STUDIES ON INFECTIOUS BURSAL DISEASE VIRUS (IBDV) dsRNA EXTRACTED FROM FORMALIN FIXED PARAFFIN EMBEDDED TISSUE

by

MOHAMED MAMDOUH HAMOUD

(Under the Direction of Pedro Villegas and Barry Harmon)

ABSTRACT

In first study, sequencing the hypervariable region of IBDV from formalin fixed paraffin embedded tissues (FFPET) that originated from chickens experiencing immunosuppression; located both in the USA and abroad, allowed accurate strain identification of IBDV. This allows direct correlation between viral identity and tissue lesions. Several new emerging viruses that don’t group with other known IBDV along with a unique variant virus that had 63 nucleotides missing from its hypervariable region were identified.

The second project investigated why some positive acute +4 lesions of FFPET yielded no RT-PCR detectable IBDV RNA. It was hypothesized that different formalin fixation conditions have negative impact on RNA detection from FFPET. To study this, bursas with high viral loads and maximum histological IBDV lesion score of 4 were fixed in formalin under various conditions. Only tissues fixed in formalin with a pH of 7.0, 5 or 10% formaldehyde, storage temperature of 25°C or less, and kept up to 2 weeks in formalin yielded detectable IBDV RNA upon extraction. No RNA could be detected from tissues fixed under extreme temperature, pH or formalin concentrations. Optimal fixation conditions for IHC detection of IBDV were in 10% formalin, pH 7.0 and 4°C, where maximum intensity of immunostaining was observed.
The third project’s goal was to produce a subunit vaccine against IBDV, without manipulation of live or killed viral particles, to protect chicken against IBDV infection. The Edgar strain of IBDV VP2 gene was obtained from frozen bursas, while the hypervariable region of VP2 was obtained from FFPET. The two genes were cloned into *Pichia pastoris* and protein production was confirmed by western blot analysis. Specific pathogen free 1-day old chicks vaccinated with recombinant VP2 showed best protection against 3 week-old challenge with Edgar IBDV. There was no morbidity or mortality in the VP2 vaccinated birds compared to 90% morbidity and 50% mortality in placebo vaccinated birds. Recombinant vaccines don’t totally protect against viral replication in bursa as confirmed by high histopathological lesion scores and immunohistochemical detection of IBDV in infected tissue. This was probably due to the high viral dose and virulence of the challenge virus used.

**INDEX WORDS:** Infectious bursal disease, Chicken, VP2, formalin fixed paraffin embedded tissue, immunohistochemistry, *Pichia pastoris*, recombinant vaccine production, phylogenetic analysis
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DEDICATION

I would like to dedicate this work to my parents, my wife Rasha Tawfik, my two wonderful daughters Salma and Yasmin for their unconditional love and support. I also would like to dedicate this work to my twin brother and two sisters for helping me be the person I am. It is their love and support that has made this work possible. They have put up with me and my pursuit for this doctorate for so long. I hope this dedication can be a simple thank you for all what they have gone through.
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Chapter I

INTRODUCTION

Purpose of the Study

Infectious bursal disease virus (IBDV) is the etiological agent of Gumboro disease or infectious bursal disease (IBD). IBD is an acute highly contagious viral disease of young chickens. This virus causes severe immunosuppression by destroying the B-lymphocytes precursors found within the bursa of Fabricius, followed by bursal atrophy. The virus is ubiquitous, highly stable in the environment and has a tendency to persist in the environment despite thorough cleaning and disinfection, thus IBDV is endemic in most poultry producing areas of the world. IBD is one of the major economically important diseases of poultry worldwide despite wide usage of vaccination programs. Most commercial chickens are exposed to IBDV early in life. In unprotected flocks, the virus causes mortality and immunosuppression. Although mortality can be quite significant, the major economic loss is the ability of IBDV to produce immunosuppression. Immunosuppressed flocks have poor performance which results in reduced economic return (230)

Controlling infectious bursal disease (IBD) and its associated immune suppression is critical to the broiler industry. This control is achieved by either vaccinating breeder hens with conventional live attenuated and/or inactivated IBD vaccines, or by the use of live IBD vaccines in broiler chicks, layers or young pullets to provide active protection against IBDV, or a combination of both strategies may be used.

There is significant antigenic, immunogenic, and pathogenic variation between IBDV strains which determines disease outcome. Some IBDV strains cause an
immunosuppressive, subclinical form of disease with less than 5% mortality, while others can cause a clinical form with up to 100% mortality such as very virulent strains. The clinical signs and the degree of immunosuppression can also vary significantly.

Vaccination is the primary means for control; and thus most efforts for protection against IBDV by the commercial poultry industry are focused on developing efficient vaccination programs. Successful immunization requires reliable IBDV field and vaccine strain characterization.

The first project was designed to detect of IBDV RNA from formalin fixed paraffin embedded tissues from field cases that showed signs of IBDV or immunosuppression. Characterization of extracted RNA allows direct correlation between viral identity and lesions present in infected tissues. Molecular identification of the hypervariable region of VP2 allows for strain identification which when aligned with sequences of vaccine strains will be a tool that can assist veterinarians in choosing which vaccines to include in their control programs against IBDV. This technology has the ability to detect newly emerging viruses that have a unique genomic sequence. For example, one strain of IBDV missing 21 amino acids from the hypervariable region was able to replicate and produce very severe lesions. Detection of unique viruses that differ from available vaccine strains can lead to use of these strains for vaccine development in the future.

Our second goal was to explore optimal RNA extraction from formalin fixed paraffin embedded tissue as well as refine detection of IBDV by use of immunohistochemistry. We sought to investigate the effect of various fixing and storage conditions on RNA extraction and real time RT-PCR identification of IBDV from
paraffin embedded tissues as well as the immunohistochemistry (IHC) detection of IBDV in the infected tissues. Bursa of Fabricius from experimentally exposed chickens with histopathological lesion scores of +3 or more were always positive for extracted RNA and identification of IBDV with real time RT-PCR. However, this was not always the case for bursal tissues that originated from field submissions. Some samples that had acute +4 lesion scores histologically were negative for IBDV with real time RT-PCR. This finding occurred more often in samples submitted from outside the United States than those originating from the United States. The cause of the discordance between severe histologic lesions and negative RT-PCR results is unknown. We hypothesized that differences in sample collection and storage methods might be partially responsible. We assumed that samples are fixed in 10% buffered formalin and kept at room temperature, but pH analysis, and microscopic artifacts suggests otherwise as some of the samples were fixed in 37% formalin with pH 2.4 or were subjected to high temperatures. We experimentally simulated these field conditions in terms of storage time, storage temperature, pH of unbuffered formalin, and different concentrations of buffered formalin to test their effect on RNA extraction from paraffin embedded tissue from a known positive sample.

Our third aim was to produce a recombinant protein or polypeptide from IBDV genomic material extracted from formalin fixed paraffin embedded tissue (FFPET) without actual manipulation of live or killed virus particles. This will allow us to use archival FFPET as a valuable source of genomic material for production of proteins or actual viruses using reverse genetics. Our focus is on protein production and not reverse genetic reproduction of viruses. This method may allow us to produce an efficient subunit
vaccine to IBDV in a system that would enable mass production for vaccination of chickens against IBDV.

We are hopeful our work may lay the foundation for improving current techniques developed for rapid characterization of IBDV field isolates and determining their antigenic properties. Production of a subunit vaccine that provides effective protection against IBDV, will allow differentiation between vaccinated birds and field challenged birds which may provide another tool to controlling this disease by the commercial poultry industry.
Chapter II

Literature Review

Part 1: Infectious Bursal Disease

Infectious bursal disease virus (IBDV) is the etiological agent of Gumboro disease or infectious bursal disease (IBD). IBD is an acute and highly contagious viral disease of young chickens. This virus causes severe immunosuppression by destroying the B-lymphocytes precursors found within the bursa of Fabricius, followed by bursal atrophy. The IBD virus is ubiquitous, highly stable and may persist in the environment despite thorough cleaning and disinfection, thus IBDV is endemic in most poultry producing areas of the world. There are two serotypes of IBDV: 1 and 2. All viruses capable of causing disease in chickens belong to serotype 1, whereas serotype 2 viruses are non-pathogenic for both chickens and turkeys (112, 157). Chickens are the only avian species known to be susceptible to clinical disease and lesions produced by IBDV. Turkeys, ducks and ostriches are susceptible to infection with IBDV but are resistant to its clinical manifestations (148, 158). IBDV has also been isolated from African black-footed and Macaroni penguins and have been serologically identified as serotype 2 IBDV (71), and further confirmed as serotype 2 by molecular identification (115).

IBD is one of the major economically important diseases of poultry worldwide despite wide usage of vaccination programs. Most commercial chickens get exposed to IBDV early in life. In unprotected flocks, the virus causes mortality and immunosuppression. Although mortality can be quite significant, the major economic
concern is the ability of IBDV to produce immunosuppression. Immunosuppressed flocks have poor performance and show reduced economic return (211).

1.1 History:

Infectious bursal disease (IBD) was first reported by Cosgrove in 1957. It was initially recognized as “avian nephrosis”, and the syndrome became known as “Gumboro disease” because the first outbreaks occurred in the town of Gumboro, Delaware, USA. The clinical picture of the syndrome includes white or watery diarrhea, followed by anorexia, depression, trembling, severe prostration, and death. At necropsy, gross pathology findings commonly include dehydration, leg and thigh muscles hemorrhages, urate deposits in kidneys and enlargement of the bursa of Fabricius (37).

Initially avian nephrosis or Gumboro disease was thought to be caused by the Gray strain of infectious bronchitis virus (IBV) because of gross changes in the kidney. This misconception arose because the IBV and IBDV infections were concurrent in many cases and IBDV was difficult to isolate with the available methods at the time of discovery (135). In later studies, Winterfield et al (256), succeeded in isolating the causative agent in embryonating eggs, and later Hitchner proposed the term “infectious bursal disease” for the disease (85).

In 1972, it was reported that IBDV infections at an early age were immunosuppressive (1). The recognition of this immunosuppressive capability of IBDV greatly increased the interest in the control of this disease. The existence of serotype 2 IBDV was reported in 1980 (156).

The Delmarva Peninsula broiler growing area experienced a significant increase in mortality and higher percentage of condemnations in 1984 and 1985. The clinical
syndrome had significant variability, but often was respiratory in nature. Lesions ranged from moderate to severe, with death usually being attributed to *E. coli* infection (38). Rosenberger *et al.* isolated four isolates designated as A, D, G, and E using vaccinated sentinel birds (205). 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 provide complete protection against these four new Delaware isolates. The Delaware isolates, A, D, G and E were designated as antigenic variants and killed vaccines were developed, tested and proven effective against them (205). Currently these and other similar variants are widely distributed in the United States (220, 221). Snyder et al. first described variant viruses as newly emergent viruses due to a major antigenic shift within serotype 1 (221, 223). The terminology given to these newly emergent viruses was “IBDV variants” as they were the result of a major antigenic shift within serotype 1 (224, 221), while the older serotype 1 viruses discovered prior to these newly emergent viruses were called standard or classical strains of IBDV.

