted by asparagine. Serine and asparagine are both polar amino acids, but asparagine is more hydrophilic than serine. Because of its polarity and hydrophilicity, asparagine is often found on surfaces of proteins where the interaction with water molecules is possible (11). According to the data provided by the predicted antigenic index, this substitution did not modify the Jameson and Wolf antigenic profile (19) and is almost identical of that seen in variant E strain. However,

because of the change of serine to asparagine, an eventual modification in protein folding and tertiary structure of VP2 in these six isolates that is different from variant E cannot be completely ruled out.

Vakharia *et al*. (35) used monoclonal antibodies (Mab), to correlate antigenic variations with amino acid sequence substitutions in hypervariable region of VP2. They suggested that residue 249 glutamine, which is conserved in most of the standard strains, may be involved in the binding with antibody B69. Because variant strains have 249 lysine, they do not bind this Mab. They also used another Mab denominated 67, which is specific and neutralizes the Delaware variant E strain. They concluded that residues 286 isoleucine, 318 aspartate, and 323 glutamate may be involved in the binding with Mab 67. In this study, isolates 625 and 11153 had asparagine as residue 318, and isolates 625, 849, 853, and 11153 showed aspartate as residue 323. Eventually, these substitutions may modify the folding of the protein and, as a consequence, modify the antigenic make up of these strains to make them antigenically different from variant E.

Isolate 9109 had a glutamine at residue 249 and glycine at residue 254 as is characteristic of IBDV standard strains. In addition, isolate 9109 exhibited high nucleotide sequence similarity with STC strain (98%), and were closely related upon phylogenetic analysis. However, isolate 9109 exhibited a unique RFLP pattern, different than that of STC. Isolate 9109 was digested with *Sac* I, *Bst* NI, and *Ssp* I, whereas the RFLP pattern of the STC strain includes positive digestion by *Sac* I, *Sty* I, and *Bst* NI (12). It was previously reported that the restriction site for *Ssp* I is characteristic of vvIBDV. This feature has been used for molecular identification of such strains (12, 17, 21). The presence of the *Ssp* I restriction site in isolate 9109 corresponds to the non-

synonymous substitution at nucleotide sequence that results in the substitution at residue 294 (leucine for isoleucine). This substitution is peculiar because isoleucine at amino acid 294 is conserved in virtually all vvIBDV strains. Amino acid residues conserved in the VP2 hypervariable region for the vvIBDV strains include alanine at position 222, isoleucine at positions 256 and 294, and serine at position 299 (2, 8, 21).

The clinical significance of the substitution at residue 294 in the protein sequence of the isolate 9109 is still not clear. This isolate was associated with a broiler flock with problems of poor performance and recurrent respiratory problems most likely as consequences of immunosuppression. However, the birds did not exhibit clinical signs or mortality rates that are characteristic of vvIBDV strains. Preliminary clinical studies in SPF birds and commercial broilers with this isolate produced a subclinical form of IBDV with bursal atrophy (data not published). There have been no previous reports of any IBDV viruses detected in United States where this change appears.

Potential antigenic sites within the predicted 230 amino acid hypervariable region were compared by calculating an "antigenic index" that reflects the influence of several different parameters such as hydrophilicity, surface probability, backbone flexibility, and secondary structure (19). Significant differences in the antigenic index profile between standard and variant IBDVs have been previously determined in the VP2 hypervariable region. Lana *et al*. (20), reported that the variant A virus was missing a peak in an antigenic index within the hypervariable domain (amino acids 284 – 288), which is present in all the other classical viruses. Although the isolates 619, 625, 849, 850, and 11153 exhibited unmatched RFLP patterns and nucleotide and amino acid residues substitutions occurred, these isolates exhibited identical antigenic profile indexes to that

of variant E strain. In the case of isolate 9109, it exhibited a profile similar to that of the STC strain.

In this study, a method for IBDV typing was tested by the amplification of a 248-bp PCR product, which includes the hypervariable region of the VP2 gene and the RFLP assay using six RE (12). Further characterization of the isolates was based on the nucleotide sequence analysis of a 698-bp PCR product. The sequence analysis of a larger PCR product was included to determine the possible substitutions in the two flanking hydrophilic regions. The RT-PCR/RFLP method used in this work has proved to be a reliable method for typing IBDV. The set of enzymes used in this work allowed for discrimination of specific nucleotide discrepancies within the hypervariable region of the VP2 gene (positions 804 to 1051), where most of the changes among the different strains of IBDV occur. Moreover, when the relationship of different IBDV strains was determined by sequence analysis using either 248-bp or 698-bp PCR products, no differences in the classification of the isolates were observed (data not shown). RT-PCR/RFLP is a valuable tool to type IBDV strains. However, there are some strains whose genomes do not hold the characteristic restriction sites. Thus, nucleotide and amino acid sequence analysis are needed to completely identify this type of field isolates.

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Table 3.1. RFLP patterns of seven IBDV field isolates, Edgar and variant E strains. Fragments lengths are shown according their respective position in the 248-bp PCR product. Based on sequence where position 1 of the 248-bp fragment correspond to nucleotide position 804 (D00868) as described by Bayliss *et al* .(1).

	Expected DNA fragments sizes (bp)[a]					
IBDV sequence	***Sac* I**	***Dra* I**	***Sty* I**	***Bst* NI**	***Taq* I**	***Ssp* I**
619	**65 183**	**-**	**-**	**-**	**185 63**	**-**
625	**-**	**-**	**-**	**-**	**185 63**	**-**
849	**65 183**	**-**	**-**	**-**	**125* 60 63**	**-**
850	**65 183**	**-**	**-**	**-**	**185 63**	**-**
853	**-**	**-**	**-**	**-**	**185 63**	**-**
9109	**65 183**	**-**	**-**	**147 101**	**-**	**206 42**
11153	**65 183**	**72 176**	**-**	**-**	**185 63**	**-**
Edgar	**65 183**	**-**	**84 164**	**-**	**-**	**-**
Variant E	**65 183**	**-**	**-**	**-**	**185 63**	**-**

[a] The fragments sizes are calculated based on the restriction enzymes digestion sites
* An additional *Taq* I RE site was detected in position 928 to 931 in isolate 849.
(-) = Negative digestion .

Table 3.2. Nucleotide sequence similarities and divergence percentages calculated by Clustal method from the pairwise nucleotide differences of the VP2 (positions 567 to 1264) of seven field isolates, variants A and E, STC, Ehime91, UK661 and 5270 strains.

	619	**625**	**849**	**850**	**853**	**11153**	**Var. A**	**Var. E**	**9109**	**STC**	**Ehime91**	**UK661**	**52-70**
619	***	94.5	95.6	97.2	94.6	94.7	93.4	96.3	91.6	92.2	90.6	90.9	91.9
625	5.4	***	97.6	96.2	98.8	96.9	95.0	97.0	92.9	93.8	91.7	92.0	93.6
849	4.4	2.3	***	97.0	97.9	96.9	95.0	97.5	93.3	93.8	91.6	91.9	93.8
850	2.7	3.8	3.0	***	96.5	96.6	95.2	98.0	94.3	94.5	92.4	92.7	93.9
853	5.2	1.2	2.0	3.6	***	96.8	95.2	97.2	92.9	93.7	91.7	92.0	93.7
11153	5.2	2.9	2.7	3.5	3.3	***	95.2	97.2	93.3	94.0	92.2	92.5	94.1
Var. A	6.3	5.2	5.1	4.7	4.9	5.1	***	97.3	95.2	96.0	94.3	94.6	95.6
Var. E	3.3	3.0	2.4	1.8	2.7	2.9	2.8	***	95.3	96.1	94.3	94.6	96.0
9109	7.9	7.4	6.6	5.9	7.4	6.9	5.0	4.8	***	98.0	93.6	94.0	96.7
STC	7.4	6.6	6.2	5.5	6.3	6.4	4.0	3.8	2.0	***	94.7	95.0	97.4
Ehime91	9.3	8.6	8.4	7.7	8.5	8.1	5.9	5.9	6.6	5.4	***	99.1	94.4
UK661	9.0	8.2	8.1	7.3	8.2	7.7	5.6	5.6	6.2	5.1	0.9	***	94.7
52-70	7.6	6.5	6.1	6.0	6.1	6.0	4.5	4.0	3.3	2.6	5.9	5.6	***

Figure Legends

Fig. 3.1. Phylogenetic analysis of a segment of the amplified 698 bp IBDV VP2 product (positions 567 to 1246 numbering according to Bayliss *et al*). The relationship among seven field isolates, variant Delaware E, the classic strains STC and 52-70, and the vvIBDV strains Ehime91, UK661 is shown. The tree was generated by parsimony method with heuristic search and 1000 bootstrap replicates replicates using parsimony method. The tree was rooted to an outgroup sequence of infectious pancreas necrosis virus (IPNV). The length of each branch represents the number of nucleotide changes between sequences.

Fig. 3.2. Deduced amino acid sequences of VP2 from residues 200 to 330 (numbering of the segment A polyprotein of Bayliss *et al*.) (1) of field isolates 619, 625, 849, 850, 853, 11153, and 9109, as well as 52/70, variant E, STC, Ehime 91 and UK 661 strains of IBDV. A dot indicates position where the sequence is identical to that of the 52/70 strain. Aligments were prepared using Megalin package (Dnastar Inc.).

Fig. 3.3. Identification of potential antigenic sites within the amino acid sequence of hypervariable region of VP2 from field isolates 625, and 9109, and from the strains 52/70, variant E, and UK 661 strain. This sequence was predicted from the amplified 698-bp IBDV VP2 product from nucleotides 567 to 1264 of VP2 gene. A- D show the differences in comparison with the 52-70 strain.

Figure 3.1.

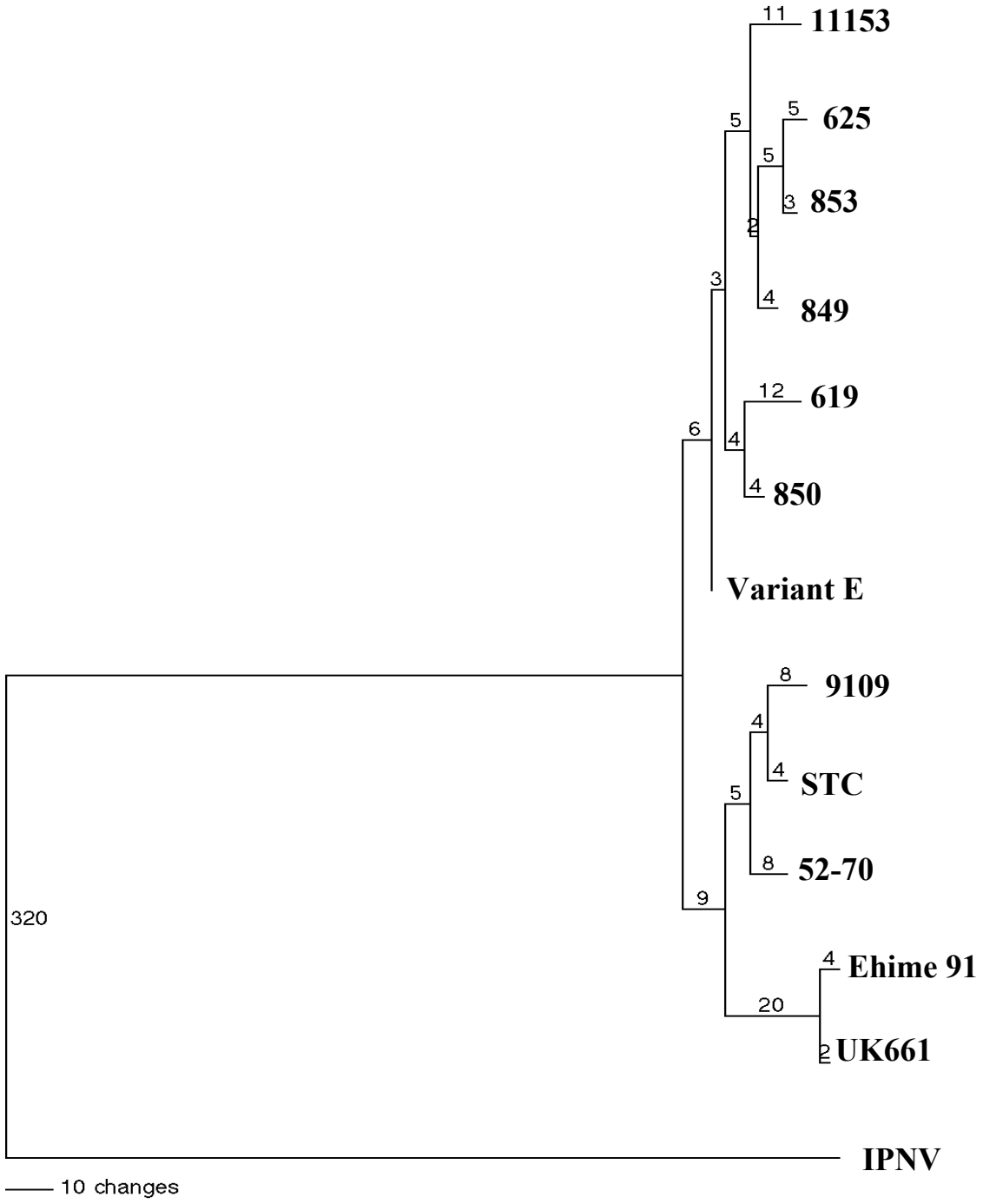

11
11153
5
5
625
5
3
853
2
3
4
849
12
619
6
4
4
850
Variant E
8
9109
4
4
STC
5
8
52-70
9
320
4
Ehime 91
20
2
UK661
IPNV
10 changes

Figure 3.2.