Acute IBDV outbreaks exhibiting 30% to 60% mortality in broiler and pullet flocks, respectively, have been commonly reported in Europe since 1987. The first reports were made by Chettle *et al.* (32), and van den Berg *et al.* (243). Some of these acute outbreaks occurred in broiler flocks where appropriate hygienic and prophylactic measures had been taken. Although no antigenic drift was detected, these strains of increased virulence were identified as very virulent IBDV (vvIBDV) strains (242, 243). The European situation has been dominated for a decade by the emergence of vvIBDV strains. These strains have now spread all over the world (57). In the Americas, acute IBD outbreaks due to vvIBDV strains have already been reported in Brazil (46, 93), and
the Dominican Republic (5). To date the terminology given to serotype 1 IBDV is standard or classic, variant, and very virulent IBDV.

**1.2 Etiology:**

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: *Drosophila* X virus or DXV) (49). IBDV is a small, non-enveloped virus whose genome consists of two segmented dsRNA segments (121). These two segments were designated A and B. Other birnaviruses have been isolated from bivalve mollusks such as Tellina virus (235), and Oyster Virus (49, 128), and Japanese eels(138). To date, there has been no report of Birnavirus capable of causing disease in mammals, although dogs have been found to be carriers for vvlIBDV (233).

The virion has a single capsid shell of icosahedral symmetry composed of 32 capsomeres and a diameter of 60 to 70 nm (49, 73, 81, 176). By cryomicroscopy, it was shown that the IBDV capsid is an icosahedron with a $T = 13$ lattice composed of trimeric subunits. The outer face of the particle is composed of 260 trimeric VP2 clusters. Closely apposed to the inside of this protein layer are 200 Y-shaped trimeric VP3 structures (19, 29).

**1.3 Viral genome structure and replication:**

The genome of IBDV is formed by two segments of double-stranded RNA (dsRNA) with the two segments detected by polyacrylamide gel electrophoresis (49, 103). The molecular weight of the double stranded segment A is $2.2 \times 10^6$ Da with a length of approximately 3.2 kb, while the molecular weight of the double stranded
segment B is $1.9 \times 10^6$ Da with a length of approximately 2.8 kb (166, 92).

The larger segment A contains two partially overlapping open reading frames. The first encodes a nonstructural polypeptide of 17 kDa known as VP5, which is not essential for replication in vitro but important for virus-induced pathogenicity (171, 172). The second ORF encodes a 110 kDa polyprotein (NH$_3$-VPX-VP4-VP3-COOH) that is autoproteolytically cleaved into three polypeptides, VPX or pVP2 (48 kDa), VP3 (32 kDa) and VP4 (28 kDa). pVP2 is further processed to produce a polypeptide known as VP2 (38 kDa) (4, 91, 119, 163). VPX, VP2, and VP3 are the major structural proteins that form the virus capsid (19), while VP4 appears to be responsible for the proteolytic maturation of the polyprotein (116, 119, 139).

Segment B encodes VP1, a 95-kDa protein which is the RNA-dependent RNA polymerase responsible for the replication of the genome and synthesis of mRNAs (48, 226). VP1 shares a number of primary sequence features with RNA polymerases from diverse origins (22).

There are direct terminal and inverted repeats at the 5’ and 3’ ends in both genome segments of IBDV 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 (173). 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 (120), which are involved in the processes of RNA replication, translation and encapsidation like other RNA viruses such as poliovirus (215).

The synthesis mechanism of both virus-specific ssRNA and dsRNA during infection with IBDV has not been clearly determined. An RNA-dependent RNA
polymerase has been demonstrated in IBDV (225). Genome-linked proteins have been demonstrated in three different Birnaviruses, (165, 192, 202), indicating that they replicate their nucleic acid by a strand displacement (semi conservative) mechanism (14, 161, 226).

1.4 Viral Proteins:

Four mature viral structural proteins designated VP1, VP2, VP3 and VP4 are detected in infected cells (9, 47, 176). A non-structural protein designated VP5 has been identified, the function of this protein is under investigation, and was found to be non-essential for viral replication in vitro (171, 172). Lombardo et al. reported that VP5 plays an important role in in vivo virus egression and pathogenesis (146).

During the processing of the polyprotein precursor NH3-pVP2-VP4-VP3-COOH into pVP2, VP3 and VP4, the existence of two sites, essential for the cleavage of the VPX-VP4 and VP4-VP3 precursors respectively, has been reported (208). These sequences are highly conserved among IBDV strains from both serotypes 1 and 2.

VP1, the RNA-dependent RNA polymerase of the virus, is present in small amounts in the virion, both as a free polypeptide and covalently linked to the two double-stranded RNA genomic segments as a genome-linked protein (VPg) (17, 119, 164). It plays a key role in the encapsidation of the viral particles (147). Boots et al. reported that enhanced virulence of vvIBDV is partly determined by its B-segment which encodes VP1 (17), particularly due to the presence of eight conserved amino acid differences (16).

VP2 is the most abundant among IBD viral proteins, accounting for 51% of the virus proteins of the serotype 1 IBDVs. This protein is the major component of the viral capsid, and is the host-protective antigen; with most of the neutralizing epitopes
occurring in the hypervariable region (amino acids 224–314) of VP2 (17) i.e. VP2 contains the antigenic region responsible for the induction of neutralizing antibodies and for serotype specificity (60). Rescue of recombinant IBDVs from chimeric A-segments revealed that amino acids (at positions 253 and 284) within the hypervariable region are responsible for the cell culture adaptation (18, 20, 140, 169, 247). 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 (63).

VP3 which is a structural protein also, accounts for 40% of the virion proteins (121). VP3 is found only on the inner surfaces of virus-like particles (152). This protein plays a role in the assembly of viral particles, and packaging of the viral genome (33, 147, 153, 228). Chevalier et al. found out that what actually controls capsid assembly of Infectious Bursal Disease Virus is the last C-terminal residue of VP3, glutamic acid 257 (33). VP3 is a group-specific antigen that is recognized by non-neutralizing antibodies, some of which cross-react with both serotypes 1 and 2 (11). It is likely that the outer subunits in the viral capsid consists of VP2, carrying the dominant neutralizing epitope, and that the inner trimers consist of protein VP3, as it was shown that the IBDV capsid is an icosahedron with a $T = 13$ lattice composed of trimeric subunits. The outer face of the particle is composed of 260 trimeric VP2 clusters. Closely apposed to the inside of this protein layer are 200 Y-shaped trimeric VP3 structures (19, 29).

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 (15). The integrity of VP4 is essential for the
proteolytic processing of the polyprotein (51, 116) and either itself, or through proteins under its control, plays a role in the trans-activation of the synthesis of VP1 (15).

VP5 is a class II membrane protein with a cytoplasmic N-terminus and extracellular c-terminus domain (146). VP5 was the last IBDV protein identified. This protein is not essential for IBDV replication in vitro or in vivo (171), however, it plays an important role in viral pathogenesis as a reverse genetics system with VP5 knockout mutant did not cause bursal lesions after experimental infections (264). VP5 expression results in the alteration of cell morphology, the disruption of plasma membrane and drastic reduction of cell viability. VP5 acts as a death protein, modifying cell membrane and promoting the release of newly formed IBD virions (146).

1.5 Natural and Experimental Hosts:

For many years, the chicken (Gallus gallus) was considered the only species in which natural infections occurred (76), where all breeds of chicken are affected. White Leghorns exhibit the most severe reactions and have the highest mortality rate. Turkeys (Meleagris gallopavo) may be infected with serotypes 1 and 2 but do not exhibit clinical signs of the disease (113, 158). There is, however, considerable potential for immunosuppression or interaction with other diseases under commercial conditions in turkeys (134). Serotype 2 was originally identified in clinically unaffected adult turkeys in Ireland (156). Currently chickens and turkeys are considered the natural hosts of the virus (148). Ducks (Cairina moschata) may develop IBDV infection and antibodies are detectable by serum virus neutralization, but neither gross nor microscopic lesions have been observed. Antibodies have been detected in wild birds like weavers (Ploceus cucullatus), rooks (Corvus frugilegus) and finches (Uraeginthus bengalus) (27, 175) as
well as in Antarctic adelie penguins, but the source of IBDV exposure has not been defined (65). The IBDV in African black-footed and Macaroni penguins has been serologically identified as serotype 2 IBDV (71), and was confirmed molecularly as serotype 2 IBDV by using reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing (115). Guinea fowl (*Numida meleagris*) inoculated with the virus did not develop lesions or antibodies (184, 186). A serotype 1 virus was isolated from two 8-week-old ostrich chicks (*Struthio camelus*) that had lymphocyte depletion in the bursa of Fabricius, spleen, and/or thymus (148). van den Berg experimentally inoculated pheasants (*Phesanus colchicus*), partridges, quails, and guinea fowl with vvIBDV and reported no clinical signs or lesions in these species (240).

1.6 Transmission:

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. There are neither experimental data nor naturally-occurring observations to suggest that IBDV is transmitted vertically by the transovarian route (148).

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 (12); IBDV is very persistent in the environment of a poultry house. Houses from which infected birds were removed, were found to harbor infective virus 54 and 122 days later (12). The lesser mealworms, *Alphitobius diaperinus* may be reservoir hosts (155, 219). IBDV has also been isolated from *Aedes vexans* mosquitoes (89), and antibodies against IBDV have been detected in rats found on poultry farms (185). No further evidence
supports the conclusion that either mosquitoes or rats act as vectors or reservoirs of the virus. Dogs have been found to be carriers for vvIBDV (233).

**1.7 Resistance to chemical and physical agents:** Without proper disinfection the virus can survive on the premises for more than 4 months. The virus is extremely resistant to disinfection and pH changes between pH 2-12. The virus remains viable following incubation for 5 hours at 56 °C and 30 min at 60 °C. The infectivity can be reduced by treatment with 0.5% formalin for 6 hours or 1% formalin for 1 hour (13).

**1.8 Clinical forms of IBDV:**

The classical form, as described since the early 1960s, is caused by the classic moderately virulent strains of IBDV. The incubation period of IBD ranges from 2 to 4 days post 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 also produces acute onset of depression, reluctance to move, ruffled feathers, white or watery diarrhea, soiled vent feathers with urates, trembling, closed eyes and prostration. The feed intake is depressed but water consumption may be elevated. Severely affected birds become dehydrated and in terminal stages of the disease have subnormal temperature, after which the bird dies (37).

The variant form, described initially in the United States, is caused 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 (220).

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 usually approaching 100% and mortality reaching 20-30% in broilers and 60% in pullets over a 7-day period (32), (179). Several isolates of vvIBDV caused mortality rates of 90-100% in 4-week old susceptible leghorn chickens (243).

1.9 Gross lesions:

Chickens which die from IBD infection as a primary cause show dehydration of the subcutaneous fascia and musculature of the thigh, inguinal and pectoral areas (37, 148). Age-related coagulation disorders coincide with the mortality and lesion severity. Insignificant changes in birds infected with field IBDV isolates at 17 days were observed compared to severe effects on birds infected at 42 days (228). Petechial hemorrhages occur occasionally in the mucosa of the intermediate zone between the proventriculus and the gizzard, as well as on leg, thigh and breast muscles. There is increased mucus in the intestine. Kidneys show enlargement and pallor with accumulation of crystalline urate in tubules (37). Renal lesions were more prominent in early outbreaks in the United States, perhaps due to co-infection with nephropathogenic strains of avian infectious bronchitis (Gray strain) (148).