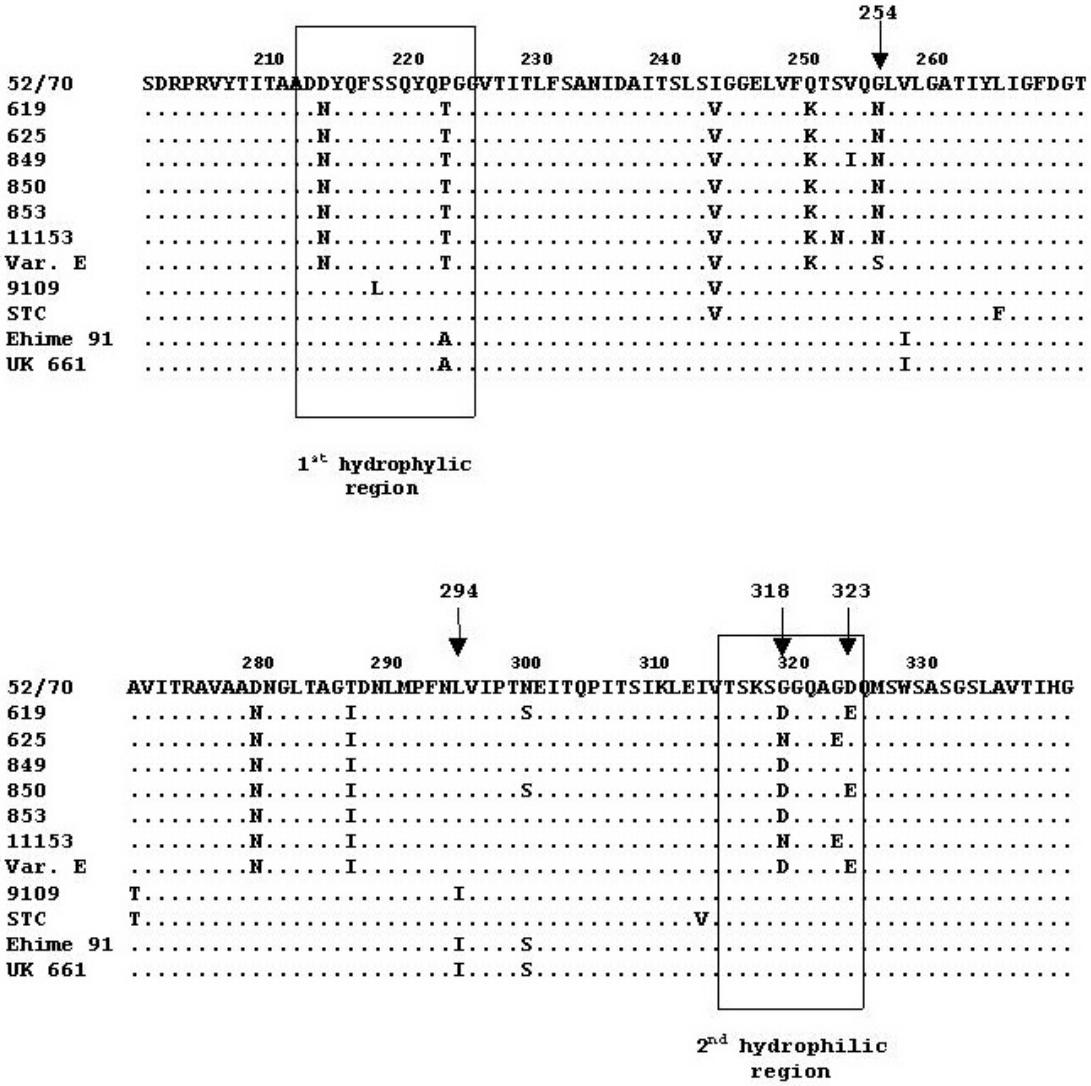
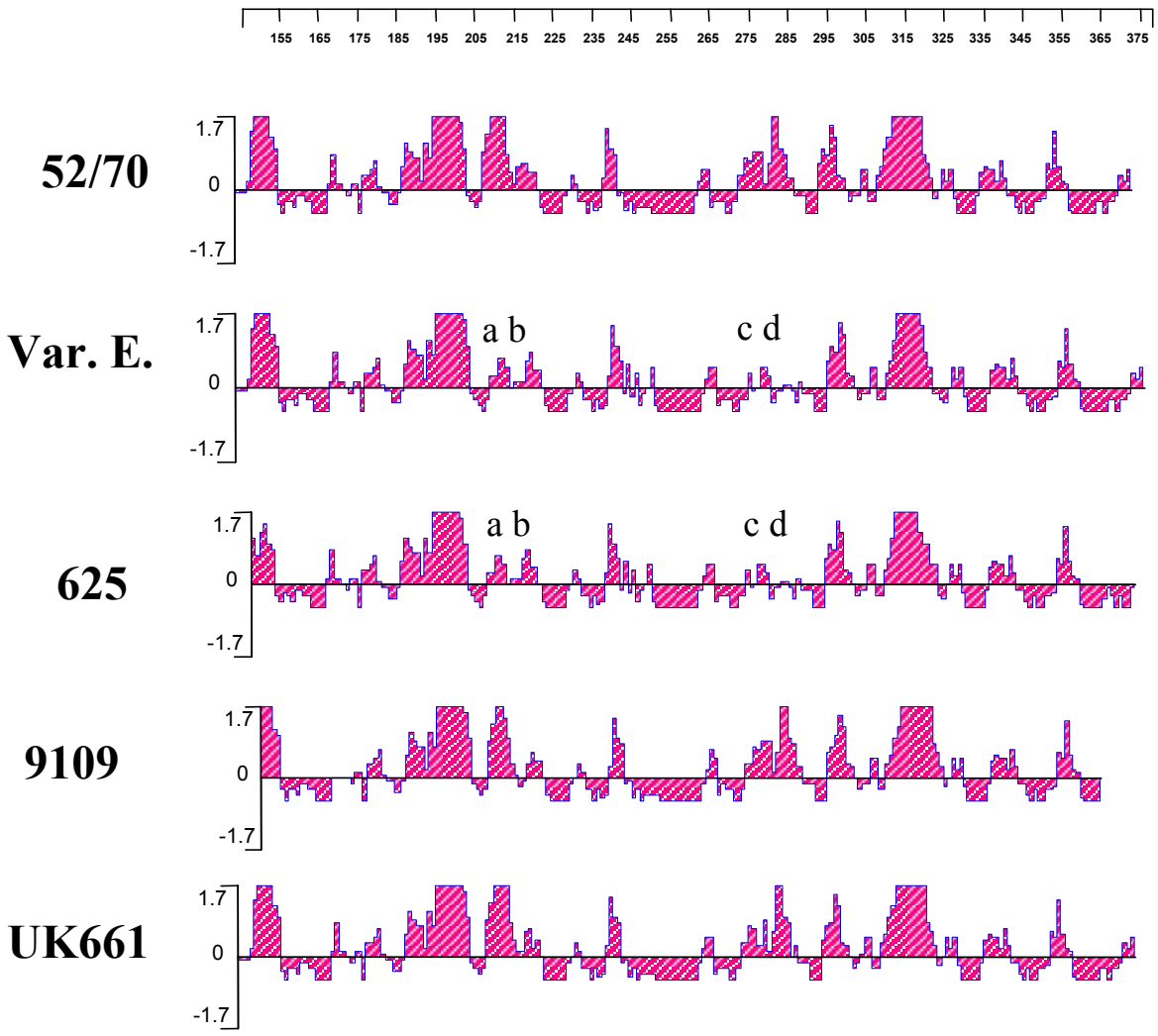

254
210 220 230 240 250 260
52/70 SDRPRVYTITAADDYQFSSQYQPGGVTITLFSANIDAITSLSIGGELVFQTSVQGLVLGATIYLIGFDGT
619 .....N.....T.....V.....K....N.....
625 .....N.....T.....V.....K....N.....
849 .....N.....T.....V.....K..I.N.....
850 .....N.....T.....V.....K....N.....
853 .....N.....T.....V.....K....N.....
11153 .....N.....T.....V.....K.N..N.....
Var. E .....N.....T.....V.....K....S.....
9109 .....L.....V.....
STC .....V.....F.....
Ehime 91 .....A.....I.....
UK 661 .....A.....I.....
1st hydrophylic region
294
318 323
280 290 300 310 320 330
52/70 AVITRAVAADNGLTAGTDNLMPFNLVIPTNEITQPITSIKLEIVTSKSGGQAGDQMSWSASGSLAVTIHG
619 .....N.....I.....S.....D....E.....
625 .....N.....I.....N...E.....
849 .....N.....I.....D.....
850 .....N.....I.....S.....D....E.....
853 .....N.....I.....D.....
11153 .....N.....I.....N...E.....
Var. E .....N.....I.....D....E.....
9109 T.....I.....
STC T.....V.....
Ehime 91 .....I....S.....
UK 661 .....I....S.....
2nd hydrophilic region

Figure 3.3.


155 165 175 185 195 205 215 225 235 245 255 265 275 285 295 305 315 325 335 345 355 365 375
52/70
1.7
0
-1.7
Var. E.
a b
c d
625
9109
UK661

CHAPTER IV

MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS FROM COMMERCIAL POULTRY IN THE UNITED STATES AND LATIN AMERICA[1]

[1]Banda, A., P. Villegas, and J. El-Attrache. Submitted to Avian Diseases, 5/9/02.

SUMMARY

From June 1999 to September 2001, 216 bursal samples from broiler farms in the United States and from various countries of Latin America were submitted to the Poultry Diagnostic and Research Center at the University of Georgia with the purpose of genotyping field infectious bursal disease viruses (IBDV). The reverse transcriptase-polymerase chain reaction (RT-PCR) was used to amplify a 248-bp product, encompassing the hypervariable region of VP2 gene. The genotyping was conducted by restriction fragment length polymorphism analysis (RFLP), using the six restriction endonucleases: *Dra* I, *Sac* I, *Taq* I, *Sty*, *Bst*, NI and *Ssp* I. For the 150 samples received from the United States, 125 samples (83.3 %) were RT-PCR positive for the presence of IBDV. One hundred positive samples, (80%) had an RFLP identical to the variant Delaware E strain, whereas ten samples (8.0%) exhibited a RFLP pattern similar to this antigenic variant. Other IBDV strains such as GLS, Lukert, PBG-98, Delaware A, and the vaccine strains Sal-1 and D-78 were also detected. Two samples exhibited a pattern similar to the standard challenge strain (STC strain) and seven strains (5.6%) were not classified by RFLP. Sixty-six bursal samples previously inactivated with phenol were received from Latin American countries. Standard strains were predominantly detected in Mexico. IBDV strains similar to variant E were detected in Colombia and Ecuador. Peru and Venezuela exhibited a higher heterogeneity of IBDV strains because of the detection of standard, Delaware type as well as GLS variant strains. IBDV strains detected from Brazil and Dominican Republic exhibited RFLP patterns identical to very virulent IBDV strains (vvIBDV) prevalent in several countries in Europe, Asia, and Africa.

Key words: RT-PCR – RFLP, infectious bursal disease virus, field strains, detection, genotyping commercial broilers, United States, Latin America.

Abbreviations: BF = Bursa of Fabricius; DEPC = Diethyl pyrocarbonate; DMSO = Dimethyl-sulfoxide; dsRNA = Double stranded ribonucleic acid; GLS = Grayson Laboratory strain; IBD = Infectious bursal disease; IBDV = Infectious bursal disease virus; RE = Restriction endonuclease; RFLP = Restriction fragment length polymorphism; RNA = Ribonucleic acid; RT-PCR = Reverse transcriptase- polymerase chain reaction; STC = Standard challenge strain; VN = Virus neutralization test; vvIBDV = very virulent infectious bursal disease virus.

INTRODUCTION

Infectious bursal disease (IBD) or Gumboro disease is an acute, highly contagious viral disease of young birds characterized mainly by severe lesions in the bursa of Fabricius followed by immunosuppression (6, 26, 32). Infectious bursal disease virus (IBDV) is a member of the *Birnaviridae* family with a genome consisting of two segments of double stranded RNA (dsRNA). IBDV is a nonenveloped icosahedral virus with a diameter of about 55- 60 nm (13, 30). Two serotypes of IBDV have been recognized and designated as I and II, but only serotype I causes naturally occurring disease in chickens (13, 20, 28).

Because of the high rate of mutations and genetic variability, the different strains of IBDV present in the field display diverse antigenic and pathogenic properties. The initial IBDV strains detected were characterized by the induction of depression, anorexia,

ruffled feathers, trembling, whitish or watery diarrhea, prostration, and mortality. These strains also induced severe nephritis, and the distinctive lesion was the presence of bursitis with further bursal atrophy. Currently, these strains are classified as standard or classic (26). In the mid eighties new types of IBDV strains were recognized. These strains suffered an antigenic shift that enabled them to induce immunosuppression in birds with adequate levels of humoral immunity; thus, these new strains were identified as antigenic variants (34, 36). The presence of these variants in the commercial flocks increased the susceptibility of other viral diseases such as infectious bronchitis, Newcastle disease, and laryngotracheitis, as well as the occurrence of opportunistic infections. In addition, birds exhibited a reduction in body weight gain along with poor flock uniformity (25, 26, 27). Very virulent strains of IBDV (vvIBDV) have been identified in severe outbreaks with high mortality and generalized hemorrhages (9, 37). The effects of theses strains have been devastating in several countries (29, 38).

To establish the proper control procedures, it is important to characterize the antigenic and pathological properties of the strains prevalent in a geographic area; thus, it is necessary to develop rapid and accurate methods for typing the different strains of IBDV. The conventional typing methods include the virus neutralization assay (VN) and antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) using monoclonal antibodies (31, 36). For conducting the VN test, the field strains should be adapted to grow *in vitro,* but several field isolates are difficult to adapt to grow *in vitro*; furthermore, the antigenic and pathological characteristics of the virus may be modified during the adaptation procedures (26). Utilization of the AC-ELISA has demonstrated that variant

strains predominantly lack one neutralizing epitope in the viral protein VP2, which is the major neutralizing eliciting antigen (35).

The use of molecular techniques has readily increased because of the assay's existing rapidity, accuracy, and versatility as well as possible correlations with the antigenic properties of the IBDV strains. The nucleic acid-based methods are useful tools for detecting viruses because the virus can be detected and typed without isolation and propagation in cell cultures or embryonated eggs. Several researchers have reported the use of reverse transcriptase – polymerase chain reaction (RT-PCR)-based techniques used along with restriction endonuclease digestion (RE), and restriction fragment length polymorphism, (RFLP) for the detection and genotyping of IBDV field strains (12, 15, 16, 21, 22, 23).

Ikuta *et al*. developed an RT-PCR technique to amplify a 248-bp VP2 fragment and further characterization was achieved by RFLP utilizing the six REs *Dra* I, *Sac* I, *Taq* I, *Sty* I, *Mva* I, and *Ssp* I (12). This procedure allowed the detection of known IBDV strains from the United States along with field isolates and commercial vaccines produced in Brazil. From 48 RT-PCR positive bursal samples from Brazil, 12 flocks exhibited vaccine RFLP pattern, a pattern compatible with a classic IBDV strain was observed in one flock, four new different RFLP were detected in 31 flocks, and a pattern compatible with vvIBDV was detected in four flocks.

The purpose of this study was to characterize field IBDV isolates from the United States and various of Latin America, based upon differences in the hypervariable region of VP2 gene by RFLP analysis according to previously described procedures (3, 12).

MATERIALS AND METHODS

Diagnostic samples. Bursal tissues from commercial broiler farms suspected of having IBDV were submitted to the Poultry Diagnostic and Research Center (PDRC) from June 1999 to September 2001. The ages of the flocks ranged from 14 to 50 days of age. One sample generally consisted of one to five bursas pooled from one flock. Samples obtained from outside the United States were placed in 1.4 to 1.5 ml aliquots of phenol: chloroform (5:1), pH 4.3 ± 0.2 (Fisher Chemicals, Fair Lawn, N.J.) in order to inactivate the infectivity of the IBDV and preserve the viral genome to be amplified by RT/PCR (18). All the foreign samples were inactivated with phenol-chloroform before they were introduced to the United States.

From a total of 150 samples from the United States, 129 samples were received from farms in Georgia (86%), the origin was unknown in 21 samples (14%). Fifty-five samples were from birds between three and four weeks of age, (36.7%), and 38 samples (25.3%) were obtained from birds between 5 and 6 weeks of age. Two samples (1.3%) were received from birds between one and two weeks of age and seven samples (4.67%) from birds around seven weeks of age. In 48 samples (32%), no data on age were available.

RNA extraction method. RNA from frozen or phenol:chloroform inactivated bursas was extracted using an acid-guanidium-phenol-chloroform RNA extraction method according to previously published methodology (7). The resulting RNA pellet was resuspended in 75% ethanol, sedimented, dried, and dissolved in 50 μl of 90% dimethyl-sulfoxide (DMSO) in diethyl pyrocarbonate (DEPC) water.

Primers. Two 23-nucleotide primers (IBDVP3 and IBDVP4) were developed by Simbios Biotecnologia (Porto Alegre, Brazil) (12). The OLIGO TM primer analysis software (National Biosciences Inc., Plymouth, MN) was used to design the primers. These primers were specific for the common sequences located on both sides of the VP2 gene hypervariable region and amplify a fragment of 248 bp at positions 804 to number1051, according to the numbering system of Bayliss *et al*. 1990 (4).

RT-PCR method. RNA from bursal samples was reverse transcribed to cDNA and amplified by a one step RT-PCR (12). After amplification, samples were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide.

Molecular identification of IBDV by RFLP. The 248 bp products obtained from the RT-PCR procedure were digested with the REs *Dra* I, *Sac* I, *Taq* I, *Sty* I, *Bst* NI, and *Ssp* I (New England Biolabs Inc., Beverly, MA) according to the manufacturer's instructions. Digested fragments were subjected to electrophoresis on a 12.5% polyacrylamide gel and visualized by rapid silver staining. Electrophoretic patterns were compared with reference IBDV RFLP patterns as well as undigested controls.

Nucleotide and deduced amino acid sequence analysis. Selected 248 bp RT-PCR products were purified by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Purified PCR products were sequenced by the dideoxy-mediated chain-termination method (33). Sequence data were assembled using Seqman ^{TM} (Dnastar Inc., Madison, WI). Nucleotide sequence analysis was performed by Clustal method using the package Megalin ^{TM} (Dnastar Inc., Madison, WI). Phylogenetic analysis of the hypervariable of VP2 gene was performed by parsimony method with heuristic search and 1000 bootstrap replicates using the package PAUP 4b2 ^{TM} (Sinauer Associates Inc. Sunderland, MA).

The tree was rooted to an outgroup sequence of the segment A of the OH strain of IBDV(accession NC 001915), which is one reference strain of the serotype II of IBDV.

GenBank sequence accessions of reference strains used for comparisons included the following antigenic variants: Delaware variant A (M64285), Delaware variant E (X54858), the isolates 625 (AY012678), 849 (AY012679), and 850 (AY012680). The standard strains included the Lukert strain (AY095230), STC (D00499), 2512 (AF279288), 52-70, (D00869), Edgar (AY095229), the isolates 9109 (AY012683), and 586 (AF305739), as well as the vvIBDV strains 849VB (X95883), and UK661 (X92760). The nucleotide sequence data of the foreign field IBDV reported in this study have been deposited with GenBank Data Libraries. The assigned accession numbers are AF498624 (BR-5, Brazil), AF498623 (DR3237, Dominican Rep.), AF498626 (DR-2, Dominican Rep.), AY100319 (EC-3, Ecuador), AF498927 (C-278, Mexico), AF498618 (Mx7499, Mexico), AF498619 (Mx7502, Mexico), AF498620 (Mx7504, Mexico), AF498621 (Mx7506, Mexico), AF498622 (Mx7997, Mexico), AY099997 (Ven-1, Venezuela), AY099998 (Ven-2, Venezuela), AY099999 (Ven-3, Venezuela), AY100000 (Ven-4, Venezuela), AY010001 (Ven-5, Venezuela), AY095534 (Mor, Venezuela), AY100320 (Mta, Venezuela), and AY100321 (Pad, Venezuela).

RESULTS

Molecular characterization of IBDV strains from the United States. From 150 samples, 125 (83.3%) were IBDV positive using RT-PCR, and 25 samples (16.7%) were found negative. After RFLP analysis, the Delaware variant E was identified in 100 of the 125 positive samples (80%). The RFLP pattern of variant E is characterized by the

presence of the RE sites for *Sac* I and *Taq* I. An RFLP pattern consisting of the site for *Taq* I only was observed in 10 cases (8%), these strains were classified as variant E-like. In two cases, the prevalent virus was characterized by positive digestion with *Sac* I, *Bst* NI, and *Ssp* I. Other strains analyzed in this study included the D-78, variant Delaware A, Grayson Laboratory strain (GLS), Lukert, PBG-98, and Sal-1, which were detected in only one case each. Finally, seven samples (5.6%) were positive for the presence of IBDV by RT-PCR, but no digestion was detected with any of the endonucleases used in this study; thus, these samples remained untypeable by RFLP.

Molecular characterization of IBDV strains from Latin American countries. The molecular characterization of different samples received from Latin American countries are displayed in Table 4.1. Sixty-six samples were received at the PDRC (University of Georgia). From the eight samples received from Brazil, seven samples exhibited the genotype characteristic of vvIBDV strains, six samples were similar to the UK-661 strain, and one was similar to the 849 VB strain. A virus similar to the Edgar strain was also detected from one sample. Seven samples were received from the Dominican Republic, six of these samples exhibited RFLP patterns identical to the UK-661 strain, one sample matched the RFLP pattern characteristic of Delaware variant E. A total of 10 samples were received from Mexico. The IBDV identified in these samples consisted mainly of standard strains such as Lukert in three samples. The Edgar, STC, and CU-1 were identified in one sample each. However, three samples contained viruses, which RT-PCR fragments were digested only with *Sty* I. This pattern did not match with any known strain; thus, these strains of IBDV were not classified by RFLP.

Samples from Peru and Venezuela exhibited several RFLP patterns; Delaware type variant strains as well as other standard strains were detected. From the 22 samples received from Peru, 14 were positive for the presence of IBDV. A RFLP identical to variant Delaware A was detected in 10 samples. Two samples exhibited a RFLP pattern characteristic of variant Delaware E, and two samples exhibited RFLP patterns that matched the classic strains PBG -98 and STC-like, respectively. Fourteen samples were received from Venezuela. Four samples exhibited RFLP patterns characteristic of the GLS strain. RFLP patterns identical to Lukert and Edgar strains were observed in two samples. Six RT-PCR products were digested with *Sac* I, *Taq* I, and *Ssp* I. This unusual pattern did not match with any known reference strain and therefore were not classified by RFLP.

Seven samples were obtained from Ecuador. From six IBDV positive samples, four samples contained virus exhibiting Delaware type A or E variant-like RFLP. Two positive samples were not classified because of the lack of digestion with any RE. Two samples were received from Colombia with RFLP patterns similar to that of Delaware type variant E –like strains.

Nucleotide sequence and phylogenetic analysis. The phylogenetic analysis of the nucleotide sequences of some bursal samples that were not completely characterized by RFLP analysis, along with reference nucleotide sequences, is shown in Figure 4.1. The nucleotide sequences of Delaware variant E, as well as from the isolates 625, 849 and 850, were located in the main stem along with the nucleotide sequences from Venezuelan samples Ven-1, Ven-2, Ven-3, Mta, Mor, and Pad. The percentages of nucleotide identity between the Delaware variant E strain and these six Venezuelan

IBDV range from 96% (Ven-3) to 98.4 (Ven-2). The Delaware Variant A and the sample EC-3 from Ecuador were grouped together, and they share a nucleotide identity of 98%.

A large branch was formed with nine different strains. The isolate 9109 (USA) and the sample Ven-4 were grouped closely to the 2512 and STC-1 standard strains. The nucleotide identity between isolate 9109 and the Venezuelan sample Ven-4 is 99.2%. The percentages of identity between the sample Ven-4 and the strains STC-1 and 2512 are 98% and 100%, respectively. The Mexican samples Mx7504, Mx7506, and Mx7997 were close related to the Lukert strain, with nucleotide identities ranging from 97.6 (Mx7504) to 98% (Mx7506 and MX7997) when they were compared with the Lukert strain. The Edgar strain was also located in this branch. However, the Mexican samples C-278, Mx7499, and Mx7502 were grouped together in a distinct and separate branch. The IBDV contained in these Mexican samples exhibited a nucleotide identity with the isolate 586 (Puerto Rico) ranging from 91.1% (Mx7502) to 92.3% (C-278). The samples from Brazil (BR-5) and from Dominican Republic (DR-2 and DR-3237) were grouped together with the vvIBDV strains 849VB and UK661. The identities between theses Latin American samples in comparison with the European strain UK661 strain ranged from 92.2 (DR-2) to 98.4 (BR-5).

DISCUSSION

An RT-PCR/RFLP assay where a 248-bp fragment that includes the hypervariable region of the VP2 gene followed by digestion with six RE was successfully used for genotyping field strains from the United States and several Latin American countries

(12). The amplified region is located within the highly variable domain between amino acid residues 239 to 332 of the VP2 gene (1).

Jackwood *et al*. (1997) developed an RT-PCR assay to amplify a 743-bp fragment of the VP2 gene. The RT/PCR products were digested with REs *Bs*t NI, *Mbo* I, or *Ssp* I. On the basis of RFLP profiles, vaccine viruses were placed into different molecular groups (17). The results indicated that little genetic heterogeneity of the VP2 gene existed among vaccine strains of IBDV. However, in another study a relatively large amount of genetic variability was observed in commercially reared chickens from 11 different states in the U.S. From 70 bursal samples, two samples (2.8%) corresponded to the variant Delaware A, 18 samples (25.7%) matched the Delaware E strain, two viruses exhibited a RFLP profile identical to the Lukert strain, and finally the remaining 48 samples (68.5%) exhibited 19 new different RFLP profiles (21). However, the significance of these new and unique RFLP patterns is still not determined.

In the present study, we found that the variant Delaware E strain was the most predominant genotype (80%) from commercial broiler farms in the United States. However, ten bursal samples (8%) contained IBDV virus, which exhibited a RFLP pattern characterized only by positive digestion with *Taq* I. These viruses were though to be similar to Delaware type variants. The similarity was confirmed by nucleotide sequence analysis of the VP2 gene. In a previous study it was determined that viruses exhibiting this genotype exhibited a similarity of about 97% with the variant Delaware E (3). Thus, these viruses were classified as variant E-like strains. The prevalence of strains similar to Delaware variant E in Georgia, reported in this work, agrees with the results from a survey conducted across the United States, using monoclonal antibodies and

antigen-capture ELISA. In the southeastern region of the United States, the Delaware type variants were the most frequently identified isolates and accounted for 46% of the isolates. Classic viruses accounted for 30%, and GLS variants accounted for 24% of the isolates (34).

Seven samples from the United States contained viruses, which were not digested by any RE used in this system. Most of these samples exhibited very faint DNA bands after the RT-PCR reaction. The amount of viral RNA might have been too low in the sample, and the yield of RT-PCR was not sufficient for enzyme digestion. DNA cloning of these RT-PCR products for further sequence analysis is necessary to achieve a definite characterization of these strains.

Two samples from the United States were digested by *Sac* I, *Bst* NI, and *Ssp* I. This pattern did not match with any reference strains used in this study. However, by sequence analysis, viruses exhibiting such RFLP pattern were 99.2% similar to the 2512 strain and 98% similar to STC strain. Previously, these strains were classified as STC-like (3). The positive digestion with the enzyme *Ssp* I has been used by several researchers (11, 12, 22, 24) to determine the presence of vvIBDV. With the genotyping system used in this study, the characteristic RFLP pattern for vvIBDV strains is characterized by digestion with *Taq* I, *Sty* I, and *Ssp* I (12). The isolate 9109 is a strain with *Ssp* I positive digestion that was isolated from a broiler flock exhibiting poor performance and recurrent respiratory problems. However, the birds did not exhibit clinical signs or mortality rates that are characteristic of vvIBDV strains. After the isolation in SPF chickens, this *Ssp* I positive isolate produced only the subclinical form of IBDV with bursal atrophy in SPF birds and commercial broilers (2). The presence of the

Ssp I restriction site corresponds to the non-synonymous nucleotide substitution that results in the substitution of leucine for isoleucine at residue 294. This substitution is conserved for the vvIBDV strains and was considered a distinctive feature of these strains (5, 10, 24). It seems that the presence of the *Ssp* I site does not correlate with the pathogenicity among U.S. strain as previously observed with strains for other countries. It appears that the clinical and pathological significance of IBDV strains exhibiting positive digestion with *Ssp* I is still unknown.

Ssp I positive IBDVs were also detected in Brazil (BR5), the Dominican Republic (samples DR2 and DR 3237), and Venezuela (Ven-1, Ven-2, Ven-3, Mta, Mor, and Pad). The strains detected in Brazil and the Dominican Republic showed identical RFLP patterns to European vvIBDV strains, as well as high nucleotide sequence identity with these vvIBDV strains detected in several countries of Europe, Asia, and Africa. The IBDVs detected in Venezuela (samples Ven-1, Ven-2, Ven-3, Mta, Mor, and Pad) are particularly interesting. These six samples were obtained from broiler flocks suffering severe bursal atrophy around 28 days of age. No severe disease or mortality was observed in the birds. The virus from these samples exhibited a RFLP with positive digestion by *Sac* I, *Taq* I, and *Ssp* I. By nucleotide sequence analysis they were closely related to the variant Delaware E and related strains, (96% to 98.4% nucleotide identity); however, these six viruses exhibited *Ssp* I positive RE sites. The molecular characteristics of these strains are intriguing because generally *Ssp* I sites are only in some standard strains such as vvIBDV strains (11, 22, 24). Unfortunately no antigenic characterization was possible because the samples were inactivated with phenol:chloroform prior to be received in our laboratory. The evolutionary significance of this change is uncertain. However, it is

plausible that the viruses in the field are undergoing genetic selection in order to infect birds that have some level of immunity against IBDV. These conditions might be exerting pressure forcing variant strains to display features observed in the standard viruses.

There are two previous reports on the presence of vvIBDV strains in Brazil (8, 12). The clinical signs and gross lesions observed in the chickens agree with those seen in other parts of the world. The Brazilian vvIBDV strains had 98% identity with the vvIBDV strains prevalent in Europe and Asia. The predicted amino acid sequence of Brazilian strains (data not shown) demonstrated an identity between 98.8% and 100% compared with the European and Asiatic vvIBDV strains.

A total of ten samples were analyzed from Mexico, six of them corresponding to typical IBDV standard strains such as Lukert strain, Edgar, STC, and CU-1, as determined by RFLP. However, three samples (C278, 7499, and 7502) exhibited a unique RFLP pattern, where only the digestion with *Sty* I was observed. After nucleotide sequence and phylogenetic analysis, these samples were classified in a separate and distinct branch (Figure 1). The reported sequence that is most related to these Mexican strains is isolate 586 from Puerto Rico (14). The viruses from Mexico had identities between 91.1% and 92.3% when they were compared with isolate 586.

Seven of the 125 positive samples (5.6%) were not identified with this system. This percentage of not classified samples is low, since the molecular assay used in this study uses six different REs, allowing for the analisys of a multitude of RFLP patterns. This minimizes the number of genetically variable IBDVs detected that do not match an established RFLP.

The detection and genotyping of several strains of IBDV was successfully accomplished by RT-PCR/ RFLP from phenol:chloroform inactivated bursal tissues submitted from some countries of Latin America. The inactivation of IBDV with this procedure has been effective and preserves the integrity of the viral RNA for RT-PCR/RFLP analysis (18). However, because of the inactivation of the virus in the submitted samples, further *in vivo* studies were not possible, thus limiting the possibility of conducting clinical and pathological studies as well as virus neutralization or cross-protection studies to determine their antigenic characteristics.

The number of samples obtained from Latin American countries analyzed in this study is limited. The samples were not selected randomly; thus, further and comprehensive epidemiological studies are needed to draw more realistic conclusions on the prevalence or incidence of IBDV in Latin America countries. However according to our findings, there is a high variety of IBDV strains prevalent in Latin American countries.

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ACKNOWLEDGMENTS

Appreciation is extended to Drs Bruce S. Seal and Maricarmen García for helpful reviews of the manuscript.

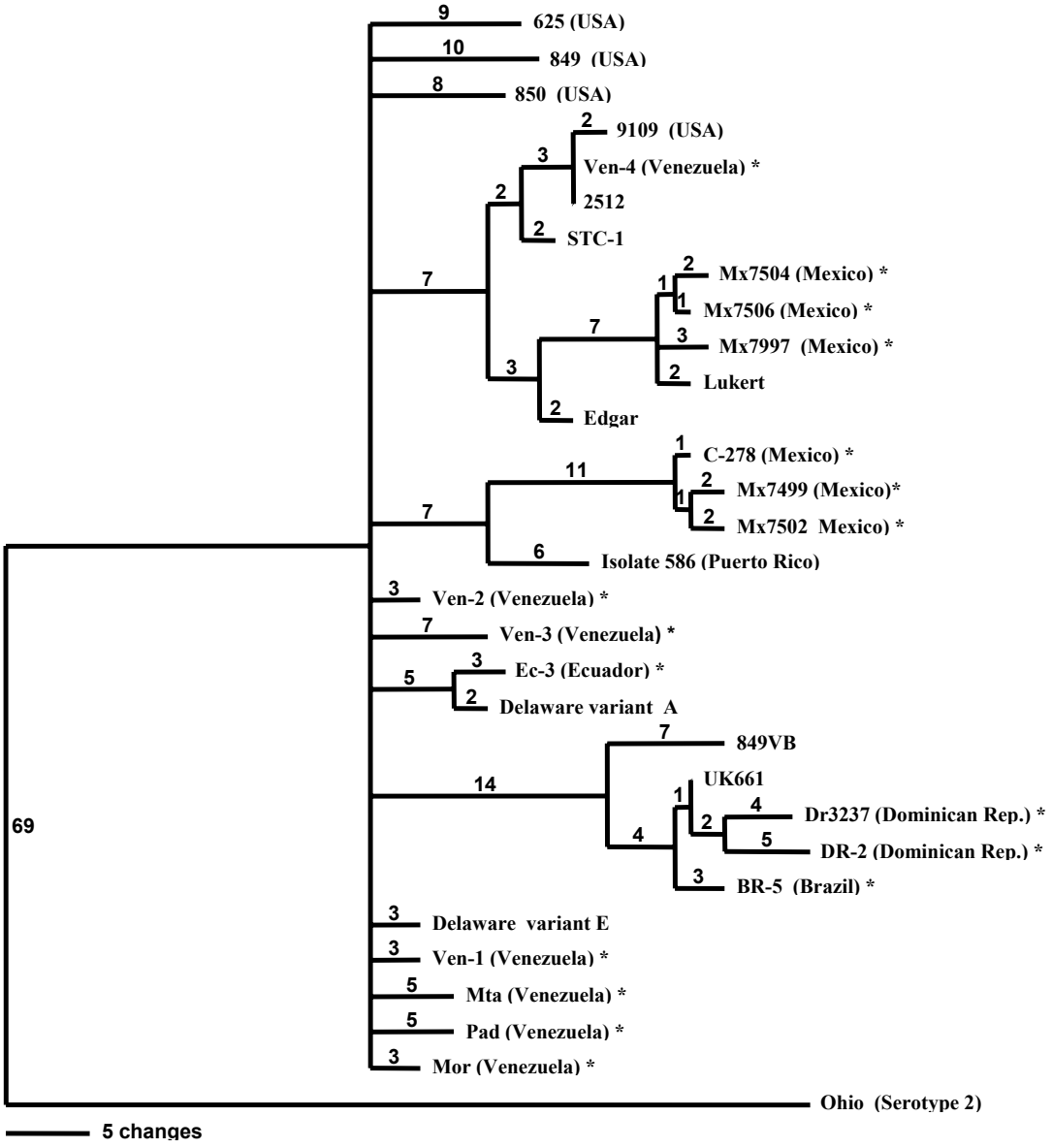
Table 4.1. Molecular characterization of IBDV detected in phenol:chloroform inactivated bursal samples received from several countries in Latin America. The molecular characterization was achieved by RT-PCR and RFLP analysis.

Country	Total of samples	Genotype similar to strain:	Positive samples
Brazil	**8**	UK-661 849 vB Edgar	**6** **1** **1**
Colombia	**2**	Variant E- Like	**2**
Dominican Republic	**7**	UK-661 Variant E	**6** **1**
Ecuador	**7**	Variant E-Like Untypeable	**4** **2**
Mexico	**10**	Lukert Edgar STC CU-1 Untypeable	**3** **1** **1** **1** **3**
Peru	**22**	Variant A Variant E Variant E-Like PBG-98 STC-Like	**10** **1** **1** **1** **1**
Venezuela	**14**	GLS Lukert Edgar Untypeable	**4** **1** **1** **6**
TOTALS	**66**		**58**

Figure Legends

Fig. 4.1. Phylogenetic analysis of a segment of the amplified 248 bp IBDV VP2 product (positions 804 to 1051 numbering according to Bayliss *et al*). The relationship among field isolates from the United States and Latin America was determined. The tree was generated by parsimony method with heuristic search and 1000 bootstrap replicates. The tree was rooted to an outgroup sequence of the Ohio (OH) strain, which represents serotype II. The length of each branch represents the number of nucleotide changes between sequences.