The bursa of Fabricius is the primary target organ following exposure to IBDV (34). When Cheville studied the bursal weights for 12 days postinfection, he noticed that by the 2nd or 3rd day the bursa has a gelatinous yellow transudate covering the serosal surface giving the bursa a creamy color instead of the normal white color. The bursa also had prominent longitudinal striations on the serosal surface. By the 4th day after the infection there was a doubling in size and weight of the bursa due to edema and hyperemia. By the 5th day the bursa returns to normal weight, but it continues to atrophy,
and from the 8th day forward it is approximately one-third its original weight (148).

Variant strains isolates have been reported that do not induce an acute inflammatory response (205, 213). Although one variant strain (IN) was reportedly able to induce such acute inflammatory response (74). The variant strain’s disease is primarily subclinical resulting in immunosuppression. Field challenge is likely a result of mild or variant strains of IBDV, such as Delaware variant E (107, 224).

Spleenomegaly has been documented, with small gray foci uniformly dispersed through the parenchyma (148, 162). The vvIBDV strains cause greater decrease in thymic weight index and more severe lesions in cecal tonsils, thymus, spleens, and bone marrow when compared to moderately pathogenic strains of IBD virus, but the bursal lesions are similar (148).

1.10 Histopathological lesions:

Histopathological lesions of IBD occur primarily in the lymphoid structures (i.e., cloacal bursa, spleen, thymus, harderian gland, and cecal tonsil). Infection with standard or variant serotype 1 IBDV strains can result in death of bursal B lymphocytes. Degeneration and necrosis of lymphocytes in the medullas of bursal follicles can be detected within one day of infection, and were soon replaced by heterophils, lymphocytes associated with karyorrhexis and pyknosis, and hyperplastic stromal histiocytes. By the third day an inflammatory response with edema, heterophil infiltration, congestion and hemorrhage were present in infections due to 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 were cleared by phagocytosis, cystic cavities develop in the medullary areas of follicles; necrosis and
phagocytosis of heterophils and plasma cells occur; and there may be fibroplasia in interfollicular connective tissue and the covering epithelium becomes infolded and irregular (6, 34, 199). 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 (213). The lack of inflammation associated with variant infections may be due to the variant viruses killing B-lymphocytes by apoptosis instead of necrosis (248).

The development of lesions by IBDV in thymus depends on the pathotype of the virus (94, 231). IBDV-induced cortical thymic lymphocyte depletion is caused by apoptosis (94). The highly pathogenic vvIBDV strains from Europe and Japan are associated with severe thymic lymphocyte loss when compared to less pathogenic strains (231). Although the thymus undergoes marked atrophy and extensive apoptosis of thymic cells during the acute phase of virus infection, there is no evidence that the virus actually replicates in T cells (230). Gross and microscopic lesions in the thymus are quickly resolved and the thymus returns to its normal state within a few days of virus infection (211).

The spleen may have hyperplasia of stromal histiocytes around the adenoid sheath arteries in early stages of the infection, and lymphoid necrosis in the germinal follicles and the periarтерiolar lymphoid sheath by the third day (148). The Harderian gland may also be affected. Normally, this gland is infiltrated and populated with plasma cells as the chicken ages. Infection with IBDV prevents this infiltration (227). In cecal tonsils, there may be acute heterophil inflammation, destruction of lymphocytes, and regeneration on the fifth day after infection (76).
Histological lesions in 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, and also glomerular hypercellularity (76). The liver may have slight perivascular infiltration of monocytes (193).

The vvIBDV strains can cause severe lesions in the cecal tonsils, thymus, and bone marrow (149). Bursal lesions caused by very virulent strains are even more severe, and are characterized by cortical lymphocyte necrosis, depletion of medullary lymphocytes with interstitial inflammation and hyperplasia of epithelial and reticular cells. The thymus, spleen, liver, and bone marrow are also affected (79). Other very virulent strains cause severe lysis of heterophil myelocytes with pyknotic nuclei (94). Thymic cortical lymphocyte necrosis and depletion of lymphocytes are more severe when caused by vvIBDV strains, than lesions caused by classical virulent strains (95).

1.11 Ultrastructural lesions in the bursa of Fabricius:

Naqi and Millar (174) followed the sequential changes in the surface epithelium of the bursa of Fabricius of IBDV-infected chicks by scanning electron microscopy. Forty eight hours post inoculation they observed a reduction in number and size of microvilli on epithelial cells. There was gradual loss of the button follicles normally seen at the surface, and by 72 hours, most had involuted. By 96 hours, there were numerous erosions of the epithelial surface. The surface was intact by day 9 postinoculation, but follicles were involuted, leaving deep pits.
1.12 Diagnosis of IBDV:

1.12.1 Differential diagnosis:

Diagnosis of the clinical forms of IBD is based on typical signs of the disease and on the histopathological lesions of the bursa of Fabricius. Differential diagnosis should include velogenic viscerotropic Newcastle disease, chicken infectious anemia, vitamin K deficiency and mycotoxicosis. In subclinical and immunosuppressive forms of IBD, Marek’s disease, chicken anemia and mycotoxicosis should be considered (134, 148). Due to the immunosuppressive nature of IBDV, it may serve as a predisposing factor to other conditions, such as gangrenous dermatitis, hemorrhagic aplastic anemia syndrome, inclusion body hepatitis, and respiratory disease. Involvement of IBDV in disease complexes of these types is usually diagnosed retrospectively by demonstrating persistent bursal lesions or serologic evidence of previous IBDV infection (206).

1.12.2 Histopathological diagnosis:

Diagnosis by histopathology of the bursa is frequently used, since the lesions caused by IBDV infection is well characterized in the bursa (34, 199). 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. Virulence of IBDV strain is measured by a lesion severity score, where a lesion score of 1 represented no lesions, 2 represented mild reduction in overall follicle size, 3 represented moderate reduction in size of follicles, and 4 represented either necrosis or follicle atrophy. Time of infection is calculated based on type of inflammatory cells present or type of inflammatory response seen in tissue.
1.12.3 Virus identification:

For virus identification, the agar gel precipitin (AGP) test can be used to detect IBDV group-specific antigen. The virus-neutralization (VN) test could be used to identify the virus in embryos or in tissue culture after adaptation to these host systems (206). An antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) has been described for detecting and characterizing IBDV isolates (223). Polyclonal antibodies can also be utilized in the AC-ELISA and may be more effective for general screening of tissue samples for IBDV (148). Direct and indirect immunofluorescent assays as well as immunocytochemistry have been shown to be highly reliable for detection of viral antigens in infected tissues. (206).

Strains of IBDV can be differentiated on the basis of pathotype and antigenic configuration. There are at least two distinct serotypes and several antigenic subtypes that can be identified by cross-neutralization and cross challenge tests. Isolates of IBDV may vary in pathogenicity from being nonpathogenic to virulent (108,122, 148).

1.12.4 Serological diagnosis:

Serologically the AGP test can be used to detect and quantitate antibodies in convalescent birds. To quantitate the antibodies, usually two fold serum dilutions are made to obtain an endpoint. The antigen is prepared from bursal homogenates of experimentally infected birds collected 3-6 days postinfection (206). The virus neutralization (VN) test is also used for antibody quantitation. The test is routinely performed in microtiter systems using cell-culture-adapted virus and chicken embryo fibroblasts. Neutralization titers are expressed as the reciprocal of the highest dilution of serum that prevents cytopathic effect. The VN test is more sensitive than the AGP test
and accordingly should be utilized when antibody titers are low or when quantitation of antibody is important (206). But for the past decade enzyme-linked immunosorbent assay (ELISA) has been widely used because it is a sensitive and rapid method. ELISA make it easy to handle large number of samples and only requires small quantities of serum. ELISA results do not always correlate with VN test findings. Using serological techniques it is possible to detect the immunologic response in an outbreak or evaluate vaccination programs (180).

1.12.5 Molecular diagnosis:

Molecular identification of IBDV has been used to diagnose IBDV. The reverse transcription-polymerase chain reaction (RT-PCR) allows for the detection of viral RNA from infected clinical samples (106, 137, 262). Using the RFLP procedure and BstN1 and Mbo1 restriction enzymes (RE) IBDV were classified into 6 molecular groups (103, 105, 110). IBDV strains that do not match any of the 6 defined molecular groups have been identified and in the USA 32 new molecular patterns have been found (111). Ikuta et al. using 6 REs (DraI, SacI, StyI, TaqI and MvaI) classified IBDV into 20 molecular groups. RT-PCR amplicons were also identified by sequencing (5, 7). Other molecular techniques include the use of RNA probes (109, 114). Using real-time RT/PCR Sequence homology or mutations were detected using real-time RT/PCR and four mutation probes. The melting temperature (Tm) of the mutation probes is an indicator of sequence homology (102). An in situ RT-PCR was developed to investigate early stages of infection in the IBDV-infected BF (265). Extraction of fragmented viral genomic RNA from formalin-fixed paraffin-embedded tissues allowed subsequent sequencing, classification, and when
correlated with lesions present in those tissues histologically, allowed identification of candidate vaccine viruses (188).

**1.13 Immunity:**

Infectious bursal disease virus is highly infectious and very resistant to inactivation. Therefore, despite strict hygienic measures, vaccination is unavoidable under high infection pressure and it is necessary to protect chickens against infection during the first weeks after hatch. The immunization of breeder flocks is especially important to confer passive immunity to their progeny (148). Antibodies transmitted from the hen via the yolk of the egg can protect chicks against early infections with IBDV, thus protecting against the immunosuppressive effect of the virus (148). Therefore, it is important to induce high titers of maternally derived antibodies that persist over the whole laying period in laying breeders, to do so breeder hens are vaccinated two times once with a live vaccine and once with inactivated oil-emulsified vaccines or vaccinated twice with inactivated oil-emulsified vaccines (86). After hatching, chickens are immunized with live vaccines, and because maternal immunity interferes with vaccination with live vaccines, the major problem with active immunization of young maternally immune chicks is determining the proper time of vaccination. This determination is aided by monitoring antibody levels in a breeder flock or its progeny (242) or by formulas like the one developed by de Wit to determine best time of vaccination(45). The titers may vary considerably within a flock and revaccinations may be necessary. It has also to be taken into consideration that vvIBDV will break through immunity provided by highly attenuated vaccine strains.
1.14 IBDV Vaccines:

1.14.1 Live vaccines:

It is well known that less attenuated strains may cause lesions in the bursa follicles and cause immunosuppression even in vaccinated birds. To avoid live vaccine side effects, a number of alternative vaccines have been produced. Examples include, a subunit VP2 vaccine produced in yeast and an immune complex vaccine, composed of the live vaccine virus complexed in vitro with antibodies (167). Satisfactory protection against IBDV can be achieved by immunization with live or inactivated vaccines. Classical live vaccines achieve lifelong and broad protection, but posses residual pathogenicity and a proportional risk of reversion to virulence (241). Many choices of live vaccines are available that differ in virulence and antigenic diversity. According to virulence, vaccines are classified as mild, mild intermediate, intermediate, intermediate plus, or hot. Vaccines that contain Delaware variants are also available (148). Ashraf et al (3) studied the interactions between two antigenically similar strains of IBDV one being a mild strain and the other being a pathogenic IBDV strain. They concluded that viral interference occurs in live chickens, and that the most significant interference occurring when infection with the mild and field strains occur 24 hr apart. This phenomenon might be due to competition for host receptor sites or production of cytokine(s), and likely has practical implications for vaccine usage and protection against IBDV (3).