Figure 4.1



* New nucleotide sequence data of foreign field IBDV reported in this study

CHAPTER V

GENOTYPING INFECTIOUS BURSAL DISEASE VIRUS BY HETERODUPLEX MOBILITY ASSAY[1]

[1]Banda, A., P. Villegas, and J. El-Attrache. To be submitted to Avian Diseases.

SUMMARY

A heteroduplex mobility assay (HMA) was developed for genotyping infectious bursal disease virus (IBDV). A set of primers was designed to amplify a 390 bp product via RT-PCR, encompassing the hypervariable region of the VP2 gene. The HMA was able to differentiate standard, antigenic variants and very virulent strains of IBDV. Furthermore, minor differences between antigenic variants were also detected by HMA. IBDV strains present in bursal samples from the field were compared with three commercial vaccines containing variant and standard strains. Variations in the migration patterns of IBDV present in field samples were observed by HMA. The results obtained by HMA correlated with RFLP and phylogenetic analysis of nucleotide sequences. However, the statistical correlation between HMA and percentage of nucleotide sequence identity was variable depending on the strain analyzed. The HMA proved to be a useful technique to rapidly genotype field isolates or strains of IBDV, and may be an auxiliary tool for selecting vaccine candidates to control infectious bursal disease in the field.

Key words: Infectious bursal disease virus, heteroduplex mobility analysis, genotyping.

Abbreviations: BF = Bursa of Fabricius; CEF = Chicken embryo fibroblast; DEPC = Diethyl pyrocarbonate; DMSO = Dimethyl-sulfoxide; HMA = Heteroduplex Mobility Assay; HMR = Heteroduplex mobility ratio; IBD = Infectious bursal disease; IBDV = Infectious bursal disease virus; PAGE = Polyacrilamide gel electrophoresis; RE = Restriction endonuclease; RFLP = Restriction fragment length polymorphism; RT-PCR

= Reverse transcriptase- polymerase chain reaction; vvIBDV = very virulent infectious bursal disease virus.

INTRODUCTION

Infectious bursal disease (IBD) or Gumboro disease is an acute and immunosuppressive disease of young chickens caused by a virus that is a member of the family *Birnaviridae*. The infectious bursal disease virus (IBDV) is a nonenveloped icosahedral virus with a diameter of about 55- 60 nm (15, 28). Two serotypes of IBDV have been recognized and designated as I and II, but only serotype I causes naturally occurring disease in chickens. The viruses belonging to this family are characterized by a genome consisting of two segments of double stranded (ds) RNA. Segment A contains two open reading frames (ORF). The large ORF encodes a polyprotein which is processed into VP2, VP3 and VP4 proteins (10), the small ORF encodes for VP5 which is considered a nonstructural protein, absent in the virion but detected in infected cells (26). Segment B codes for VP1, a RNA dependent RNA polymerase (20). There is a high genetic variability among different strains of IBDV, this genetic variability results in different antigenic and pathogenic types of IBDV. The most significant changes in the IBDV genome occur in the hypervariable region of the VP2 gene (13, 22).

The different strains of IBDV can be readily classified into three groups. The standard strains induce a severe clinical disease in chickens older than 3 weeks. Clinical signs include depression, ruffled feathers, dehydration, whitish diarrhea and mortality. When standard strains cause infection in younger birds they will induce a severe immunosuppression without significant clinical signs (24). IBDV variants are

antigenically different to the standard strains, they are extremely immunosuppressive and can induce severe bursal atrophy. Antigenic variants are extensively spread through the United States (23). The third group includes the very virulent IBDV (vvIBDV) strains. These strains are responsible for severe clinical outbreaks in Europe, Asia and Africa. Even though these strains are antigenically related to the standard strains, they are more virulent than previously known standard strains (31).

The heteroduplex mobility assay (HMA) is an established method to compare two PCR amplicons, which are mixed together in approximately equal amounts and heated to 94°C - 95°C to denature them into individual single strands. Upon cooling of this mixture, homoduplexes will form from single strands of the same amplicon, and heteroduplexes will form from strands of different amplicons. The heteroduplexes will contain "bubbles" due to mismatches. These mismatches will reduce the mobility of the heteroduplexes and they will exhibit a slower migration through a polyacrylamide gel matrix. Following gel electrophoresis, both homoduplexes and heteroduplexes can be visualized, and the distance between them is proportional to the divergence between the two sequences (3).

The HMA has been used successfully for the molecular characterization of several viral agents such as genotyping hepatitis C viruses (HCV) with PCR products from the 5' unstranslated region or the NS5b region (32). The patterns of heteroduplexes correlated with the genotype, as determined by sequencing. HMA was also used to simplify the identification of mixed infections with two HCV genotypes.(32). Polyak *et al*, (29) studied the evolution of quasispecies of HCV by analyzing the hypervariable region 1 by HMA. They observed higher rates of mutations in interferon-treated patients

in comparison with controls (29). For measles, two distinct wild-type lineages and one group of vaccine like viruses were detected by analyzing the carboxy-terminal 590 nucleotides of the nucleocapsid (N) gene using HMA (21). Ellis and Zambon (12) observed that HMA was sensitive and specific for the detection of human, avian, and swine influenza A viruses, and the differentiation of subtypes between 1.9% and 21.4% nucleotide divergence (12). Other viruses that have been genotyped by HMA, are Norwalk-like viruses (25), and the GB virus C (hepatitis G) (33). Berinstein *et al*. (5) were able to distinguish vaccine-like viruses from virulent isolates of Newcastle disease viruses by analyzing the F protein cleavage site coding sequences from several isolates using HMA. Results obtained by HMA correlated with phylogenetic analysis of nucleotide sequences (5).

The HMA is an easy and economical method for analyzing viral genomes, and has been used successfully for genotyping several human diseases, and may offer the same advantages in the genotyping IBDV strains. The objective of this study was to analyze theVP2 gene hypervariable region of IBDV by HMA and to compare results obtained by this technique with data obtained by RFLP and nucleotide sequence analysis.

MATERIALS AND METHODS

Diagnostic samples. Bursal tissues from commercial broiler farms suspected of having IBDV were obtained and kept frozen until processing. Bursal samples obtained from outside the United States were placed in 1.4 to 1.5 ml aliquots of phenol: chloroform (5:1), pH 4.3 ± 0.2 (Fisher Chemicals, Fair Lawn, N.J.) to inactivate the infectivity of the IBDV and preserve the viral genome to be amplified by RT/PCR (18).

IBDV reference strains and samples analyzed in this study are listed in Table 1. The standard reference Edgar strain was propagated in specific pathogen free (SPF) birds. The CEF-Lukert and the Ohio strains were propagated in chicken embryo fibroblast cultures.

RNA extraction method. RNA from frozen or phenol:chloroform inactivated bursas was extracted using an acid-guanidium-phenol-chloroform RNA extraction method according to previously published methodology (7). The resulting RNA pellet was resuspended in 75% ethanol, centrifuged, dried, and dissolved in 50 µl of 90% dimethyl-sulfoxide (DMSO) in diethyl pyrocarbonate (DEPC) water.

RT-PCR method. RNA from bursal samples was reverse transcribed to cDNA and amplified by a one step RT-PCR procedure (12). After amplification, samples were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide.

Reference PCR amplicons. IBDVs detected in bursal samples from the United States and Latin America were previously typed by RT-PCR/RFLP (2), (1). Selected samples were amplified by RT-PCR using the following set of primers 5'-TCTGCAACAGCCAACATCAACG-3', as forward primer, and 5'-TCAGGATTTGGGATCAGCTCGA-3', as reverse primer. This set was designed to amplify a product of 698 bp from nucleotide position 567 to 1264 of the VP2 gene. The 698 bp-RT-PCR product, were purified by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and inserted into the pCR 2.1 vector and cloned according to the manufacturer's specifications (Life Technologies, Carlsbad, CA). Recombinant plasmids were used for nucleotide sequencing, and amplified by PCR and used as reference PCR products for HMA. The reference IBDV genotypes utilized in this study included examples of standard, antigenic variants and vvIBDV strains.

Nucleotide sequence analysis. Recombinant plasmids were sequenced by the dideoxy-nucleotide chain-termination method (30). Sequence data were assembled using Seqman ™ (Dnastar Inc., Madison, WI). Nucleotide sequence analysis was performed by the Clustal method using the package Megalin ™ (Dnastar Inc., Madison, WI). Phylogenetic analysis of the hypervariable region of the VP2 gene was performed by the parsimony method with heuristic search and 1000 bootstrap replicates using the package PAUP 4b2 ™ (Sinauer Associates Inc. Sunderland, MA). The tree was rooted to an outgroup sequence of Ohio strain of IBDV which is one reference strain of the serotype 2 of IBDV.

The nucleotide sequences of the RT-PCR products obtained from the reference strains and from IBDV detected in field samples have been deposited with GenBank Data Libraries. GenBank sequence accessions are listed in Table 5.1.

Primers for HMA. Two 22-nucleotide primers were developed using the Primer Premier software (Premier Biosoft International, Palo Alto, CA). This pair of primers was specific for the common sequences located on both sides of the VP2 gene hypervariable region and amplify a fragment of 390 bp at positions 653 to 1042, according to the numbering system of Bayliss *et al*. 1990 (4). The nucleotide sequence for IBDHMF (forward primer) primer is 5-TCT TGG GTA TGT GAG RCT YGG T-3 and for IBDHMR (reverse primer) is 5-GGC TGG GTT ATC TCR YTG GTT G-3.

HMA . HMA was performed by mixing 1 µl of a reference PCR products with 1 µl of a sample PCR products and 10 µl of DEPC water. The mixtures were denaturated for 5 min. at 94C. After denaturation, the mixtures were chilled immediately on wet ice. Homoduplexes and heteroduplexes were separated at 125 V by polyacrylamide gel

electrophoresis (PAGE) using the mutation detection enhancement gel matrix MDE^{TM} gel solution (BioWhittaker Molecular Applications, Rockland, ME) at 1X concentration according to the manufacturer protocol. The HMA patterns were visualized by rapid silver staining. The heteroduplex mobility ratio (HMR) was calculated as the distance the heteroduplex migrated from the well divided by the distance the homoduplex migrated from the well. Values of HMR of 1.0 corresponded to homoduplexes, HMRs close to 1.0 indicated a close relation between the nucleotide sequences that are being compared, lower values indicated a high number of mismatches between the sequences (29).

Pairwise comparisons by HMA were conducted between nine PCR products and 13 reference PCR products that included genotypes representative of standard (LKS, Edgar, Lukert intermediate, Lukert Inter. Plus, Moulthrop HL, and 9109), antigenic variants (1084E, 89/03, U-28, 619, and K-1) and vvIBDV strains (DR-2 and BR-5. Separately HMA was performed with 14 diagnostic bursal samples from commercial broiler flocks in Georgia that have been previously characterized by RFLP. These samples were compared with the vaccine strains 89/03, 1084E and Lukert intermediate.

Statistical analysis. Nonparametric Spearman's correlation coefficient between HMR and percentage of nucleotide sequence identity was calculated according to previously described methods (8).

RESULTS

Sequence nucleotide analysis. Nucleotide sequences encompassing the hypervariable region of VP2 were aligned, followed by phylogenetic analysis (Fig. 5.1). The IBDV genotypes analyzed separated into five main branches. The first branch was

composed of the antigenic variant strains of IBDV, which included the vaccine strains 1084E and 89/03 along with four strains isolated from commercial broiler farms (619, 9865, 11153 and U-28). A second branch included the Moulthrop HL strain and the 9109 isolates. Sequences corresponding vvIBDV strains from Brazil and Dominican Republic were grouped together in a third branch. Edgar and Lukert-related strains were grouped in a fourth branch. Finally sequences from viruses detected in Venezuela (K-1 and Mor) were grouped separately as a unique group. The LKS strain and the C-278 sequences from Mexico were branched independently after phylogenetic analysis.

HMA. Pairwise HMA of nine IBDV strains with 13 reference genotypes are shown in Fig. 5.2. HMR data and nucleotide sequence identity percentages are also depicted. The CEF-Lukert strain and the vaccine strains Lukert intermediate, exhibited similar profiles of HMA, the heteroduplexes band formed between them could not be separated from the homoduplexes and resulted in HMRs of 1.0. CEF-Lukert and Lukert intermediate were clearly differentiated from other classic strains, as well antigenic variants and vvIBDV strains. They exhibited HMRs of 0.94 and 0.92 respectively, when they were compared with the Edgar strain. When CEF-Lukert and Lukert intermediate were compared to the antigenic variants, the HMRs ranged from 0.84 (CEF-Lukert compared to 1084E) up to 0.91 Lukert intermediate and isolate 619. The HMRs observed for the comparisons with the products DR-2 and BR-5 (field samples containing vvIBDV), ranged from 0.84 (Lukert int. compared with both DR-2 and BR-5) up to 0.87 (CEF-Lukert compared with DR-2).

Isolates 9865 and 11153 exhibited similar HMA profile, and both appeared evidently related with the Delaware variant genotypes. Isolate 9865 exhibited the highest

HMRs (0.94) when it was compared with the products from all variant IBDVs (1084E, 89/03, U-28, 619 and K-1). Isolate 11153 yielded higher HMRs when it was compared with the strain 89/03 (0.96) and with the isolate 619 (0.97).

Isolate 9109 appeared more related with Moultroup HL (1.0) and with the Edgar strain (0.96). When isolate 9109 was compared with itself, the heteroduplexes and homoduplexes could not be separated by PAGE. The PCR product from vvIBDV DR-1 was closely associated with the strain DR-2 (HMR of 1.0) and with BR-5, (HMR of 0.97). HMR values of 0.95 were produced when DR-1 was compared with the Moultroup HL vaccine and with isolate 9109. Larger shifts were observed with the comparison with other strains. The sample from Mexico identified as C-278 did not show a close relationship with any of the reference strains used in this study, the HMR for C-278 ranged from 0.82 when it was compared with both Lukert derivated vaccines, and 0.93 with the strain 1084E. The sample from Venezuela designated Mor, exhibited a profile similar to the Delaware variants. The sample Mor exhibited the closest relationship with the isolate 619 (HMR = 1.0) where both homoduplexes and heteroduplexes bands were indistinguishable. The sample Mor also exhibited a close relationship with the vaccine 89/03 (HMR = 0.97) and with the strain U-28 (0.95). Largest shifts produced by unpaired nucleotides occurred with the Ohio strain. The HMRs ranged from 0.27 when it was compared with the Edgar and the 1084E strains to 0.38, when it was mixed with BR-5.

The results of HMA from 14 diagnostic samples compared with vaccine strains 89/03, 1084E and Lukert intermediate are shown in Fig.5.3. These samples were previously characterized by RFLP. Twelve samples exhibited RFLP patterns characteristic of Delaware variant E (VE). By RFLP, the IBDV present in the sample

13270 was characterized as Lukert (LK), and the IBDV in the sample 15941 as STC-like (STC). There were minor differences between the 12 variant strains and the vaccine strains 89/03 and 1084E. However, the 12 variant E samples were more closely related to 89/03 than to the 1084E strain. The mean HMR obtained when they were compared with 89/03 was 0.98, while it was 0.96 when they were compared with 1084E. The differences where more evident when these strains were compared to the Lukert intermediate, the HMR ranged from 0.79 up to 0.90, with a mean HMR of 0.87.

Correlation between HMR and nucleotide sequence identity percentage. The nonparametric Spearman's correlation coefficients between HMR and nucleotide sequence identity for the nine IBDVs analyzed compared with the 13 reference PCR products are shown in Table 5.2. The statistical correlation observed, ranged from –0.69 when the Ohio strain was analyzed up to 0.90 observed with the isolate 9865.

DISCUSSION

The structural VP2 protein of IBDV has been determined to be the major antigen that elicits a neutralizing antibody response in the bird. The majority of differences between IBDV strains are located in the area between amino acid residues 206 to 305, which has been designated as the variable domain or variable region (13, 22). Since the hypervariable region of the VP2 gene exhibits a number of changes depending on the IBDV strain, most of the molecular techniques for genotyping IBDV are focused on this region (6, 11, 14, 17, 16, 19). The purpose of this study was to use HMA to analyze a 390 bp PCR or RT-PCR product encompassing this region in order to classify and characterize different IBDVs.

The HMA described in this study was a suitable method for genotyping IBDV strains. It clearly discriminated between the three main groups of IBDV strains; standard, antigenic variants and vvIBDV strains. HMA even detected differences among strains classified in the same group. The Edgar strain, the CEF-Lukert and both Lukert intermediate vaccines were branched together after phylogenetic analysis, however, the Edgar strain is somewhat distant to the three Lukert-derived strains. This feature was clearly detected by HMA. There are only two nucleotide mismatches between CEF-Lukert and Lukert intermediate (0.5% of divergence), and three mismatches between CEF-Lukert and the Lukert intermediate plus (0.8% of divergence), and the paired HMA comparisons between CEF-Lukert and both Lukert intermediate vaccines produced indistinguishable heteroduplex bands from the homoduplex ones (HMR of 1.0), indicating that two or three mismatches were not detected by HMA. However, there are 10 nucleotide mismatches between Edgar and both Lukert-derived strains (2.6% of divergence), and the pairwise HMA between the Edgar strain and both Lukert strains produced clear separate heteroduplex and homoduplex bands, indicating that divergence percentages around 2.6% are well detected by HMA. Previously reported data indicate that the nucleotide sequence divergence percentages between 1% to 2% are detected by HMA (9).

A similar situation occurred with isolates 9865 and 11153 that were genotyped as Delaware type variants by RFLP. By HMA, both isolates exhibited a closer relationship with the variant strains 1084E, 89/03, U-28, and 619. However, the HMA profile for isolate 11153 is rather different from that of isolate 9865, because isolate 11153 exhibited a closer relationship with the strains 89/03, U-28 and 619 than isolate 9865 did. This

feature agrees with the result of the phylogenetic analysis, where isolates 9865 and 11153 were branched together although, isolate 9865 is located more distant to the other variant strains in the phylogenetic tree.

The viruses present in the samples DR1, DR2 and BR5 were classified as vvIBDV strains (1). This similarity was also confirmed by HMA, but HMA indicated that 13 mismatches (3.3% of divergence) occur between the sample DR1 from Dominican Republic and BR5 from Brazil.

The Ohio strain that belongs to the serotype 2 of IBDV was also included in this study. This serotype has been associated mainly with turkeys, and no serotype 2 isolates have been demonstrated to be either pathogenic or immunosuppressive (24). The nucleotide sequence identity between the Ohio strain and the serotype 1 IBDV strains used in this study ranged from 64.1 to 69.7. In spite of the larger distances between the Ohio strain and the serotype I strains, the conditions of the HMA described here, allowed for the formation of heteroduplexes, but the largest shifts in heteroduplex migration were observed Fig 1). It has been reported that if two PCR amplicons share less than 65% similarity they are unlikely to form heteroduplexes (3).

Minor nucleotide sequence differences can be detected between strains used as vaccines and field strains. From 14 bursal samples used in this study, 12 were previously characterized as Delaware E. Pairwise HMA between these 12 variants with the vaccine strains 89/03 and 1084E demonstrated that these samples were somewhat more related to the vaccine 89/03 than to 1084E strain. This may indicate either the chickens from those flocks received that vaccine strain, or the IBDV strains resident in these flocks are genetically closer to 89/03 than they are 1084E. Broader differences were observed when

pairwise HMA comparisons were performed using the Lukert intermediate vaccine. Thus, HMA may be practical for genotyping IBDV present in the flocks, but also may be an auxiliary tool for selecting the vaccines strains that are more related to the field strains. However, more research correlating HMA relationships with antigenicity between strains is needed.

Statistical correlation between HMR and nucleotide sequence identities percentages was obtained by the nonparametric Spearman's correlation coefficient. Depending on the strain analyzed, the correlation coefficient ranged from 0.41 for isolate 9109 to 0.90 for isolate 9865. This means that there is no linear association between HMR and nucleotide sequence percentage, thus, HMR should not be used to predict nucleotide identity values. However, since HMA is an easy and economical method, it may be a useful tool for screening a large number of isolates before nucleotide sequencing. Indeed it has been used with other diseases such as measles (21).

Two bands corresponding to heteroduplexes were observed in several HMA patterns. These two different heteroduplexes are formed when two different double-stranded PCR amplicons are mixed. The sense strand of amplicon A will anneal with the antisense strand of amplicon B, but also a heteroduplex formed between the antisense strand of product A will anneal with the sense strand of product B. Thus two different double stranded DNA "hybrids" are formed. These two heteroduplexes will not necessarily migrate together despite sharing mismatched positions, due to interactions with bases flanking the mismatches (27, 33). However, other explanation for the multiple heteroduplex bands may by the presence of single stranded RT-PCR amplicons that did not reanneal because of the rapid cooling conducted in this study, some bands might.

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Table 5.1. IBDV samples analyzed by HMA

Type of Sample	Name	Country of Origin	Classification by RT/PCR-RFLP	GenBank Accession Number
Standard reference strains	Edgar*	U.S.A.	Edgar	AY095229
	CEF-Lukert	U.S.A.	Lukert	AY095230
Commercial Vaccines:	1084E	U.S.A.	Variant E	AF498628
	89/03	U.S.A.	Variant E	AF498629
	LKS	Mexico	CU-1	AY094618
	Lukert Int.	U.S.A.	Lukert	AF498631
	Lukert Int Plus	U.S.A.	Lukert	AF498632
	Moulthrop HL	U.S.A.	ND[1]	AF498633
Field Isolates (USA):	11153	U.S.A.	Variant E	AY012682
	619	U.S.A.	Variant E	AY012677
	9109	U.S.A.	STC Like	AY012683
	9865	U.S.A.	Variant E	AF498630
	U-28	U.S.A.	Variant E	AF498635
Foreign phenol:chloroform inactivated bursal samples:	BR-5	Brazil	vvIBDV	AF498624
	C-278	Mexico	Untypeable	AF498927
	DR-1	Dominican Rep.	vvIBDV	AF498625
	DR-2	Dominican Rep.	vvIBDV	AF498626
	K-1	Venezuela	GLS	AF498634
	Mor	Venezuela	Untypeable	AY095534
Serotype 2 strain	Ohio*	U.S.A.	ND[1]	AY095231

1. Strains kindly provided by Dr. Phil Lukert
2. [1] ND = Not done

Table 5.2. Correlation between heteroduplex mobility ratio (HMR) and nucleotide sequence identities of nine IBDV strains when compared with 13 reference PCR amplicons. Correlation (r) was calculated by nonparametric Spearman's coefficient.

IBDV STRAIN	**r**	**P value**
Lukert CEF-adapted	0.7472	0.0033
Lukert intermediate	0.8164	0.0007
Isolate 9865	0.8988	0.0001
Isolate 11153	0.5295	0.0627
Isolate 9109	0.4158	0.1576
DR-1	0.7663	0.0022
C-278	0.5629	0.0452
Mor	0.7417	0.0037
Ohio	-0.6917	0.0088

Figure Legends

Fig. 5.1 Phylogenetic analysis of a segment of the amplified 390 bp IBDV VP2 product (positions 653 to 1042 numbering according to Bayliss *et al*). The relationship among 20 nucleotide sequences from IBDV is shown. The tree was generated by parsimony method with heuristic search and 1000 bootstrap replicates. The tree was rooted to an outgroup sequence of infectious pancreas necrosis virus (IPNV). The length of each branch represents the number of nucleotide changes between sequences.

Fig. 5.2 Pairwise HMA analisis of nine IBDV strains compared with 13 reference PCR products. HMR = Heteroduplex mobility ratio (HMR= Heteroduplex migration distance/ homoduplex migration distance), Nuc. Sec. = Nucleotide sequence identity percentage, determined by Clustal Method.

Fig. 5.3. HMA from 14 diagnostic samples compared with vaccine strains 89/03, 1084E and Lukert intermediate. HMR = Heteroduplex mobility ratio (HMR= Heteroduplex migration distance/ homoduplex migration distance).

Figure 5.1

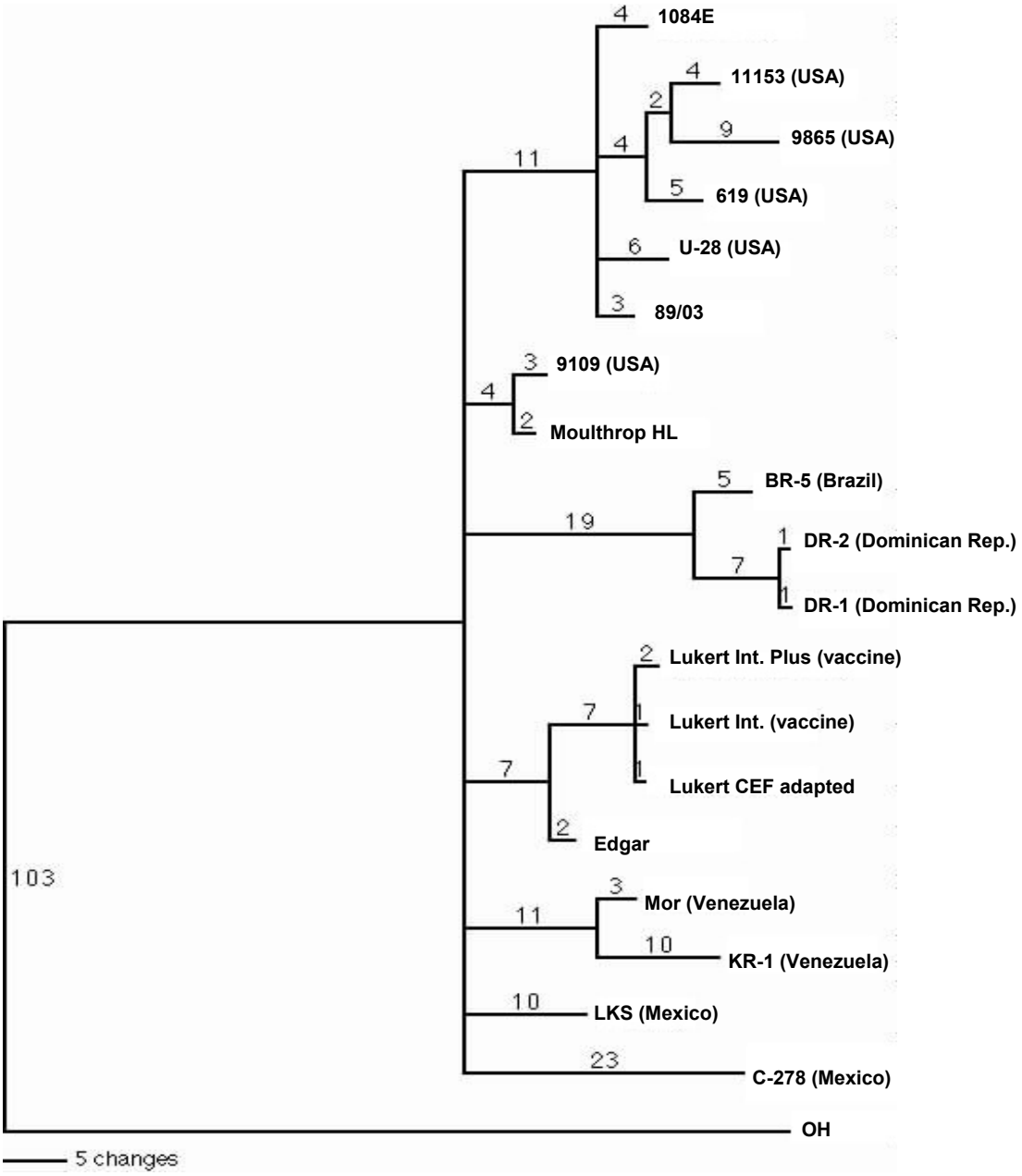

4
1084E
4
11153 (USA)
2
9
9865 (USA)
11
4
5
619 (USA)
6
U-28 (USA)
3
89/03
3
9109 (USA)
4
2
Moulthrop HL
5
BR-5 (Brazil)
19
1
DR-2 (Dominican Rep.)
7
1
DR-1 (Dominican Rep.)
2
Lukert Int. Plus (vaccine)
7
1
Lukert Int. (vaccine)
7
1
Lukert CEF adapted
2
Edgar
103
3
Mor (Venezuela)
11
10
KR-1 (Venezuela)
10
LKS (Mexico)
23
C-278 (Mexico)
OH
5 changes

Fig. 5.2

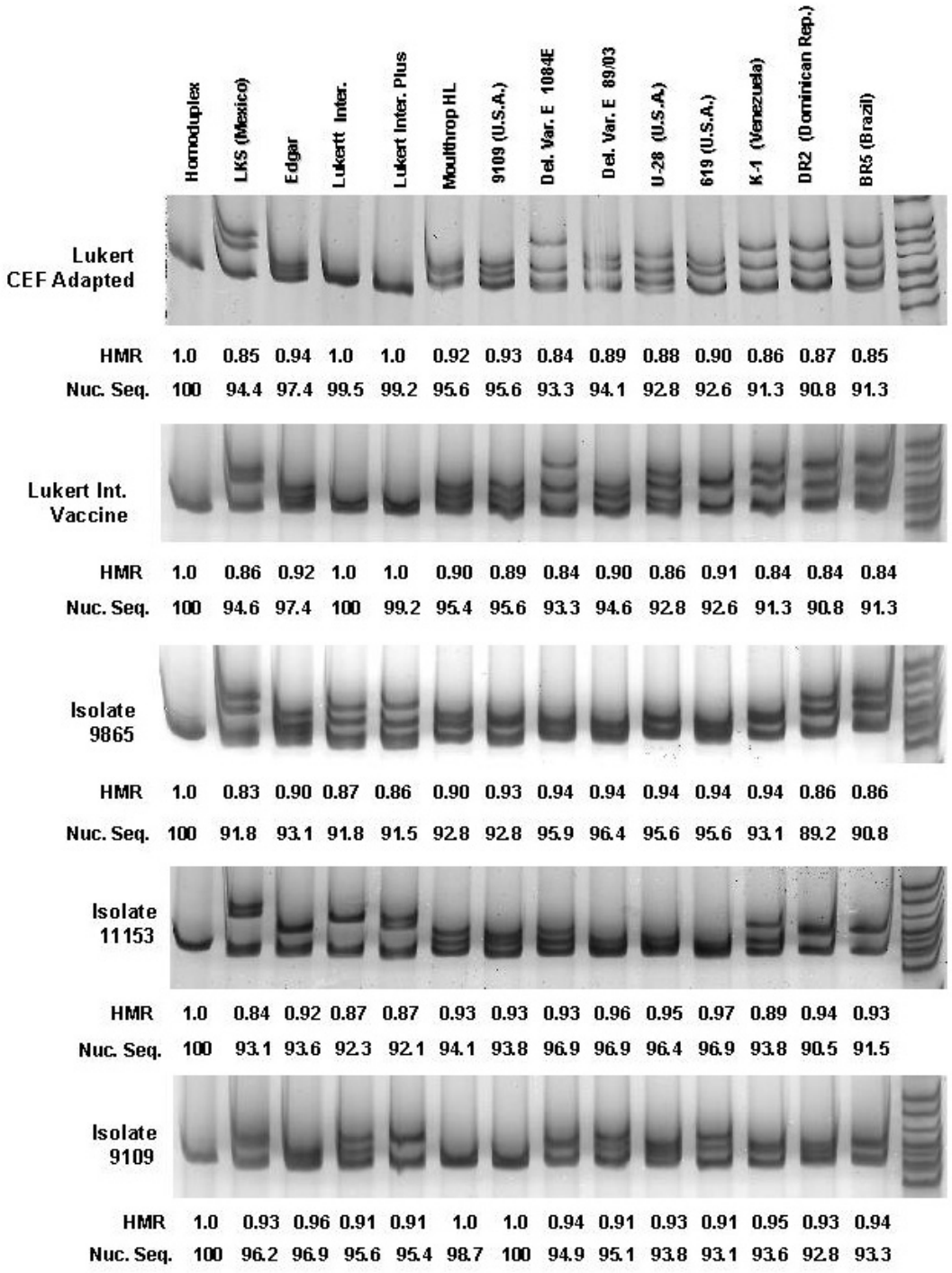

Homoduplex
LKS (Mexico)
Edgar
Lukertt Inter.
Lukert Inter. Plus
Moulthrop HL
9109 (U.S.A.)
Del. Var. E 1084E
Del. Var. E 89/03
U-28 (U.S.A.)
619 (U.S.A.)
K-1 (Venezuela)
DR2 (Dominican Rep.)
BR5 (Brazil)
Lukert CEF Adapted
HMR 1.0 0.85 0.94 1.0 1.0 0.92 0.93 0.84 0.89 0.88 0.90 0.86 0.87 0.85
Nuc. Seq. 100 94.4 97.4 99.5 99.2 95.6 95.6 93.3 94.1 92.8 92.6 91.3 90.8 91.3
Lukert Int. Vaccine
HMR 1.0 0.86 0.92 1.0 1.0 0.90 0.89 0.84 0.90 0.86 0.91 0.84 0.84 0.84
Nuc. Seq. 100 94.6 97.4 100 99.2 95.4 95.6 93.3 94.6 92.8 92.6 91.3 90.8 91.3
Isolate 9865
HMR 1.0 0.83 0.90 0.87 0.86 0.90 0.93 0.94 0.94 0.94 0.94 0.94 0.86 0.86
Nuc. Seq. 100 91.8 93.1 91.8 91.5 92.8 92.8 95.9 96.4 95.6 95.6 93.1 89.2 90.8
Isolate 11153
HMR 1.0 0.84 0.92 0.87 0.87 0.93 0.93 0.93 0.96 0.95 0.97 0.89 0.94 0.93
Nuc. Seq. 100 93.1 93.6 92.3 92.1 94.1 93.8 96.9 96.9 96.4 96.9 93.8 90.5 91.5
Isolate 9109
HMR 1.0 0.93 0.96 0.91 0.91 1.0 1.0 0.94 0.91 0.93 0.91 0.95 0.93 0.94
Nuc. Seq. 100 96.2 96.9 95.6 95.4 98.7 100 94.9 95.1 93.8 93.1 93.6 92.8 93.3

Fig. 5.2 (Cont.)