1.14.2 Inactivated vaccines:

Killed vaccines in oil emulsions stimulate high levels of maternal immunity and are extensively used in the field (148). Inactivated vaccines and live vaccines made from variant strains protect chickens from disease caused by either variant or standard strains,
whereas inactivated vaccines made from standard strains do not protect, or only partially protect, against challenge with variant strains (99). To overcome this autogenous killed vaccines are used by some companies to better prime their breeders (218). Very virulent strains of IBDV can be controlled adequately under experimental conditions by vaccination with commercial vaccines prepared from classical attenuated strains (56, 187, 242).

1.14.3 In-ovo vaccines:

In ovo vaccination may provide a way for vaccines to circumvent the effects of maternal antibody and initiate a primary immune response (64, 69). An “immune complex” vaccine has been developed, in which the vaccine virus is complexed in vitro with an optimum amount of antibodies (167). These virus-antibody complex vaccines seem very promising in protecting against IBDV(72). This new technology utilizes specific hyperimmune neutralizing antiserum with a vaccine virus under conditions that are not sufficient to neutralize the vaccine virus but which are sufficient for delaying the pathological effects of the vaccine alone. This allows chicks to be vaccinated more effectively in the presence of passive immunity even with a strain that would be too virulent for use in ovo or at hatching (72, 86, 69).

1.14.4 Molecularly engineered vaccines:

IBDV VP2, expressed in yeast (197, 259), the baculovirus system (52, 151, 196, 217, 238), recombinant NDV vaccines (90) or via transgenic Arabidopsis thaliana plants (257) have been studied for the use as subunit vaccines. An advantage of this technology is that a vaccine based on VP2 alone should allow monitoring of the field situation by the discrimination between antibody induced by vaccine (anti-VP2 only) and that induced by
infection (anti-VP2 and VP3) (239). The use of a reverse genetics system could represent a basis for the genetic attenuation of IBDV strains and for the generation of new vaccines, although interference of passive immunity would still exist. Therefore, recombinant viral vaccines expressing the VP2 protein, such as fowl pox virus (8), herpesvirus of turkey (HVT) (44, 234), or fowl adenovirus (214) might be able to mount an active immune response, as they are less sensitive to neutralization by anti-IBDV maternally derived antibodies.

1.15 Antigenic variation of IBDV:

The high mutation rate of RNA viruses and the high selection pressure generated by intensive vaccination of birds can lead to the emergence of viruses with new properties allowing them to persist in immune populations. Historically, these mutations have led to antigenic variation and modifications in virulence of circulating Infectious Bursal Disease Virus (IBDV) strains. It is therefore essential to identify and characterize new IBDV isolates as soon as they appear and compare them with previously described viruses (239, 241). The capsid protein, VP2, is the major host protective immunogen. Immunization of susceptible chickens with purified VP2 elicits neutralizing antibodies and confers protection against homologous virulent virus challenge (11, 60). Three panels of neutralizing monoclonal antibodies (mAbs) have been used in antigen capture enzyme-linked immunosorbent assays (AC-ELISA) for antigenic characterization of serotype 1 isolates of IBDV (61, 62, 54). Neutralizing mAbs have been shown to bind to VP2 within a restricted region, called the variable domain, between amino acids 206 and 350, which is highly hydrophobic but has short hydrophilic regions at each end (7). Since this epitope was denaturated by SDS, it was determined that is a conformationally-dependent epitope.
Antigenic epitopes on VP3 protein have also been reported but these antibodies are not completely neutralizing (4, 59). Antigenic diversity between IBDV serotypes has been recognized when serotypes 1 and 2 were defined on the basis of their lack of in vitro cross neutralization (156). Based on studies with monoclonal antibodies, IBDV strains belonging to serotypes 1 and 2 have been found to not share major neutralizing epitopes (9, 209). Polyvalent neutralizing antiserotype 1 monoclonal antibodies such as monoclonal antibodies 1, 6, 7, 8 and 9 (54), monoclonal antibody 8 (222), and monoclonal antibodies 6F6 and 7C9 (244) have been developed.

Antigenic differences have been shown within serotype 1, and the study of different strains has led to dividing serotype 1 into six subtypes, differentiation based on cross neutralization assays using polyclonal sera (108). Studies with monoclonal antibodies demonstrated the presence of a number of modified neutralizing epitopes among antigenically variant strains detected in the United States. Based on this evidence, there may have been an antigenic shift in IBDV viruses in the US (224). There are a minimum of at least five neutralization epitopes on the standard IBDV strains (e.g. D78 strain) as defined by the monoclonal antibodies 8, 179, B69, R63, and 10. Delaware viruses have lost the B69 site, GLS viruses lack the B69 and R63 sites but has a monoclonal 57 reactive site, the DS326 virus lacked the sites for monoclonal antibodies B69, R63 and 179, the RS593 had the 179, 8 and 67 site only, while the AL2 variant strain had 8, 57, 67 and 179 sites (222, 223). Thus based on the reactivity with various monoclonal antibodies, the IBDV viruses are antigenically grouped as classic or standard, GLS Variant, RS593 variant, AL2 variant, Y2K Variant DS326 and Delaware type variants (132, 237)
Despite their enhanced pathogenic properties, the vvIBDV strains were considered to be closely antigenically related to the standard strains such as the Faragher 52/70 strain, based on high cross-neutralization indices (56). Using neutralizing monoclonal antibodies developed by Snyder to characterize US IBDV variants, van der Marel studied twelve European isolates of IBDV. He detected no important differences between the standard strain 52/70 and vvIBDV (246). Similar data was produced by Oppling et al (187). However, Eterradossi et al. developed nine other monoclonal antibodies and using these he detected modified binding and neutralizing properties against French vvIBDV strains (54). All their monoclonal antibodies neutralized most mild or intermediate vaccines strains, whereas two monoclonal antibodies did not neutralize a French vvIBDV strain, US variant A, and the European strain Faragher 52/70. Based on their results, they suggested a neutralizing epitope may be altered in the European vvIBDV strains, causing decreased antibody neutralization. This difference could be used to differentiate vvIBDV strains (54).

1.16 Molecular basis of IBDV variability:

Sequencing of the VP2 gene of numerous different IBDV strains and escape mutants confirmed that a hypervariable domain is responsible for antigenic variation (187, 209, 237) and comparison of this region offers the best option for IBDV typing. Therefore, the nucleic acid sequencing of genes coding for VP2 and subsequent deduction of their predicted amino acid sequences is now commonly used for phylogeny. 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 acids 239 and 332 (7, 133, 237) whereas van den Berg et al.
said this hypervariable region is between amino acids 206 and 350 (245). This highly variable region falls entirely within those sequences of VP2 identified as the minimum region required for reaction with virus neutralization monoclonal antibody 5 (60, 261).

Hydrophilicity profiling of this region showed that there are two hydrophilic peaks at either ends of this region, the larger peak (Major peak A) from amino acids 212 to 224 and the other from amino acids 314 to 324 (Major peak B). 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 (75, 209). It is interesting that most of amino acid variations in this region fall within these two peaks (7, 133).

Variations in IBDV antigenicity depend on changes in hydrophilic peaks. The serotype 2 strain 23/82 (209), the North-American antigenic variants A, E, GLS and DS326 (75, 133, 237), and neutralization resistant escape mutants (209) all exhibit amino acid changes in these hydrophilic peaks. Only differences in the intervening hydrophobic domains are found between typical serotype 1 strains (237). A nucleotide sequence comparison suggested that four amino acid alterations in the VP2 protein of the Delaware F strain allowed this variant to escape neutralizing antibodies. These amino acids were located at positions 213, 222, 318, and 323 (75). By restriction enzyme and amino acid sequence analysis, point mutations have been detected at residues 222, 254 and 323. Amino acid residues 222 and 254 are consistently mutated in the variant strains (50, 105). Glycine is present in the standard strains, amino acid residue number 254, whereas the variants have serine at this position (50, 104).
Mundt (2005) showed by reverse genetics systems for IBDV, that amino acid changes in these hydrophilic peaks was accompanied by loss or acquirement of monoclonal antibody recognition sites (170). Vakharia et al. used monoclonal antibodies to correlate antigenic variations with amino acid sequence substitutions in the hypervariable region of VP2 (237). They found that the amino acid residue glutamine at position 249 might be involved in the binding of neutralizing antibody B69, which recognizes epitopes in standard strains. All the variant viruses have lysine instead of glutamine at this position, and they escape binding with antibody B69. Using a baculovirus expression system to synthesize all the structural proteins coded for in segment A of the IBDV genome, Vakharia et al., produced virus like particles (236). They mapped the antigenic sites by producing chimeric cDNA clones of IBDV using the variant GLS plasmid as a backbone and inserting fragments from the D78 and Delaware strains. At least two antigenic reactive sites are present on the surface of IBDV, one falls between amino acid residues 222 and 249, and the other between 269 and 323.

In highly virulent strains three specific amino acid residues in VP2 have been reported at position 222 (Ala), 256 (Ile), 294 (Ile) and 299 serine which differ from classical strains (21). These substitutions are also present in other strains isolated from other countries such as Bangladesh (98), Brazil (46, 93), China (28), Germany (266), Israel (195), Japan (142), (262), Malaysia (36, 88), Nigeria (266), Taiwan (144), and Vietnam (232). Positions 222-223 and 318-324 may be critical for the vvIBDV (55). These three positions have been identified as “hot spots” for mutations in several escape mutants resistant to selected neutralizing monoclonal antibodies (209, 244).

The role of VPI in the virulence of IBDV was unknown for a long time, only
recently did Liu and Vakharia (2004) by the use of reassortant viruses recovered by use of reverse genetics system discover the role VP1 plays in the virulence of IBDV \textit{in vivo}. They showed virulence was due to the effect of VP1 on viral replication kinetics \textit{in vivo} (145). The VP1 sequences of very virulent IBDV strains are genetically distinct from those 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 (97).

\textbf{Part 2: IBDV Pathogenesis and Immunosuppression:}

\textbf{2.1 IBDV Pathogenesis:}

IBD is characterized by lesions in lymphoid organs, and in chicks older than 3 weeks is characterized by morbidity and in some cases death (213, 251). Chickens that recover are severely immunosuppressed (1, 25, 84) due to the loss of B cells, especially the developing B-cell population in the bursa of Fabricius. Only serotype 1 viruses cause IBD, and pathotypes have been classified in increasing order of virulence as mild, intermediate, variant virulent (in North America), classical virulent, very virulent (vv) or hyper-virulent strains (241).

The mature bursa of Fabricius is the main target organ of IBDV as it is the source for B lymphocytes in avian species. Bursectomized chickens did not develop clinical IBD despite the presence of infection (79). The severity of the disease is directly related to the number of susceptible cells present in the bursa of Fabricius; therefore, the highest age
susceptibility is between 3 and 6 weeks, when the bursa of Fabricius is at its maximum development. This age susceptibility is broader in the case of the vvIBDV strains (99, 100, 179).

After oral infection or inhalation, the virus replicates primarily in the lymphocytes and macrophages of the gut-associated lymphoid tissues. From the gut, the virus is transported to other tissues by phagocytic cells, most likely resident macrophages (211, 239). By 13 hours post-inoculation (h.p.i.), most bursal follicles are positive for virus and by 16 h.p.i. a second more pronounced viraemia occurs with secondary replication in other organs leading to disease and death (168). It is suggested that vvIBDV causes similar disease signs to those of classical virulent strains with the same incubation time of 4 days but with an exacerbated acute phase (241, 255).