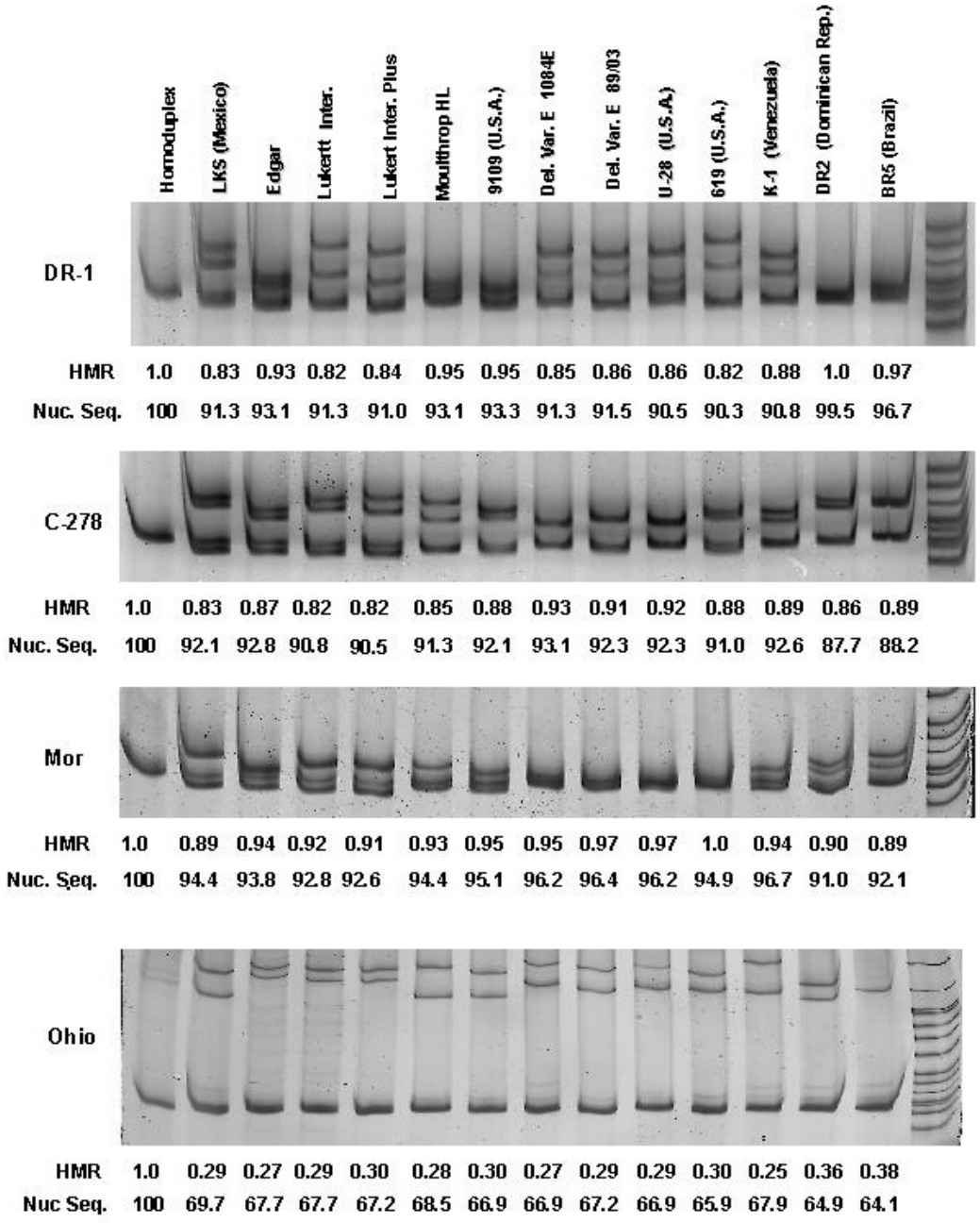
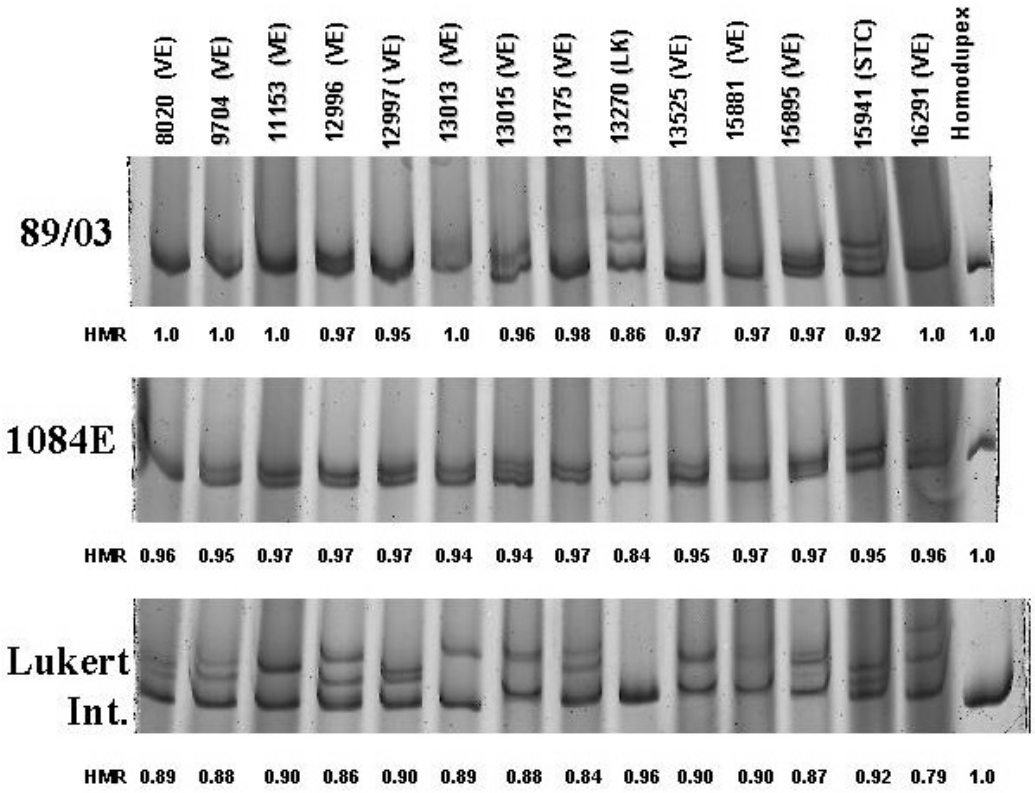

Homoduplex
LKS (Mexico)
Edgar
Lukertt Inter.
Lukert Inter. Plus
Moulthrop HL
9109 (U.S.A.)
Del. Var. E 1084E
Del. Var. E 89/03
U-28 (U.S.A.)
619 (U.S.A.)
K-1 (Venezuela)
DR2 (Dominican Rep.)
BR5 (Brazil)
DR-1
HMR 1.0 0.83 0.93 0.82 0.84 0.95 0.95 0.85 0.86 0.86 0.82 0.88 1.0 0.97
Nuc. Seq. 100 91.3 93.1 91.3 91.0 93.1 93.3 91.3 91.5 90.5 90.3 90.8 99.5 96.7
C-278
HMR 1.0 0.83 0.87 0.82 0.82 0.85 0.88 0.93 0.91 0.92 0.88 0.89 0.86 0.89
Nuc. Seq. 100 92.1 92.8 90.8 90.5 91.3 92.1 93.1 92.3 92.3 91.0 92.6 87.7 88.2
Mor
HMR 1.0 0.89 0.94 0.92 0.91 0.93 0.95 0.95 0.97 0.97 1.0 0.94 0.90 0.89
Nuc. Seq. 100 94.4 93.8 92.8 92.6 94.4 95.1 96.2 96.4 96.2 94.9 96.7 91.0 92.1
Ohio
HMR 1.0 0.29 0.27 0.29 0.30 0.28 0.30 0.27 0.29 0.29 0.30 0.25 0.36 0.38
Nuc Seq. 100 69.7 67.7 67.7 67.2 68.5 66.9 66.9 67.2 66.9 65.9 67.9 64.9 64.1

Figure 5.3


8020 (VE)
9704 (VE)
11153 (VE)
12996 (VE)
12997 (VE)
13013 (VE)
13015 (VE)
13175 (VE)
13270 (LK)
13525 (VE)
15881 (VE)
15895 (VE)
15941 (STC)
16291 (VE)
Homodupex
89/03
HMR 1.0 1.0 1.0 0.97 0.95 1.0 0.96 0.98 0.86 0.97 0.97 0.97 0.92 1.0 1.0
1084E
HMR 0.96 0.95 0.97 0.97 0.97 0.94 0.94 0.97 0.84 0.95 0.97 0.97 0.95 0.96 1.0
Lukert Int.
HMR 0.89 0.88 0.90 0.86 0.90 0.89 0.88 0.84 0.96 0.90 0.90 0.87 0.92 0.79 1.0

CHAPTER VI

TISSUE TROPISM OF TWO INFECTIOUS BURSAL DISEASE VIRUS FIELD ISOLATES IN COMMERCIAL BROILERS EVALUATED BY *IN SITU* HYBRIDIZATION[1]

[1]Banda, A., P. Villegas, J. El-Attrache and C. Brown. To be submitted to Avian Diseases.

SUMMARY

The tissue tropism of two infectious bursal disease (IBDV) field isolates was studied in commercial broilers using *in situ* hybridization (ISH). Two IBDV field isolates one identified as 9865, which is closely related by nucleotide sequence analysis to Delaware variant E strain, and isolate 9109 which is related to the standard challenge strain (STC), were inoculated orally in broilers at one day or two weeks of age. One group of chickens was inoculated with the previously characterized Edgar strain. Two 698-bp antisense digoxigenin-labeled riboprobes encompassing the VP2 hypervariable region of standard and variant strains were prepared to perform the ISH technique on formalin-fixed, paraffin-embedded tissues. Pathologically, both 9865 and 9109 isolates produced a slight decrease in bursal size in the chickens inoculated at one day of age, while a severe bursal atrophy was observed in the broilers inoculated at two weeks of age. The *in situ* hybridization technique detected the presence of IBDV in the broilers inoculated with both field isolates and the Edgar strain. All three IBDV strains used in this experiment infected the one-day old birds and replicated in bursa even in the presence of maternal antibodies. However, the amount of ISH-positive cells was decreased in one-day old compared with the birds inoculated at two weeks of age, presumably due to high levels of passive immunity in the young birds. There were some differences in the replication sites between isolates. Isolate 9865 was mainly detected in the bursa, whereas isolate 9109 and the Edgar strain were detected in bursa but some ISH positive cells were also detected in thymus, cecal tonsils, spleen, kidney and proventriculus.

Key words: infectious bursal disease virus, field isolates, tissue tropism, commercial broilers, *in situ* hybridization, riboprobe

Abbreviations: CID_{50} = Chicken Infectious Dose 50%; DIG = digoxigenin; DPI = Days postinoculation; HPF = High power field; IBD = Infectious bursal disease; IBDV = Infectious bursal disease virus; ISH = *in situ* hybridization; RNA = Ribonucleic acid; RT-PCR = Reverse transcription- polymerase chain reaction; SPF = Specific-pathogen-free; STC = Standard challenge strain.

INTRODUCTION

Infectious bursal disease (IBD) is a major immunosuppressive condition of young chickens which is widespread around the world. IBD-induced immunosuppression represents a major cause of economical losses to the poultry industry (13). The disease is highly contagious in young chickens. The clinical disease occurs between 3 and 6 weeks of age and clinically the birds appear depressed with ruffled feathers, dehydration, white colored diarrhea, hypothermia and death. The subclinical form of the disease occurs generally in chicks younger than 3 weeks of age, the birds affected by the subclinical form develop a severe immusuppression, which make the birds highly susceptible to other diseases of viral or bacterial etiology (18). Two distinct serotypes of IBDV have been identified. The serotype 1 strains are pathogenic to chickens and the strains belonging to this serotype can be divided on the basis of virulence and antigenic variation into standard or classical strains, antigenic variant strains and very virulent IBDV (vvIBDV) strains (18).

The incidence of the infection in major poultry-producing areas is high; most of the flocks are exposed to the virus during the early stages of life (18). The characteristics of IBDV as well as the clinical manifestations are different around the world. In the United States, the predominant form is the immunosuppressive subclinical disease, caused by variant strains (13, 17). The clinical disease characterized by high mortality is present in several countries in Europe, Asia, Africa (8, 22, 23), and Latin America, where vvIBDV are present (6, 10).

All the IBDV strains cause some degree of bursal atrophy because of lymphoid cell depletion, but there are marked differences between strains in their abilities to induce lesions in other organs such as cecal tonsils, thymus, and spleen. It has been suggested that pathogenicity of field strains may be associated with viral replication in non-bursal lymphoid organs (21). Thus, the use of techniques to detect the virus within lesions is not only helpful for diagnosis, but could also give clues with respect to pathogenicity to properly characterize new IBDV isolates emerging in the field. There are reports using *in situ* hybridization (ISH) for the detection of IBDV in formalin-fixed, paraffin-embedded tissues (12, 15, 19). However, the use of antisense riboprobes for ISH procedures applied to veterinary studies is increasing and the value as a diagnostic or research methods is being recognized (4).

Riboprobe ISH has been used for studying viral infections. The technique provides information similar to other molecular biology techniques such as Southern/northern blotting and polymerase chain reaction (PCR), but has the added advantage of allowing for topologic assessment of nucleic acid localization. ISH, using riboprobes, has been used in pathogenic studies of other animal viral diseases (4). In a

previous study, riboprobe ISH was used to detect sites of viral replication in chickens inoculated with three commercial vaccine pathotypes (Delaware variant A, D78 and Moulthrop). The sites for viral replication were consistent for both standard vaccines, viral replication was detected in bursa, spleen, and thymus. However, the Delaware A vaccine exhibited limited replication in the bursa (19).

The purpose of this study was to use riboprobe ISH assay to determine the tissue tropism of two field isolates of IBDV that were obtained from commercial flocks exhibiting recurrent respiratory problems likely due to immunosuppression.

MATERIALS AND METHODS

Viruses. Isolates 9865, which is closely related by nucleotide sequence analysis to Delaware variant E strain, and 9109, closely related to the Standard Challenge Strain (STC) (2) were isolated from commercial broiler flocks that exhibited recurrent respiratory problems. The pathogenic standard Edgar strain (kindly provided by Dr. Phil Lukert, University of Georgia), was also included for comparison purposes. Both field isolates and the Edgar strain, were maintained by passage in specific-pathogen-free (SPF) chickens, the viral inoculums were prepared from homogenized bursal tissue. For titration purposes, the bursal homogenizates were ten-fold diluted, and the dilutions were inoculated in SPF birds, the bursal atrophy was used as a criteria for evidence of infection. The amount of chicken infectious doses 50% (CID_{50}) present in the viral inoculums were determined by the Reed and Munch method (24). Before inoculation all the viral fluids were treated with chicken anemia virus (CAV) antisera, and the fluids were determined to be free of CAV by nested polymerase chain reaction (PCR).

In vivo trial. One hundred and forty four one-day-old commercial broilers were obtained from a local hatchery. The birds were divided into eight groups of 18 birds each and housed in Horsfall Bauer isolation units under positive pressure. Three groups were orally inoculated with 10^3 CID_{50}/0.2 ml of isolates 9865, 9109 and the Edgar strain, respectively, at one day of age, and three additional groups were inoculated at 2 weeks of age. Two groups of chickens remained as negative uninoculated controls. After inoculation, chickens were examined daily for clinical signs and mortality. At 2, 4, 6, and 8 days postinoculation, four chickens were sampled from each group. Each bird was weighed and humanely euthanatized. The bursa was weighed, and samples of bursa of Fabricius, thymus, spleen, cecal tonsils and proventriculus were harvested and fixed in 10% buffered formalin for histopathology and ISH. The bursa:body weight ratios were calculated and the bursal index was obtained according to the formula described by Lucio and Hitchner (16).

Maternal humoral immunity assessment. Levels of maternal antibodies in sera were determined before the inoculation at one day or two weeks of age by a commercial ELISA test kit (IDEXX, Laboratories, Inc Westbrook, ME).