Actively dividing, surface immunoglobulin M-bearing B-cells are lysed by infection (80, 83, 203), but cells of the monocyte-macrophage lineage can be infected in a persistent and productive manner, and play a crucial role in dissemination of the virus (24,96) and in the onset of the disease (127, 131, 212). The exact cause of clinical disease and death is still unclear but does not seem to be related only to the severity of the lesions and the bursal damage. Prostration preceding death is very similar to what is observed in acute coccidiosis, and is reminiscent of a septic shock syndrome (241). The macrophage could play a specific role in this pathology by exacerbated release of cytokines such as tumor necrosis factor or interleukin 6 (127). Macrophages are known to be activated by interferon gamma; increased secretion of interferon as has been demonstrated both in \textit{in vitro} in chicken embryo cultures and \textit{in vivo} in chickens after their infection with IBDV (66, 67).
In addition to causing necrosis in the lymphoid cells of the bursa, IBDV also induces apoptosis (63, 130, 249). Apoptosis is characterized by cell shrinkage and chromatin condensation and does not generate a local inflammatory response. Induction of apoptosis in infected cells contributes to the pathogenesis of IBDV in the bursa (118, 183), chicken peripheral blood lymphocytes (248), and in the thymus (94, 230). Virally-induced apoptosis can occur in cells in the absence of detectable virus (118, 178, 230). A direct effect of viral proteins like VP2 and VP5 has been implicated in the induction of apoptosis (63, 263). Apoptotic cells have also been observed in viral antigen-negative bursal cells, underlining the possible role of immunological mediators in this process (178, 230). And finally, apoptosis has also been observed in the proventriculus of IBDV challenged SPF leghorn chickens (189).

2.2 IBDV Immunosuppression:

Most immunosuppression studies have been restricted to vaccine or classical virulent strains of IBDV (201). Clinical and subclinical infections with IBDV may cause suppression of both humoral and cellular immune responses (211). The first indication of damage in the immune system was reported by Helmboldt and Gardner in 1964 (76). In 1970, Cho demonstrated that white leghorn chickens exposed to IBDV at one day of age were consistently more likely to develop visceral tumors and nerve enlargement by Marek’s disease virus (35). In 1972 it was reported that IBDV infection at an early age was immunosuppressive, and severely depressed the antibody response to Newcastle disease virus (1). IBDV replication in the bursa leads to extensive lymphoid cell destruction in the follicular medullas and cortices (229). The acute lytic phase of the virus is associated with a reduction in circulating IgM+ cells (83, 203). IBDV-exposed
chickens produce suboptimal levels of antibodies against a number of infectious and noninfectious antigens (62, 126, 260).

2.2.1 Humoral response to IBDV infection:

Only the primary antibody response is impaired, the secondary responses remain intact (70, 203, 213), and this humoral deficiency may be reversible (211). Although destruction of Ig-producing B cells may be one of the principal causes of humoral deficiency, other mechanisms are possible including the adverse effect of IBDV on antigen-presenting and helper T cell functions (213). A paradox associated with IBDV infections in chickens is that although there is immunosuppression against many antigens, the response against IBDV itself is normal, even in 1-day-old susceptible chickens (216). There appears to be a selective stimulation of the proliferation of B cells committed to anti-IBDV antibody production (148).

2.2.2 Cellular immune response to IBDV infection:

T-cells are resistant to infection with IBDV (82, 62), and there is no evidence that the virus actually replicates in thymic lymphocytes (213, 230). However, there is evidence that \textit{in vitro} mitogenic proliferation from T cells of IBDV exposed birds is severely decreased. This mitogenic inhibition is likely mediated by macrophages, however, how IBDV induces macrophages to exhibit this suppressor effect is not clear (211). It has been suggested that T cells are induced during an IBDV infection in the bursa, but phenotypic characterization is still required and (124, 125). Rodenberg et al. studied changes in the T-cell subsets within the blood, bursa, spleen and thymus after infection with classical virulent IBDV and suggested that, although the number of immunoglobulin (Ig)M\(^+\) cells in the bursa and spleen decreased significantly, the relative
proportions of CD4⁺ and CD8⁺ T cells did not change (203). Immunohistochemical
analysis of chicken lymphoid tissues to determine the location of the virus and various
leukocyte subsets showed an influx of T cells into the bursa (177, 251). Bursal CD4⁺ and
CD8⁺ αβ₁,TCR⁺ cells increased (251), and some expressed the activation marker CD25
(the interleukin-2 receptor-a) (124). It still remains a matter of contention whether these
cells are a migratory or resident bursal population.

The number of CD4⁺ and CD8⁺ T cells increased after infection and were first
detected at the corticomedullary boundary of the bursa, probably reflecting their route of
entry (251). The cortico-medullary boundary is rich in arterioles and venules, which
allow migratory lymphocytes entry into the follicles in response to an appropriate chemo-
attractant(s). Although CD4⁺ T cells had increased by 7 d.p.i., their location was
restricted to the cortico-medullary boundary and the cortex, even though most IBDV⁺
cells were present in the medulla. Later, CD8⁺ T cells were detected throughout the
follicles. The presence of numerous T cells suggests that cell-mediated immunity has a
very important role to play in recovery from an infection with vvIBDV. Poonia and
Charan (198) reported consistently higher titers of IBDV in chickens lacking a fully
functional T-cell-mediated immune system. This increase in bursa T cells appears to be a
consequence of migration and not an artifact due to the loss of B cells or the result of an
expansion of resident T cells (251).

Sharma et al. (211) also detected a dramatic infiltration of T-cells in the bursa
during acute IBDV infection, accompanied by the abrupt drop in the number of IgM⁺
cells. By the seventh day of infection, the infiltrating cells were predominantly CD8⁺
lymphocytes. It was suggested that T-cells modulate the infection, limiting viral
replication in the bursa in the early phase of the disease. They also promote bursal tissue
damage and delay recovery, possibly through the release of cytokines and cytotoxic
effects (135). Cytotoxic T cells may exacerbate virus-induced cellular destruction by
lysing cells expressing viral antigens. T cells may also promote the production of pro-
inflammatory factors, such as nitric oxide, increasing tissue destruction (211).

2.2.3 Innate immunity

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 (211).

2.3 Pathogenesis of vvIBDV:

As for vvIBDV, VP2 antigen was most prevalent in the bursa, followed by the
spleen and then the thymus, as reported for classical virus strains (231, 251). However,
the distribution of VP2 antigen within the bursa was different from that with less virulent
IBDV. VP2 antigen was first detected at the cortico-medullary boundary, which probably
corresponds to the site of viral entry. Migratory cells
(e.g. macrophages) probably carry vvIBDV to the bursa from the site of primary
replication (intestine), and once in the medulla, it is able to colonize individual follicles
(101, 255). It is widely accepted that IBDV primarily targets immature B cells located in
the bursal cortex. However, vvIBDV VP2 antigen was mostly present in the medulla, a
site where more mature B cells are found (190, 255); only later was UK661 strain of
IBDV disseminated into the cortex (255).

Rapid depletion of virus from the bursa is associated with distinctive spherical
bodies accumulating in the medulla that resembled highly phagocytosing, activated
macrophages (252, 154). Macrophages appear to play a major role in the clearance of
dead (apoptotic and necrotic) cells and cell debris. A large accumulation of cells
expressing the macrophage marker KUL-01 and major histocompatibility complex
(MHC) class II invariant chain was present around 3 to 4 d.p.i. (255). The corresponding
decrease in viral load could partly be related to the action of phagocytosing macrophages,
although also to the exhaustion of target cells. Depletion of medullary Bu-1+, IgM+ and
IgG+ cells, prior to their depletion from the cortex, and depletion of IgM+ cells before
IgG+ cells, is consistent with the suggestion that more immature B cells are preferred
targets, although it is important to note that both populations became depleted. This will
have profound consequences both for the immune responses against vvlIBDV itself and
the consequent immunosuppression after viral clearance. The extended range of target
lymphocytes could partly explain the increased virulence of vvlIBDV isolates (255).

After destruction of the bursal architecture by vvlIBDV, Bu-1+ B cells begin to
return to the depleted bursal follicles by 14 d.p.i. However, very few cells with surface
IgM+ or IgG+ are detected with the monoclonal antibodies M1 and G1 (255). There is
also some bursal recovery of the IgM+ cell population at 14 d.p.i. with classical virulent
IBDV (123). The number of IgM+ cells were fewer than the Bu-1+ B cells which could be
due to that IBDV induced conformational changes in the Ig molecules resulting in
alterations in those epitopes recognized by the M1 and G1 monoclonal antibodies. IBDV-
induced changes in chicken IgM giving rise to a monomeric form in the circulation of
convalescent chickens (101). It was recently reported that only B cells expressing a
surface immunoglobulin are able to colonize bursal follicles, whereas those B cells with a
non-productive V-D-J recombination lacking Ig expression are deleted (194). This
suggests that the Bu-1+ cells repopulating the bursa are likely to be non-functional and will be subsequently deleted. The lack of expression of surface Ig on bursal B cells is consistent with the convalescent chick being severely immunocompromised (101). Lack of Ig-producing B cells would explain the poor antibody response to secondary antigenic challenge in chickens recovering from IBDV infection. The situation after vvIBDV infection seems likely to be far more severe than with less virulent strains (25, 77).

An important aspect of the pathogenesis and clinical disease caused by vvIBDV is the involvement of other lymphoid organs (94). The extensive damage in the thymus caused by UK661 IBDV strain was far greater than that reported for classical virulent stains (231). At the height of thymic pathogenesis much of the cortex contained pyknotic nuclei within large vacuolated spaces, many of which merged. This suggests either that vvIBDV has a direct apoptotic or necrotic effect on thymocytes (118) or that cell death is due to bystander activity (254). Lesions in the spleen were less evident and the transient nature of detectable virus may be related to lymphocyte and macrophage populations restricting the spread of the IBDV (255). The spleen is the major secondary lymphoid organ in the chicken where most systemic T-cell and B-cell responses are initiated. The B-lymphocyte population was most severely affected, suggesting that peripheral B cells are highly susceptible to vvIBDV. This further supports the notion that immature B cells are not the only targets for vvIBDV, as both IgM+ and IgG+ B cells decreased. The IgM+ cell population recovered in the spleen, reaching a higher number than before infection, in association with germinal centers. This is most probably a consequence of expansion of the peripheral B-cell pool and not due to seeding from the bursa. The IgG+ population also recovered and was associated with germinal centers, suggesting isotype switching of
the IBDV-reactive IgM+ population. The lack of bursal architecture (and probably function) and the increase in splenic IgM+ and IgG+ cells is consistent with the B-cell compartment being severely suppressed, while at the same time IBDV-reactive B cells are present (10, 200). The T-cell compartment within the spleen seemed to be unaffected after infection (255).

In the thymus, IBDV antigen was only transiently detected at 4 to 7 d.p.i. The rapid depletion of Bu-1+ medullary B cells probably follows the loss of viral target cells and restricted viral distribution. Few, if any, Bu-1+, IgM+ or IgG+ cells were detected in the thymic cortex even though VP2 antigen was detected there. The presence of IBDV antigen on the surface of numerous, adjacent cortical thymocytes suggests that IBDV is able to adhere to immature T cells. However, it seems unlikely that IBDV directly infects and replicates within these cells as Hirai and Calnek were unable to show replication of IBDV in chicken T cells (80). The proportion of CD4+ and CD8+ T cells in the thymus altered little following infection.

**Part 3: Pichia Pastoris**

3.1 Introduction:

Geneticists have learned how to manipulate DNA, to identify, move and place genes into a variety of organisms that are quite different from the source organism. A major use for many of these recombinant organisms is to produce proteins. Since many proteins are of immense commercial value, numerous studies have focused on finding ways to produce them efficiently and in a functional form. Escherichia coli have been the choice of researchers for several reasons. It is a single-celled organism that reproduces
mainly through asexual reproduction. The organism’s simplicity makes it easy and cheap to work with. Its food source is simple, and it does not require elaborate facilities for growth and maintenance. Its rapid growth cycle allows for a quick increase in the population size of a particular strain, as an *Escherichia coli* population can double in less than an hour. In addition to its own circular DNA, *Escherichia coli* have plasmids that complement the main segment of DNA. Plasmids are easy to isolate and manipulate. The plasmid can be removed, a desired gene can be inserted into it, and the plasmid can be reintroduced into *Escherichia coli*. The *Escherichia coli* will then produce the foreign protein as if the protein were native to the bacteria. In order to express protein, a promoter from the host needs to be located immediately in front of gene to be expressed. The promoter regulates when, how much and how often the gene is transcribed. The simplicity of *Escherichia coli* makes it a desirable host for production of a foreign protein; however, it also has its disadvantages as a prokaryote. All prokaryotes do not have any of the membrane bound organelles found in eukaryotes. In eukaryotes a protein is often post-translationally modified in different organelles, such as the endoplasmic reticulum or the Golgi apparatus. Modifications often involve addition of different forms of glycosylation. Any eukaryotic protein can be mass translated in *Escherichia coli*, but many are not quite finished and hence, they are nonfunctional. *Escherichia coli* will give the same primary structure, as occurs when that protein is initially produced in its own cell type. However, the failure to modify that structure often means that the protein will not form as it would with the presence of certain organelles. Because of the problems encountered when using a prokaryote to produce eukaryotic products, researchers sought suitable eukaryotic replacements like mammalian, insect and yeast cells. Of these three
eukaryotic expression systems, yeast cells are the most desirable, as they combine the ease of genetic manipulation and rapid growth characteristics of a prokaryotic organism with the subcellular machinery for performing post-translational protein modification of eukaryotic cells (41).

The yeast *Saccharomyces cerevisiae* has been popular with molecular biologists, and many proteins have been produced using it as a protein expression system. *S. cerevisiae* has several limitations, as its product yields are low, often reaching a maximum of 1-5 percent of total protein yield. Additionally, the presence of foreign gene products puts stress on the cells. The production of the protein during the growth phase hinders growth. Even the use of inducible plasmid promoters to achieve a partial separation between the growth and protein production phase, has not been effective due to the instability of the plasmid (23). Besides the difficulties encountered with scaling up protein production to get better yields, several reports have noted the hyperglycosylation of secreted glycoproteins which may cause differences in immunogenicity and diminished activity. Also, many of the secreted proteins of *S. cerevisiae* are not found free in the medium, but rather in the periplasmic space. This leads to problems with purification and further decreases product yield (23).

*Pichia pastoris* was discovered 30 years ago when Koichi Ogata discovered its ability to utilize methanol as a sole source of carbon and energy (181). Because methanol could be inexpensively synthesized from natural gas (methane), there was immediate interest in exploiting these organisms for the generation of yeast biomass or single-cell protein (SCP) to be marketed primarily as a high protein animal feed. However, the oil crisis of the early 1970’s increased the price of methane drastically along with drop of
soybean prices rendered this goal unpractical. Use of *Pichia pastoris* as an alternative species to *S. cerevisiae* in molecular biology was appealing, as growth protocols were similar in both species. Also because *P. pastoris* has a strong inducible promoter that can be used for protein production. *P. pastoris* is capable of generating post-translational modifications such as proteolytic processing, folding, disulfide bond formation, and glycosylation that are more similar to human protein modifications than *S. cerevisiae* was capable of doing (43). Thus, many proteins that end up as inactive inclusion bodies in bacterial systems are produced as biologically active molecules in *P. pastoris*. The *P. pastoris* system is also generally regarded as being faster, easier, and less expensive to use than expression systems derived from higher eukaryotes, such as insect and mammalian tissue culture cell systems, and usually gives higher expression levels (78). The production of a functional protein is intimately related to the cellular machinery of the organism producing the protein. The yeast *Pichia pastoris* is a useful system for the expression of milligram-to-gram quantities of proteins for both basic laboratory research and industrial manufacture. The fermentation can be readily scaled up to meet greater demands, and parameters influencing protein productivity and activity, such as pH, aeration and carbon source feed rate, can be controlled (78). Compared with mammalian cells, *Pichia* does not require a complex growth medium or culture conditions, is genetically relatively easy to manipulate, and has a eukaryotic protein synthesis pathway. Because of these characteristics, some proteins such as G protein-coupled receptors that cannot be expressed efficiently in bacteria, *S. cerevisiae* or the insect cell/baculovirus system, have been successfully produced in functionally active form in *P. pastoris* (30, 141). *Pichia* can be grown to very high cell densities using minimal media (250, 253) and
integrated vectors help genetic stability of the recombinant elements, even in continuous and large-scale fermentation processes (204). Simple purification and isolation of a recombinant protein is facilitated by the fact that P. pastoris does not secrete a lot of its own proteins (41). Therefore, the powerful genetic techniques available, together with its economy of use, make P. pastoris a system of choice for heterologous protein expression. So, what began more than 20 years ago as a program to convert abundant methanol to a protein source for animal feed has developed into what are today two important biological tools: a model eukaryote used in cell biology research, and a recombinant protein production system. Pichia has gained widespread attention as an expression system because of its ability to express high levels of heterologous proteins. As a result, recombinant vector construction, methods for transformation, selectable marker generation, and fermentation methods have been developed to exploit the productive potential of this system (207).

Research Corporation Technologies (Tucson, AZ, USA) are the current holders of the patent for the P. pastoris expression system, which they have held since 1993, and the P. pastoris expression system is available in kit form from Invitrogen Corporation (Carlsbad, CA, USA).

3.2 Methanol metabolism in Pichia

Methylo trophic yeasts are able to utilize methanol as the sole carbon and energy source. All such strains identified to date belong to only four genera: Hansenula, Pichia, Candida and Torulopsis (58). They all share a specific methanol utilization pathway involving several unique enzymes. The initial reactions take place in specialized microbodies (the peroxisomes) followed by subsequent metabolic steps in the cytoplasm.
Peroxisomes play an indispensable role during growth, as they harbor the three key enzymes for methanol metabolism, viz. alcohol oxidase, catalase and dihydroxyacetone synthase. The proliferation of peroxisomes is a reflection of environmental conditions. When the cells are grown on glucose, very few peroxisomes are present. When grown on methanol, peroxisomes may take up to 80 percent of the total cell volume. Previous results clearly show that the alcohol oxidase promoter is both tightly regulated and is a strong promoter. The production of foreign protein can be repressed until the culture is saturated with colonies, and then the production of the foreign protein can begin with the de-repression and induction of the gene. The subsequent reactions of methanol assimilation and dissimilation are localized in the cytosol. The methanol metabolism is fully described by Cereghino and Cregg (31, 5, 117), Jahic and Gellisen (68).

3.3 Methanol utilization phenotypes:

There are three phenotypes of *P. pastoris* host strains with regard to methanol utilization. The Mut⁺, or methanol utilization plus phenotype, grow on methanol at the wild-type rate and require high feeding rates of methanol in large-scale fermentations (31). The Mut⁻, or methanol utilization slow phenotype, have a disruption in the *AOX1* gene. Since the cells must then rely on the weaker *AOX2* for methanol metabolism, a slower growing and slower methanol utilization strain is produced. Mut⁰ strains have been found to be advantageous for production of hepatitis B surface antigen (40). The Mut⁻, or methanol utilization minus phenotype, are unable to grow on methanol, since these strains have both *AOX* genes deleted. One of the advantages of this phenotype is that low growth rates may be desirable for production of certain recombinant products.
Currently, the majority of researchers use the Mut$^+$ phenotype (87, 217), although some researchers are also using the Mut$^-$ phenotype (2, 191).

3.4 The Pichia Expression System:

The expression of any foreign gene in $P. pastoris$ comprises three principal steps: (a) insertion of the gene into an expression vector; (b) introduction of the expression vector into the $P. pastoris$ host; and (c) examination of potential strains for the expression of the foreign gene (31, 41, 129).

The majority of heterologous protein production in $P. pastoris$ is based on the fact that enzymes required for the metabolism of methanol are only present when cells are grown on methanol (53). This has been the most successful system reported for this organism (141). The AOX promoters have been the most widely utilized promoters; however, other promoter options are available for the production of foreign proteins in $Pichia$, (31).

3.4.1 Promoters:

One of the drawbacks with $S. cerevisiae$ was that it did not have a strong inducible promoter. $Pichia pastoris$ has a strong inducible promoter. This inducible promoter is related to the fact that $P. pastoris$ is a methylotrophic yeast. The first step in the utilization of methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide (136). This step is catalyzed by the enzyme alcohol oxidase. The expression of this gene is tightly regulated. When the yeast is grown on glucose or ethanol, alcohol oxidase is not detectable in the cells. However, when the yeast is grown on methanol,
alcohol oxidase can make up to thirty-five percent of the total cellular protein. The control of the amount of alcohol oxidase is largely transcriptional (42).

There are two alcohol oxidase genes: AOX1 and AOX2. The protein coding regions of the genes are largely homologous, 92 percent and 97 percent at the nucleotide and amino acid sequence levels respectively (182). The promoters share very little homology. No mRNA of the two genes is detectable when the yeast is grown in glycerol. The promoter region for AOX2 has a repressor region that leads to the inhibition of gene expression, and an activation region that leads to the enhancement of gene expression. The AOX1 gene promoter probably has a similar mechanism (182).

The AOX1 promoter has been the most widely reported and utilized of all the available promoters for P. pastoris (141). One of the reasons that the constitutive GAP (glyceraldehyde 3-phosphate dehydrogenase) promoter has not been widely used is the belief that constitutive production of foreign proteins in P. pastoris may have cytotoxic effects (160). However, recent studies have found not only that cytotoxic effects are not necessarily observed, but also that production levels of a recombinant exo-levanase (LsdB) using the GAP promoter were similar to those using the AOX1 promoter (160). Combining the GAP and AOX1 promoters in a strain expressing human granulocyte-macrophage colony-stimulating factor (hGM-CSF) resulted in a two-fold increase in production of the recombinant protein (258). By using this combined promoter for sequential expression of a constitutively produced enzyme required for post-translational modification, an inducible recombinant protein can be modified to a specified form within the fermentation process itself (26, 258).
The \textit{YPT1} [a small GTPase involved in secretion] (210) and \textit{PEX8} [a peroxisomal matrix protein] (143) promoters have not been widely used, probably because of their low expression levels, since the goal of the majority of researchers using the \textit{P. pastoris} expression system is to obtain maximum amounts of expressed foreign proteins (31). Other promoters include the \textit{AOX2} promoter (39), \textit{FLD1} promoter (250) and the \textit{ICL1} promoter (159).