Histopathology. Portions of bursa, thymus, spleen, cecal tonsils and proventriculus were collected immediately postmortem into 10% buffered formalin and allowed to fix for 24 hours. The tissues were then processed and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. Replicate sections for ISH were sectioned and placed on electrostatically charged slides (Superfrost Plus, Fisher Scientific Pittsburgh, PA). Histologic lesions in bursa of Fabricius such as lymphoid depletion, inflammatory infiltrate, edema, lymphoid follicle atrophy, epithelial hyperplasia and

interfollicular fibrosis were scored according to the following numerical scale: 0 = no lesion, 1 = focal, mild scattered lymphoid depletion, 2 = multifocal, 1/3 to ½ of the follicles have atrophy or depletion of lymphocytes, 3 = diffuse, atrophy of all follicles.

Riboprobe preparation. Bursas from SPF chickens infected with isolate 9865 were harvested after 50 hrs postinoculation, the total RNA was extracted using an acid-guanidium-phenol-chloroform RNA extraction method according to previously published methodology (5). For the Moultroup HL riboprobe, total RNA was extracted directly from dried frozen vaccine (Schering Plough Animal Health, Union, NJ). The resulting RNA was reverse transcribed to cDNA and amplified by a one step reverse transcription-polymerase chain reaction (RT-PCR) technique (2), using two 23-nucleotide primers 5-TCTGCAACAGCCAACATCAACG-3, as forward primer, and 5-TCAGGATTTGGGATCAGCTCGA-3, as reverse primer. This set was designed to amplify a product of 698 bp from nucleotide positions 567 to 1264 according to the numbering system of Bayliss *et al*. 1990 (3), encompassing the hypervariable region of the VP2 gene. The 698 bp-RT-PCR products obtained, were purified by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA), inserted into pCR 2.1 vector and cloned according to manufacturer specifications (Life Technologies, Carlsbad, CA). Recombinant plasmids were linearized by restriction enzymes. The linearized recombinant plasmids were *in vitro* transcribed for 2 hours at 37 C using T7-RNA polymerase (Roche, Indianapolis, IN) and a mixture of nucleotides labeled with digoxigenin (DIG), (DIG RNA Labeling Mix, Roche, Indianapolis, IN) to obtain DIG-labeled antisense RNA. After *in vitro* transcription, the templates were digested using RQ1 Dnase (Promega Biosciences Inc. Madison, WI). DIG incorporation into the

riboprobe was confirmed by dot-blot. The two 698 nucleotide-long DIG-labeled antisense riboprobes, were complementary to the hypervariable region of the VP2 gene from two different antigenic subtypes of IBDV, specifically the Moulthrop strain and the Delaware type variant isolate 9865. The riboprobe made from the Moultroup strain was used for detecting isolate 9109 and the Edgar strain. The riboprobe specific to the isolate 9865 was used to detect the homologous strain. The nucleotide sequences of the RT-PCR products used to prepare the DIG-labeled antisense riboprobes were deposited with GenBank; the accession numbers are AF498630 (isolate 9865) and AF498633 (Moulthrop HL).

***In situ* hybridization**.- Tissue sections were deparaffined in CitriSolv (Fisher Scientific, Pittsburgh, PA) and rehydrated in 0.2 M Tris pH 7.5 with 0.1 M glycine, and digested with 35 μg/ ml proteinase K for 15 minutes at 37 C in a humid chamber. The *in situ* hybridization procedure was conducted according to previously published protocols (1, 19). Tissues from birds inoculated with isolate 9865 were hybridized *in situ* using a riboprobe prepared from the VP2 hypervariable region of the same isolate. Birds inoculated with isolate 9109 and the Edgar strain were *in situ* hybridized with a riboprobe containing the VP2 hypervariable region from the Moulthrop strain. Tissues from uninoculated control birds were hybridized with either the 9865 or the Moulthrop riboprobes. Following stringent washes, detection of probe was accomplished by incubating with 1:300 antidigoxigening antibody conjugated to alkaline phosphatase (Roche Indianapolis, IN). Substrate/chromogen was indolylphosphate/nitroblue tetrazolium and development progressed for 1-3 hours. Slides were counterstained with hematoxilin and coverslipped with Permount ® (Fisher Scientific, Fair Lawn, NJ) for a

permanent record. The staining was reported as: 0 = no ISH positive cells, 1 = less than five ISH positive cells/high power field (HPF), 2 = Five to 10 ISH positive cells / HPF, and 3 = more than 10 ISH positive cells / HPF.

RESULTS

Clinical signs. In the birds inoculated at one day of age no clinical signs or mortality were observed. Birds inoculated with the Edgar strain at two weeks of age exhibited slight depression, ruffled feathers but no mortality was observed. No clinical signs or mortality was observed in uninoculated control groups.

Maternal immunity to IBDV. Before inoculation at one day of age, the geometric mean antibody titer (GMT) measured by ELISA was 4739, whereas two-week-old chickens exhibited a ELISA titer of 15 before inoculation.

Bursal index. Bursal indices of chickens inoculated at one day and at two weeks of age are shown in Table 6.1. With all three IBDV strains, chickens inoculated at one day of age exhibited a slight decrease in bursal indices through the experimental period compared to the uninoculated control chickens. Some birds inoculated with the Edgar strain exhibited higher bursal indices in comparison with the control group at 8 days postinoculation (dpi). The birds inoculated at two weeks of age with all three strains experienced severe bursal atrophy that was evident at 6 and 8 dpi.

ISH in bursa. Results of ISH in the birds inoculated at one day are presented in Table 6.2. In general, the ISH positive cells were initially distributed in the germinal centers of the lymphoid follicles, appearing in the cortex at later stages. In a few cases, ISH positive cells were observed in the interfollicular connective tissue. Isolate 9109 and

the Edgar strain were detected from the second day postinoculation to 8 dpi, whereas, positive cells were detected beginning at 4 dpi with isolate 9865. The amount of positive bursas and the scores of ISH decreased at 8 days postinoculation. Furthermore, the birds inoculated with isolate 9865 exhibited lower number of ISH positive cells in comparison with isolate 9109 and the Edgar strain. With all the IBDV strains used, increased numbers of ISH positive cells were found in the chickens inoculated at two weeks of age compared to the birds inoculated at one day of age. In the birds inoculated at two weeks of age, there were positive ISH cells at every sampling period. There were the greatest number of ISH positive cells at 6 dpi. The bursal tissues from all uninoculated control birds were consistently negative by ISH.

ISH in thymus. Some sections exhibited ISH positive cells in thymus and these cells were located at the junction between the cortex and medulla. A few ISH positive cells were detected at 4 dpi in the birds inoculated with isolates 9865 and 9109 at one day of age, and at 8 dpi with the Edgar strain. In the chickens inoculated at two weeks, isolate 9109 was detected in the thymus in two birds at 2 and 4 dpi, whereas isolate 9865 was detected in one bird at 8 dpi.

ISH in spleen. Only one bird inoculated at one day with isolate 9109 showed positive cells in the white pulp, in few centers of nonencapsulated diffuse lymphoid tissue at 4 dpi. No birds inoculated at two weeks exhibited ISH positive cells in spleen.

ISH in cecal tonsils. The presence of positive ISH in lamina propria cells of cecal tonsils was observed at 4 dpi and 8 dpi in birds inoculated at one day of age with the Edgar strain. From the birds inoculated at two weeks of age, a small number of positive ISH cells were detected with isolates 9865, 9109 and the Edgar strain (Fig 6.2A) at 8 dpi.

ISH in Proventriculus. Birds inoculated at one day of age, exhibited few ISH positive epithelial cells at 6 dpi with isolate 9109, and with the Edgar strain at 8 dpi (Fig 6.2B). In the chickens inoculated at two weeks, only the Edgar strain was detected in the proventriculus at 2 dpi and 4 dpi.

ISH in kidney. The ISH-positive cells in kidney were distributed as scattered clusters of tubular cells. In the birds inoculated at one day of age, isolate 9109 was detected in kidney at 2 dpi and 6 dpi. Four birds inoculated with the Edgar strain exhibited few cells at 6 dpi (Fig. 6.2C) and at 8 dpi. In the birds inoculated at two weeks of age, isolate 9109 was detected at 4 dpi in two birds.

Histopathology. Results of lymphoid depletion by histopathology are shown in Table 6.3. Predominantly mild lymphoid depletion was observed in the birds inoculated at one day of age. The Edgar strain induced lymphoid depletion beginning at 4 dpi. All three viruses induced lesions at 6 and 8 dpi. Mild mononuclear inflammatory infiltrate was observed with the Edgar strain at 4 dpi, and with isolate 9865 at 6 dpi. Epithelial hyperplasia was observed with isolate 9109 at 4 dpi and at 6 dpi, whereas moderate epithelial hyperplasia was observed with the Edgar strain and isolate 9865 at 6 and 8 dpi respectively.

In the chickens inoculated at two weeks of age, the lymphoid depletion was more severe in comparison to the birds inoculated at one day of age. The lymphoid depletion peaked at 6 dpi for isolate 9865 and at 8 dpi for both isolate 9109 and the Edgar strain. Mild inflammatory infiltrate was observed only in the birds inoculated with isolate 9865 at 4 dpi and 6 dpi. However, edema was observed more frequently, beginning at 2 dpi in

the birds inoculated with isolate 9109 and was also observed at 4 and 6 dpi with both 9865 and 9109 isolates.

DISCUSSION

The inoculation of the IBDV isolates 9865, 9109 and the Edgar strain in commercial broilers either at one day or at two weeks of age resulted in a subclinical disease similar to that observed under field conditions. Most of the chickens inoculated with the two isolates did not exhibit severe clinical signs or mortality, however, those inoculated with the Edgar strain at two weeks of age, showed slight and transient depression, and ruffled feathers. The birds inoculated at one day of age exhibited some decrease in bursal size, whereas the birds inoculated at two weeks developed severe bursal atrophy.

Both IBDV isolates 9865 and 9109 were obtained from commercial broiler flocks exhibiting poor performance and recurrent respiratory problems. By nucleotide sequence analysis, isolate 9865 is closely related to the Delaware variant E, whereas isolate 9109 is closely related to the STC strain, and exhibited the presence of a site for the restriction endonuclease *Ssp* I (2). The presence of the restriction site for *Ssp* I has been considered a molecular marker characteristic of vvIBDV and is related to the amino acid isoleucine at residue 294, which is characteristic of vvIBDV (10, 11, 14). In experimental conditions, *Ssp* I-positive IBDV strains have induced severe clinical signs with high morbidity (100%) and mortality (45%) with the presence of severe bursal hemorrhages, congestion and paint brush hemorrhages in the breast, thigh and legs, as well as hemorrhages at the junction of the proventriculus and gizzard (9). These lesions were not observed in the

chickens inoculated with this isolate, suggesting that the sole presence of the *Ssp* I restriction site should not be used as indicator of pathogenicity. Thus, other genetic characteristics may play a role in determining the pathogenicity of IBDV strains. The clinical significance of the presence of *Ssp* I-positive IBDV field strains in the United States is not completely clear, thus, further research is needed.

The main replication site for the three strains used in this experiment was the bursa of Fabricius, however, positive ISH cells were detected in other organs in the birds inoculated either at one day or two weeks of age with isolate 9109 and the Edgar strain. The replication of variant isolate 9865 was more restricted to the bursa, whereas the replication in other lymphoid and non-lymphoid tissues was almost negligible. These differences in tissue tropism between standard and variant strains are similar to that observed by Sellers *et al*. (2001), where the hybridization was less extensive and limited to the bursa in the chickens inoculated with Delaware variant A in comparison with the birds inoculated with the standard vaccines strains D78 and Moulthrop (19). Elankumaran *et al* (2002) described that inoculated birds inoculated with the Delaware variant E were consistently positive for IBDV RNA in the bursa of Fabricius, cecal tonsils and bone marrow by RT-PCR, but spleen and thymus yielded virus-specific RNA occasionally (7). Finally, the early studies on Delaware variant A by Sharma *et al*., (1989), demonstrated that although both standard Moultroup and Delaware A strains caused a rapid bursal atrophy, Moultroup strain produced well-pronounced thymic lymphocyte necrosis, whereas the thymus remained unaffected in chickens inoculated with variant A (20). The presence isolate 9109 and Edgar strain in the tubular cells in kidney is important may be important in terms of viral shedding.

However, the effect of maternal antibodies on the replication of isolate 9865 has to be considered. Possibly their specificity limited more effectively the replication of isolate 9865 in comparison with the other two strains studied. The protective effect of passive immunity by maternal antibodies has been previously documented (16). In this study, all the three IBDV strains may have overcome the passive immunity present at one day of age, since IBDV was detected in the bursa and other organs such as thymus, kidney and proventriculus. Maternal antibodies may have exerted a protective effect, since in the birds inoculated at one day of age, only slight decrease in the bursal size was observed, whereas severe bursal atrophy was evident in the chickens inoculated at two weeks of age. Furthermore, higher amount of ISH positive cells in bursa were observed in the birds inoculated at two weeks, where maternal antibodies had decreased considerably. Histopathological findings in the bursa of Fabricius also confirmed that the lymphoid depletion was more severe in the chickens challenged at two weeks in comparison with the chickens inoculated at one day of age.

To our knowledge, this is the first report on the use of DIG labeled antisense riboprobes to detect and determine the tissue tropism of IBDV field isolates inoculated under experimental conditions in commercial broilers. By the use of riboprobe ISH, valuable and complete information on the tissue tropism and pathogenic properties of the emerging strains of IBDV can be obtained.

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ACKNOWLEDGEMENTS

We thank Drs. James Stanton and Erica Behling-Kelly for their valuable technical assistance.

Table 6.1. Results of bursal index[A], at indicated days postinoculation, of commercial broilers experimentally infected with isolates 9865, 9109 and the Edgar strain.

		Days after inoculation			
	IBDV Strain	**2**	**4**	**6**	**8**
One-day inoculation	**Control**	1.0	1.0	1.0	1.0
	9865	0.78	0.74	0.89	0.93
	9109	0.80	0.79	0.84	0.88
	Edgar	0.81	0.69	0.76	1.26
Two-week inoculation	**Control**	1.0	1.0	1.0	1.0
	9865	1.0	1.0	0.50	0.52
	9109	0.94	1.0	0.46	0.44
	Edgar	1.14	1.0	0.69	0.79

[A] Bursal index calculated according to Lucio *et al*. (16)

Table 6.2. Results of *in situ* hybridization of bursal tissues taken from commercial broilers experimentally infected at one day or two weeks of age with isolates 9865, 9109 and the Edgar strain.

	IBDV Strain	Days after inoculation: 2		4		6		8	
		Positive/Total	Average score[A]	Positive/Total	Average score	Positive/Total	Average score	Positive/Total	Average score
One-day inoculation	**9865**	0/4	0.0[A]	2/4	0.5	1/4	0.25	1/4	(0.25)
	9109	4/4	1.0	3/4	0.75	2/4	0.5	1/4	(0.25
	Edgar	1/3	0.3	1/4	0.25	4/4	1.0	1/4	(0.25)
Two-week inoculation	**9865**	3/4	0.75	3/4	1.0	4/4	1.5	4/4	(1.0)
	9109	3/4	1.5	3/4	1.0	4/4	1.0	2/4	(0.75)
	Edgar	1/4	0.25	2/4	0.5	4/4	1.25	3/4	1.25)

[A] Average score: 0 = No ISH positive cells, 1 = less than five ISH positive cells/high power field (HPF), 2 = five to ten 10 ISH positive cells / HPF, and 3 = more than ten ISH positive cells / HPF.

Table 6.3. Scores of lymphoid depletion by histopathology of bursal tissues, taken from commercial broilers experimentally infected at one day or two weeks of age with isolates 9865, 9109 and the Edgar strain.

		Days after inoculation							
	IBDV Strain	**2**		**4**		**6**		**8**	
		Positive/Total	Average score[A]	Positive/Total	Average score	Positive/Total	Average score	Positive/Total	Average score
One-day inoculation	**Control**	0/3	0.0	0/4	0.0	0/4	0.0	3/4	0.75
	9865	0/4	0.0	0/4	0.0	2/4	0.5	3/4	0.75
	9109	0/4	0.0	0/4	0.0	3/4	1.0	2/4	0.5
	Edgar	0/4	0.0	2/3	1.3	4/4	1.75	3/4	0.75
Two-week inoculation	**Control**	4/4	1.0	1/4	0.25	3/4	0.75	4/4	1.0
	9865	4/4	1.5	4/4	1.75	4/4	3.0	4/4	2.75
	9109	4/4	1.25	4/4	1.5	4/4	2.5	4/4	3.0
	Edgar	4/4	1.0	3/4	0.75	4/4	1.75	4/4	2.5

[A] Average score: 0 = No lesion; 1 = mild; 2 = moderate, 3 = severe

Figure Legends.

Fig. 6.1. Riboprobe *in situ* hybridization (ISH) of bursa of Fabricius in commercial broilers experimentally infected with infectious bursal disease virus. (A) Cells showing positive ISH distributed in lymph follicles in the bursa, from a bird inoculated at two weeks with isolate 9865, 4 dpi 10X; (B) Severe lymphoid depletion, the intrafollicular epithelium is hyperplastic (arrow) with positive ISH cytoplasm cells. Bird inoculated at two weeks of age with isolate 9109, 6 dpi, 40X; (C) Abundant number of positive cells, from a bird inoculated at two weeks with the Edgar strain, 6 dpi, 10X: D) Detail of lymphoid follicle from photograph 1C 40X.