3.4.2 Selectable markers:

All \textit{P. pastoris} expression strains are derived from NRRL-Y11 430 (Northern Regional Research Laboratories, IL, USA), the wild-type strain. One or more auxotrophic mutants are often present in these strains, allowing for selection during transformation of strains containing the appropriate selectable marker gene. A number of selectable marker genes are known for the molecular genetic manipulation of \textit{P. pastoris}, they include \textit{HIS4} (histidinol dehydrogenase gene), \textit{ARG4} (argininosuccinate lyase gene), \textit{Zeo}$^R$ (zeocin resistance gene), Blasticidin S deaminase gene, \textit{ADE1-PR-amidoimidazolesuccinocarboximide synthase}, \textit{URA3-orotidine 5$'$-phosphate decarboxylase} and \textit{Sor}$^R$-acetyl-CoA carboxylase (31).

The genetic manipulation of \textit{P. pastoris} for the production of various heterologous proteins is simplified by the use of a wide range of selectable markers and promoters. Choosing the correct markers and promoters is essential for obtaining a high productivity (150).

3.5 Post-translational modifications:

The post-translational modifications made by \textit{P. pastoris} are more suitable for use in humans. The structure of carbohydrate added to secreted proteins is known to be
very organism specific. Many proteins secreted from *S. cerevisiae* have been demonstrated to be antigenic when introduced into mammals thus; the use of glycoprotein products synthesized by yeast for therapeutic purposes has been avoided. A comparison of a *S. cerevisiae* protein secreted from *S. cerevisiae and P. pastoris* has shown distinct differences between N-linked oligosaccharide structures added to proteins secreted from these yeast. The majority of the N-linked oligosaccharide chains are high mannose. However, the length of the carbohydrates chains is much shorter in *P. pastoris*. Even the longest chains of protein produced in *P. pastoris* contained only approximately thirty mannose residues, which is significantly shorter than the 50 to 150 mannose residue chains typically found on *S. cerevisiae* glycoproteins. The second major significant difference between the glycolation by *S. cerevisiae* and *P. pastoris* is that glycans from *P. pastoris* don't have alpha 1,3-linked mannose residues that are characteristic of *S. cerevisiae* (41). The enzyme that makes alpha 1,3 linkages is alpha 1,3 mannosyl transferase and it is undetectable in *P. pastoris*. It is significant, because the alpha 1,3 linkages on *S. cerevisiae* glycans are primarily responsible for the highly antigenic nature of glycoproteins used for therapeutic products (41).

### 3.6 Production of heterologous proteins in *P. pastoris*:

The *Pichia* expression system has been widely used to produce a variety of different heterologous proteins (31). *P. pastoris* grows on a simple mineral media and does not secrete high amounts of endogenous protein. Therefore the heterologous protein secreted into the culture is relatively pure and purification is easier to accomplish (58). Cell growth is particularly important for secreted protein production in bioreactors, since the concentration of the product in the extracellular medium is roughly proportional to
the concentration of cells in the culture in many instances. The yields of proteins expressed intracellularly in bioreactors are also high, due to the efficiency of the AOX1 promoter (31). Production of large amounts of heterologous proteins in shake-flask culture is difficult, due to the limitations of volume, oxygen transfer, substrate addition and an inability to monitor these factors efficiently (150). The use of bioreactors is preferable, since all of these parameters can be monitored and controlled simultaneously, allowing more efficient production of the desired heterologous protein. Cereghino et al. reviewed the use of bioreactors for the production of recombinant proteins from P. pastoris (30).

*Pichia pastoris* as a yeast expression system was chosen because it combines the simplicity of prokaryotic growth requirements, growth potential and ease of manipulation along with the post-translational modifications of eukaryotes to ensure that the expressed protein is functional and has the proper conformation.
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Chapter III

Identification of infectious bursal disease viruses from RNA extracted from paraffin embedded tissue.

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1Mohamed M. Hamoud and Pedro Villegas accepted in Avian Diseases
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Abstract:

After histopathological screening bursa of Fabricius for the presence of infectious bursal disease virus (IBDV), the hypervariable region of the VP2 gene for IBDV was extracted from formalin fixed paraffin embedded tissue blocks. Using real-time RT-PCR and sequencing, IBDV was identified in 227 different blocks. The ability to identify the actual virus strain associated with the lesions observed microscopically in the bursa of Fabricius allowed for direct correlation between viral identity and lesions, which may help in designing vaccination strategies. Several new emerging viruses that do not group with other known IBDV in phylogenetic tree analysis were identified, as well as a unique variant virus that had 63 nucleotides missing from its hypervariable region.

Introduction:

Infectious bursal disease virus (IBDV) is the etiological agent of Gumboro disease or infectious bursal disease (IBD). IBDV destroys the B lymphocyte precursors found within the bursa of Fabricius, followed by bursal atrophy in young chickens (32). In unprotected flocks, the virus may cause mortality and/or immunosupression (16, 26). There are two serotypes of IBDV: 1 and 2. All pathogenic viruses belong to serotype 1, whereas serotype 2 viruses are non-pathogenic for both chickens and turkeys (12, 21). Very virulent (vv) IBDV strains emerged in Europe in the late eighties, causing up to 60% mortality (4, 30). Strains of vvIBDV have also spread to other continents (1, 7, 28). The epidemiological origin of vvIBDVs is still unknown (17).

IBDV belongs to the family Birnaviridae (genus Avibirnavirus) which includes viruses with bisegmented dsRNA genomes (5). Genome segment A has 2 overlapping open reading frames (ORF) the first encodes a precursor polyprotein in a large ORF, the
product of which is cleaved by autoproteolysis to yield mature VP2 (outer capsid), VP4 (protease), and VP3 (inner capsid) (15). The other ORF of segment A encodes a non structural protein, VP5 and is possibly involved in virus release (18, 33). Genome segment B encodes the virus polymerase, VP1 (20). VP2 is the major host-protective antigen of IBDV. It contains at least three independent epitopes responsible for the induction of neutralizing antibody (2), thus the antigenic characterization of IBDV has been based mainly on the study of the VP2 gene where some amino acid changes have been shown to be the basis for antigenic variation (29), or have been proposed as putative markers for vvIBDV (3, 22, 31).

Controlling infectious bursal disease (IBD) and its associated immune suppression is critical to the broiler industry. This control is achieved by either vaccinating breeder hens with conventional live attenuated and/or inactivated IBD vaccines, or by the use of live IBD vaccines in broiler chicks, layers or young pullets to provide active protection against IBDV, or a combination of both.

Using a histopathology archive can be very important in molecular research. Bursal tissue specimens that have been preserved as paraffin blocks for many years represent a historical collection of IBD. Paraffin embedded tissues have proved to be a valuable source of DNA or RNA for molecular genetic analysis and the identification of infectious agents like hepatitis C, and polio viruses (8, 10, 24). The use of tissues that are formalin fixed and embedded in paraffin as a source of genetic material may allow a direct correlation between the IBDV strain present in the tissue and the pathological lesions visualized by microscopy. Therefore, the information obtained by this technology
may help refine vaccination programs for IBDV, as well as to detect any new emerging IBDV strains.

The objective of this study was to utilize RNA extraction from formalin fixed paraffin embedded tissue for rapid identification of nucleic acids from field strains of IBDV that are causing actual lesions in commercial chickens, and comparing amino acid sequences and nucleic acid sequences of the hypervariable region of VP2 of these field strains to known vaccine and common IBDV strains.

Materials and methods

Tissue and Histopathological evaluation. Bursal tissues mainly from commercial broiler flocks experiencing IBD-related problems from various regions of the world were formalin fixed and paraffin embedded. Paraffin-embedded tissue samples were sectioned, mounted, stained using hematoxylin and eosin (HE), and examined using light microscopy. All sections of bursa of Fabricius were assigned a lesion score, where a score of 1 represented no lesions, a score of 2 was defined as mild variation in follicle size, 3 as moderate variation in follicle size, and 4 as either necrosis or follicle atrophy (23). Tissues came from chickens with an age range from 14 to 84 days; tissues from older birds were from broiler breeders.

Viruses: IBDV strains Edgar, Lukert, Variant A, Variant E, GLS, STC kept in our laboratory were used as reference strains. Commercial IBDV vaccines S706™, SVS-510™, IBD Blen™ and Bursa Blen™ (Merial Select Inc. Gainesville, GA), Clonevac D78™ (Intervet Inc. Kenilworth, NJ), Univax™ (Schering-Plough Animal Health, Millsboro, DE) and Bursine 2™ (Fort Dodge Animal Health, Fort Dodge, IA) were obtained from each company.
RNA Extraction. RNA was extracted from formalin-fixed, paraffin-embedded bursas and examined for IBDV nucleic acid by real-time RT-PCR (23). Sections totaling 50 µm in thickness were cut from each formalin-fixed, paraffin embedded tissue block, deparaffinized in HemoDe (Fisher Scientific, Pittsburgh, PA), washed with 100% ethanol, and digested with 25 µg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 1 hr at 50 C. RNA was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s recommendations, diluted in 25 µl of 90% dimethyl sulfoxide, and frozen at -80 C until assayed. RNA from commercial vaccine viruses was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD) according to the manufacture’s recommendations, and then sequenced as mentioned below.

Real-time RT-PCR. Extracted RNA was denatured at 95 C for 5 min and kept on ice. An RT-PCR was performed using reagents from the Light Cycler RNA Amplification SYBR Green I Kit (ROCHE Molecular Biochemicals, Indianapolis, IN). The primers used were designed to amplify a 276-base pair (bp) segment of the IBDV genome shared by all strains, which represents the hypervariable region of the VP2 gene (primers synthesized were different from those in cited reference). Amplification and detection of specific products were also performed using a Light Cycler (ROCHE Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s recommendations. Briefly, RT-PCR was done at 55 C for 30 min., followed by denaturation at 95 C for 30 sec. Fifty five PCR cycles were performed, consisting of denaturation (95 C for 1 sec), hybridization (55 C for 10 sec), and extension (72 C for 13 sec). A melting curve analysis was done with an initial denaturation at 95 C. DNA melting was accomplished with an initial temperature of 65 C for 10 sec and a gradual temperature increase of 0.1 C/sec
until 95 C was reached. The melting temperature of the expected 276-bp amplicon was
between 82 C and 84 C. This estimated melting temperature was used to confirm the
identity of IBDV specific products obtained using real-time RT-PCR. Additional
confirmation of specific amplification was done using routine gel electrophoretic
techniques of the PCR products on 2% agarose (Sigma Chemical Co., St. Louis, MO),
followed by ethidium bromide staining (23).

**Nucleotide sequence analysis:** Extracted genomic material that was positive for IBDV
by real-time RT-PCR melting curve analysis was submitted to Integrated Biotech
Laboratories (IBL) at The University of Georgia (Athens, GA) for sequencing. The
nucleotide sequences were retrieved using Chromas program
were generated using EditSeq™ (DNASTAR inc. Madison, WI). All nucleic and
amino acid sequences were aligned using the clustal W method using the MegAlign™
program (DNASTAR inc. Madison, WI) and sequence distances calculated. Phylogenetic
analyses were conducted using MEGA version 3.0 software
([http://www.megasoftware.net/](http://www.megasoftware.net/)) by Neighbor-Joining, 1000 bootstrap replicates and p-
distance. This was used to compare the field IBDV strains to common vaccine strains
either sequenced in our lab or available on GenBank, as indicated by accession numbers
written before the strain name.