Fig. 6.2. A) *In situ* hybridization positive cells in cecal tonsil lymphoid follicles from a chicken inoculated at two weeks with the Edgar strain, 8 dpi 40X; B) Epithelial cells with positive ISH in proventriculus, from a chicken inoculated with Edgar strain at one day of age, 8 dpi 40X; C) Kidney, glomerular and tubular cells positive to ISH from a chicken inoculated with the Edgar strain at one day of age, 8 dpi 40X.

Figure 6.1

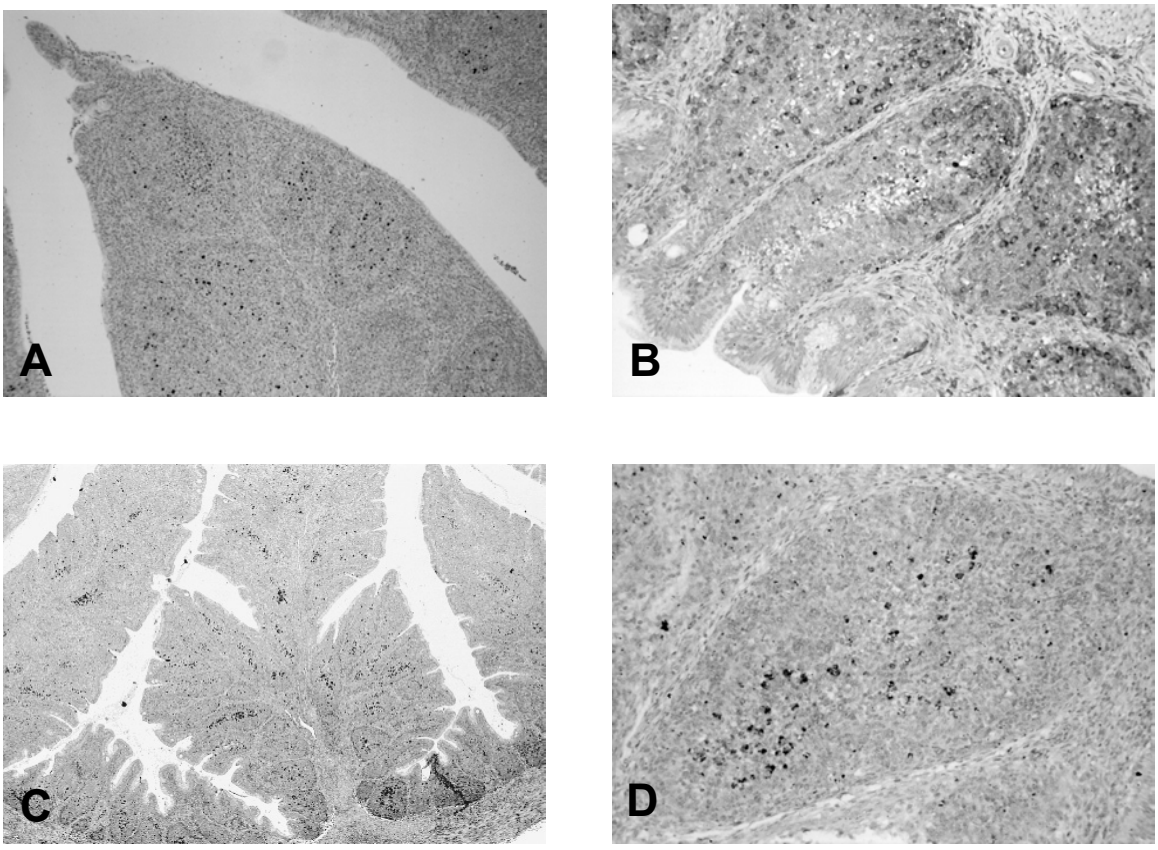
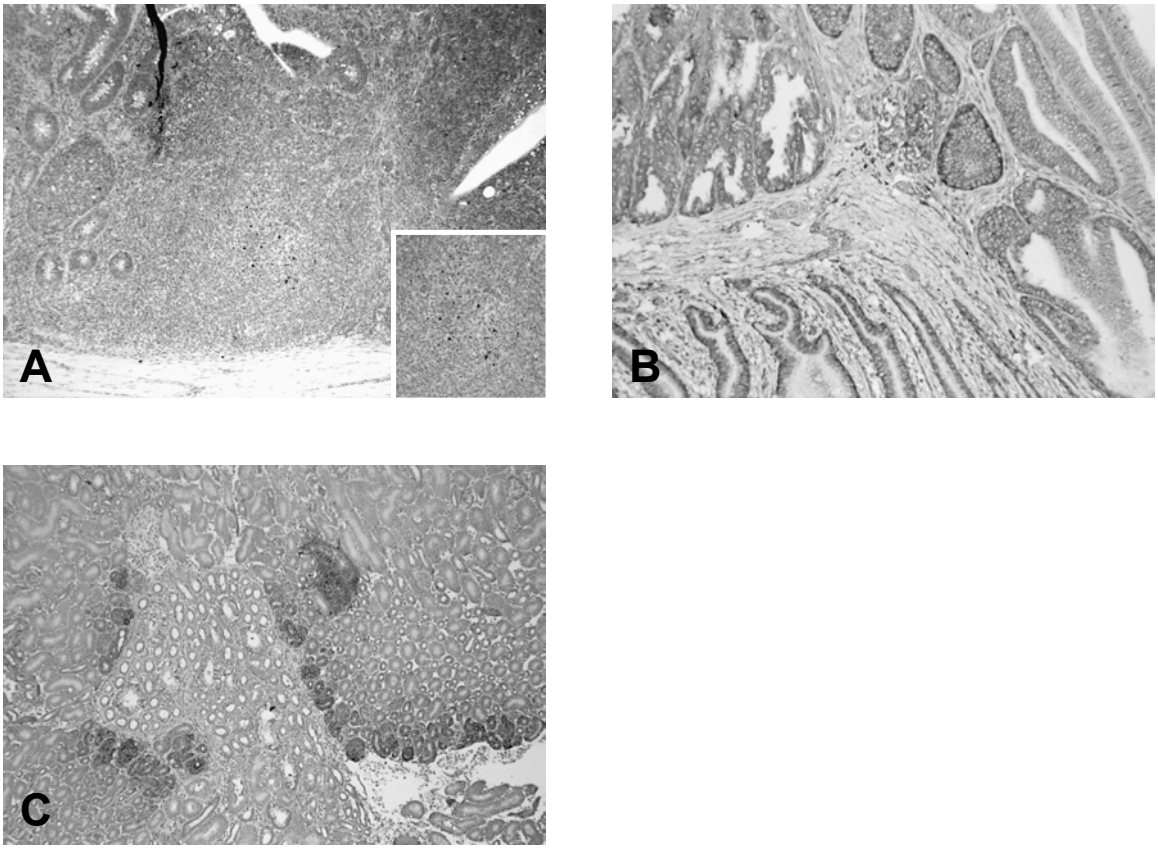


Figure 6.2



CHAPTER VII

DISCUSSIONS AND CONCLUSIONS

Different molecular approaches were applied to the detection and characterization of IBDV strains present in the field. Such molecular techniques included the reverse transcriptase-polymerase chain reaction/restriction fragment length polymorphism, (RT-PCR/RFLP), nucleotide and amino acid sequence analysis, heteroduplex mobility assay (HMA) and *in situ* hybridization (ISH).

Detection and characterization of IBDV by RT-PCR RFLP

The structural viral protein 2 (VP2) of IBDV is the major capsid protein and has been recognized as the major antigen that induces neutralizing antibody response in the host. The VP2 sequence is conserved with exception of the central area between residues 206 to 305, where the majority of amino acid differences among IBDV strains appear. This area has been denominated the variable domain or variable region. Most of the genotyping techniques for IBDV are based on the analysis of this area of the viral genome.

In the present study, an RT-PCR technique followed by a RFLP analysis was applied successfully to genotype IBDV strains present in United States and Latin America. The RT-PCR technique was designed to amplify a 248-bp fragment of the IBDV-VP2 gene from bursal samples. The fragment encompassed the variable region of

VP2. The RFLP was based on the use of the RE *Dra* I, *Sac* I, *Taq* I, *Sty* I, *Bst* NI, and *Ssp* I.

Most of the field IBDV detected in the Eastern part of the United States exhibited genotypes similar to the Delaware variant E (73.3%). This data agrees with previously published data by Snyder *et al*. (1992) using monoclonal antibodies, and by Jackwood *et al*. (1997) with RT-PCR/RFLP. However, after nucleotide and amino acid sequence analysis of six recent IBDV isolates of these, differences with the sequence of the Delaware variant E previously reported by Dr. Vakharia (1994) were observed.

The amino acids lysine 249 and serine 254 are conserved in the Delaware variants, and they are considered identifiable signatures for these IBDV strains. Amino acid sequence analysis of the VP2 region of six isolates demonstrated that serine at position 254 was substituted by asparagine. This change was consistently observed in the six variant isolates analyzed. Both amino acids, serine and asparagines are polar, but an eventual change in the protein folding and antigenic properties may not be ruled out.

Potential antigenic sites within the predicted amino acid hypervariable region were compared by calculating the "antigenic index". The antigenic index is an algorithm that reflects the influence on the antigenicity of several different parameters such as hydrophilicity, surface probability, backbone flexibility, and secondary structure of the amino acids. The antigenic index of the six variant isolates was compared with the Delaware E, and no differences in the patterns were observed. However, to characterize the antigenic properties of these isolates, serological studies such as serum virus neutralization test or ELISA are needed. These changes from the original sequence of the variant E indicate that the IBDV strains are still undergoing genetic selection in the field.

Further studies should be pursued to understand the evolutionary changes occurring in the IBDV populations present in the field.

The isolate identified as 9109 was obtained from a broiler flock with recurrent respiratory problems and poor performance. After nucleotide sequence analysis, isolate 9109 was classified as a standard strain closely related to the standard challenge strain (STC). However, this isolate exhibited a unique RFLP pattern with the presence of the restriction site for the enzyme *Ssp* I. The presence of the restriction site for *Ssp* I has been characteristic of very virulent IBDV (vvIBDV), and this feature has been used as a molecular marker for identification of such strains. The presence of the site for the enzyme *Ssp* I, corresponds to a nonsynonimous substitution that results in the substitution of leucine by isoleucine. This amino acid substitution is one of the three substitutions consistently observed in vvIBDV.

Under experimental conditions, *Ssp* I-positive IBDV strains from Europe, Asia and Africa have induced severe clinical signs with high morbidity and mortality. The inoculated birds exhibited severe bursal hemorrhages, congestion and generalized hemorrhages. After conducting clinical and pathological studies, isolate 9109, induced the subclinical form of the Gumboro disease, and bursal atrophy similar to that observed with the antigenic variants. No severe disease or mortality was observed with this isolate. This indicates that not all the *Ssp* I-positive strains have the characteristics of vvIBDV and the diagnostic value of using the presence of the *Ssp* I restriction site for detecting vvIBDV may not be accurate in 100% of the strains. The clinical significance of the presence of *Ssp* I-positive IBDV field strains in the United States is not completely clear.

Seven of the 125 positive samples (5.6%) were not identified with this RFLP system. Most of these samples exhibited very faint DNA bands after the RT-PCR reaction, which indicates that the amount of viral RNA might have been too low in the sample, and the yield of RT-PCR product was not sufficient to allow the detection of the enzyme digestion. This percentage of unclassified samples was minor in comparison with other commercially used molecular assays. The use of several restriction endonucleases, allows for a multitude of RFLP patterns to be analyzed. This minimizes the number of genetically variable IBDVs that do not match an established RFLP. However, nucleotide sequence analysis should be considered as the "gold standard" technique for characterizing IBDV strains that are not classified by RFLP.

A variety of different genotypes determined by RFLP were observed in Latin American countries. The most interesting findings were the vvIBDV detected in Brazil and Dominican Republic, as well as the untypeable IBDV detected in Mexico and Venezuela.

The strains detected in Brazil and the Dominican Republic showed identical RFLP patterns to European vvIBDV strains, as well as high nucleotide sequence identity with these vvIBDV strains detected in several countries of Europe, Asia, and Africa.

The *Ssp* I positive IBDVs detected in Venezuela are particularly interesting. These samples were obtained from broiler flocks showing severe bursal atrophy around 28 days of age. No severe disease or mortality was observed in the birds. The virus from these samples exhibited a RFLP characteristic of the Delaware variant E, (positive digestion with *Sac* I and *Taq* I) but with positive digestion with *Ssp* I. The molecular characteristics of these strains are intriguing because of the presence of *Ssp* I site has

been observed only in standard strains such as vvIBDV strains. This is the first report related to the detection of Delaware-like variant strains exhibiting the *Ssp* I site. Unfortunately, it was not possible to conduct clinical and pathological studies *in vivo* with these viruses, because the samples were inactivated with phenol: chloroform.

There were some IBDV from Mexico that exhibited unique RFLP and it was not possible to classify these samples by RFLP. After phylogenetic analysis of the nucleotide sequences, these strains were grouped together in a separate branch. Their nucleotide sequences were somewhat similar to the nucleotide sequence from isolate 586 from Puerto Rico characterized previously by Jackwood and Smiley (2001).

It is important to comment that the number of samples obtained from Latin American countries analyzed in this study was limited. Further and comprehensive epidemiological studies are needed to draw more realistic conclusions.

Genotyping of IBDV by HMA

An HMA was developed to genotype strains of IBDV. HMA has been used to genotype different viral agents. HMA is an easy and economical method; it may be a useful tool for screening a large number of isolates before nucleotide sequencing. Nucleotide sequence relationships can be drawn from HMA. The HMA described in this study was a suitable method for genotyping IBDV strains. It clearly discriminated between the three main groups of IBDV strains; standard, antigenic variants and vvIBDV strains. HMA was able to detect differences between sequences as low as 10 nucleotide mismatches. The results obtained by HMA were supported by the results obtained by RFLP.

In pairwise HMA the relationship between the PCR amplicons was quantified by the heteroduplex mobility ratio (HMR). The statistical correlations between HMR and nucleotide sequence identities percentages were calculated by the nonparametric Spearman's correlation coefficient. The correlation coefficients ranged from 0.41 to 0.90 depending of the samples analyzed, thus it was determined that there is no linear association between HMR and nucleotide sequence percentage, therefore, HMR should not be used to predict nucleotide identity values.

Minor nucleotide sequence differences between vaccines strains and field isolates were detected. Bursal samples previously characterized as Delaware E by RFLP were compared by HMA with the variant strains 89/03 and 1084E as well with the Lukert strain. The bursal samples were somewhat more related to the vaccine 89/03 than to the 1084E strain, indicating either the chickens were vaccinated with 89/03 or that the IBDV strains resident in these flocks are genetically closer this vaccine strain. Broader differences were observed when pairwise HMA comparisons were performed using the Lukert intermediate vaccine.

Therefore, HMA may be practical for genotyping IBDV present in the flocks, its main advantage over RFLP is that the complete sequence of the PCR amplicons can be analyzed, while with RFLP only some spots in the sequence are analyzed. HMA may be an auxiliary tool for selecting the vaccine strains that are more related to the field strains. However, more research correlating HMA relationships with antigenicity between strains is needed.

Tissue Tropism of IBDV field isolates by ISH

Clinical and pathological studies were conducted in order to compare the pathogenic properties of the *Ssp* I-positive isolate 9109 with one variant isolate (isolate 9865) and the Edgar strain. The inoculation of these IBDV in commercial broilers either at one day or at two weeks of age resulted in a subclinical disease similar to that observed under field conditions.

The ISH technique was applied to detect the tissue tropism of the IBDV strains studied. No mayor differences in the clinical and pathological characteristics were observed between these two isolates. However, the replication of isolate 9865 was more restricted to the bursa, whereas the replication in other lymphoid and non-lymphoid tissues was almost negligible.

Both IBDV field isolates and the Edgar strain overcame the passive immunity present at one day of age, since IBDV was detected by ISH in the bursa and other organs such as thymus, kidney and proventriculus. However, the maternal antibodies may have exerted a protective effect, since in the birds inoculated at one day of age, only slight decrease in the bursal size was observed, whereas severe bursal atrophy was evident in the chickens inoculated at two weeks of age. Furthermore, a higher amount of ISH positive cells were observed in the birds inoculated at two weeks, where maternal antibodies had decreased considerably.

The use of riboprobe ISH offers some advantages over other molecular techniques, and may provide valuable and more complete information about tissue tropism and pathogenicity of the emerging strains of IBDV. The molecular techniques are valuable tools for the identification and characterization of IBDV strains. They can give a

good idea about their antigenic and pathological properties. However, in some cases clinical and pathological studies *in vivo* are necessary to support the results obtained by molecular techniques.