**Selection of sample for evaluation:** Tissues (bursa, spleen and thymus) were collected
globally from problem farms with immunosuppressive and performance issues. The
tissues were then fixed in 10% buffered formalin and shipped to a histology lab where the
tissues were processed and put into paraffin blocks. The blocks were cut with a
microtome and slides were prepared for histological evaluation. The tissues were evaluated by an avian histopathologist and the tissues with severe acute IBDV lesions were identified and selected for genomic extraction. Real-time RT-PCR was carried out on extracted genomic material and IBD was identified in samples by melting curve analysis. The IBDV genomic material was sequenced and the sequences analyzed.

Results

Histopathological examination. There were 452 paraffin embedded blocks from IBDV cases submitted to our laboratory from the beginning of January 2003 until the end of June 2005 for IBDV testing. A majority of those blocks were characterized by the presence of lesion scores of +3 or +4 (characterized by having severe acute bursitis); while very few had +1 and +2 lesion scores.

Real time RT-PCR. Melting curve analysis detected that IBDV genomic material was extracted from 227 of these blocks. The other 225 blocks yielded genomic material that was negative for IBDV on real time RT-PCR testing, in spite of having severe histological lesions characteristic of IBDV.

Cases from the United States of America totaled 207, of those 181 (87%) contained IBDV genomic material. There were 245 international samples, of those only 46 (19%) were positive on real time RT-PCR testing. The extracted genomic material expressed as location [number of positive cases] represented samples from Alabama [16], Arkansas [10], Colorado [4], Delaware [22], Georgia [22], Iowa [2], Maryland [1], Michigan [1], Mississippi [45], North Carolina [19], Ohio [4], Pennsylvania [4], South Carolina [2], Texas [1], Virginia [13], and unknown domestic origin [15]. While international samples represented Argentina [4], Brazil [4], Dominican Republic [2], El-
Salvador [7], Guatemala [4], Mexico [6], Peru [7], Venezuela [12], China [6] and Japan [1] (Tables 2 and 3).

Some of the submitted samples were autolytic, had chronic lesions, or contained mild lesions (lesion score \( \leq +2 \)) and yielded no detectable genomic material upon extraction with an exception of one block that had a +2 lesion score and was identified as a Variant E virus.

**Nucleotide and deduced amino acid analysis.** The nucleotide sequences of extracted genomic material from 227 cases were determined across 276 base pairs (bp) of the VP2 hypervariable region. The most dominant viruses present in the U.S.A were the Variant E viruses while internationally they were the classic strains (Table 1). The geographic origins of different strains of IBDV are illustrated in Tables 2 and 3. These 227 cases included 29 classic, 15 Variant A, 160 Variant E, 4 vvIBDV, 7 that could not be sequenced (Real time melting curve analysis was positive but sequence results - from 2 different independent labs - had too many unknown nucleotides to be properly identified) and 12 unique viruses that did not fall in any particular group (table 1).

Several virus strains exhibited major differences from other IBDV strains were identified and therefore are referred here as “unique viruses”. Examples for these unique viruses included cases identified as 077 and 266. Case 077, in the absence of case 266 falls on a branch of its own (Figure 2), but in the presence of case 266, it clusters with Nobilis Gumboro 228E™ (Figure 1) with 94.2% similarity in nucleic acid and 94.5% in deduced amino acid analysis. Case 266 was on a branch of its own on both nucleic and amino acid phylogenetic trees and did not cluster with any other IBDV strains (figure 1). Sequence distance data showed that case 266 was closest to Variant E with 96.0%
similarity in nucleotide sequence but was closest to STC and AF457106 Univax G603 (Schering Plough has G603 under the trade name Bursavac) with 92.3 % similarity in deduced amino acid analysis.

Another example for unique viruses was case 087 (figure 1). Extraction and identification of its genomic material revealed that this IBD virus strain lacked 63 nucleotides from its VP2 hypervariable region. In spite of this, the virus was apparently still capable of replication in the bursa and caused acute follicle necrosis with no regeneration (+4 lesion score). Nucleic acid analysis indicated that this was 98.1% similar to Variant A virus while its deduced amino acid analysis concluded it was 95.7% similar to both Variant A and Variant E viruses.

One Variant E virus was identified from a block containing tissue with +2 lesion score, and this was an extremely rare result; as all other +2 blocks from experimentally infected birds and commercial submissions did not yield genomic material for IBDV.

In multivalent vaccines, all the different strains present could not be identified. Only the viral strain with the highest viral titer was identified in these multivalent vaccines.

**Discussion:**

Controlling infectious bursal disease (IBD) and its associated immune suppression is critical to the broiler industry. This control is achieved by either vaccinating breeder hens and/or progeny to provide protection against IBDV. The use of these vaccines may have caused selection pressure on field viruses that has led to the emergence of variant strains of IBDV (27). It is also speculated that the very virulent
strains of the IBDV (vvIBDV) may have emerged for the very same reasons that caused the variant strains to emerge (25).

Because IBD is primarily controlled using vaccination, the failure of vaccines to generate adequate immunity in young chicks is a major concern. One cause of vaccine failure can be attributed to genetic drift of field viruses, which has lead to antigenically divergent IBDV strains known as variants (9, 11). Current variant and classic IBDV vaccines are able to protect against many of the antigenically divergent IBDV strains (9, 14). However, new antigenic variants of the virus have been reported to break through maternal and active immunity generated using commercially available vaccines (14).

The purpose of this study was to rapidly identify field isolates of IBDV by using formalin fixed paraffin embedded tissues as a source of genomic material. This is of importance because it will allow a direct correlation between the virus strain present and the lesions produced by this virus in a timely fashion. It will also allow analysis of foreign strains of IBDV with no importation and biosecurity restriction issues because of the inactivated nature of the antigen. Therefore, the information obtained by this technology will help refine vaccination programs for IBDV, as well as detect any new emerging IBDV strains both domestically and internationally.

Analysis of 452 paraffin embedded tissue blocks for presence of IBDV was based on histopathological lesion scores for IBDV. These 452 samples were not selected at random. Instead, they were submitted because IBD was suspected in the flock or on the farm. This study was therefore biased toward IBDV on farms experiencing immune suppression-related problems. When the viral genomes identified were from viruses
associated with disease in commercial poultry operations, they had a greater potential to be genetic and antigenic mutants (13).

In chickens experimentally infected with IBDV, all paraffin embedded blocks with bursas having acute +3 or +4 bursitis had extractable genomic material that was positive for IBDV based on melting curve analysis, indicating high sensitivity of primers used. Most of the 452 blocks analyzed had +3 or +4 acute lesion scores for IBDV, yet only 227 were positive for IBDV extractable genomic material. IBDV genomic extraction rate from samples originating from the U.S.A. was 87%, while that from overseas was only 19%. A possible explanation is that most of the cases that had acute +3 or +4 histological lesion score for IBDV but were negative on real-time RT-PCR melting curve analysis for IBDV, were inadequately fixed. This conclusion was based on experimental simulation of different fixation conditions that could occur in the field, where bursas positive for extractable IBDV RNA were rendered negative just by changing fixation conditions. Improving fixation conditions will improve extraction of RNA from paraffin embedded tissue (6). The seven samples that could not be sequenced were sequenced repeatedly in 2 different laboratories and yielded similar non specific results. They were not false positives as they had segments of IBDV sequence, yet they could not be aligned due to the presence of too many non specific nucleotides (Ns). Also this genomic material was extracted from bursa’s with specific +4 acute lesions of IBDV.

Nucleic acid and deduced amino acid analysis of extracted IBDV genomic material has shown that the Variant E strain of IBDV is the predominant strain present in the U.S.A. as it has also been observed by Jackwood and Sommer-Wagner (13). Classical strains found in the United States were mainly of vaccine origin. There were no vvIBDV
strains found in the U.S.A. Internationally, four vvIBDV strains from both Venezuela and China were identified. Variant A strains were found in both Peru and Venezuela. Variant E strains were identified in El Salvador, Guatemala, Mexico, Peru and Venezuela. Internationally, more classical than variant strains were identified, which is similar to what Lukert and Saif have reported (19).

Phylogenetic analysis shows that serotype 1 viruses grouped together in several different groups. These groups were identified as Lukert, Edgar, vvIBDV, STC, 2512, Websters, various classic, Variant A and Variant E groups. Previous knowledge of strain origin and identity along with location of viruses on phylogenetic trees was the basis used for grouping these viruses. The Faragher 52/70 (Gallivac) and the 228E strains fell on different branches and did not cluster with any other classic IBDV strains (Figure 1 and 2). It was noticed that BursaVac™ fell into two different clusters, the first (GenBank accession number AF498633) being in the STC cluster and the second (AF148075) was in the Websters cluster. The same observation was seen for Bursine 2™, where the Bursine 2 sequenced in our laboratory fell in the Lukert group (similar to the Bursine 2 with GenBank accession number AF498631 - not in figure) while Bursine 2 with accession number AY332561 fell in the group named various classic (Figure 1 and 2). The explanation for this may be the use of different strains for production of the same vaccine, different strains are being given the same trade name by different companies and researchers, or mislabeling occurred and sequences were posted incorrectly on GenBank. Therefore, extreme caution has to be made when advising which vaccine to use, because although two vaccines may have the same trade name, they may contain different IBDV strains which may not cross protect against the same field isolates of IBDV. To overcome
this problem, it is best if the vaccines that are used for comparison to field isolates be sequenced by the researchers themselves. Variant viruses clustered into Variant A and Variant E. Variant E had three different branches, with strain 89/03 on one branch, RS593 on the second branch and Delaware E on the third branch.

Due to the large number of field strains analyzed in this study, only representative samples are discussed. Several unique virus strains that did not group with the common IBDV strains and fell on branches of their own were identified; examples for this are cases 077 and 266. Sequence distance data showed that case 266 had a peculiar property of being closest to a Variant E strain (96.0% similarity) when analyzing its nucleotide sequence but its deduced amino acid sequence distance data concluded it was closest to STC and Bursavac G603 strains (92.3 % similarity). The reason for the shift from variant to classic strain based upon deduction of amino acid sequence from a nucleotide sequence is unknown. There may be a need to include these strains (077 and 266) in future IBDV vaccines depending on how widespread these viruses become among chicken flocks, as each of these strains was isolated from one farm only.

Genomic material was isolated and identified from a unique IBD virus strain that was missing 63 nucleotides from its hypervariable region in the VP2 gene, but was apparently able to replicate in the bursa and cause lymphocytic damage. This unique virus was given case number 087. Nucleic acid analysis indicated that the virus in case 087 was 98.1% similar to Variant A virus while its deduced amino acid analysis concluded it was 95.7% similar to both Variant A and Variant E viruses. VP2 is important for virus attachment to cell receptors and is the viral protein that is recognized by the chicken’s immune system (2, 29). Missing these 21 amino acids apparently did not
affect the virus’s ability to attach and enter the susceptible B-lymphocytes. Constant vaccination pressure in the past may have contributed to the emergence of variant strains and vvIBDV (25, 27). Case 087 may have resulted from random mutations by the virus in an attempt to escape from the immunoglobulins produced in vaccinated chickens; or maybe this virus was always there in the population of IBDV viruses and constant vaccination against variant IBD viruses suppressed other viruses, giving this virus a chance to infect and propagate in the bursas. Also, the equal base pair distances from both Variant A and Variant E viruses may provide cross protection against both Variant A and E viruses, however, this needs to be investigated.

The strain identified from the bursa of Fabricius with a lesion score of +2 needs to be further investigated to see if it is truly apathogenic, and if the virus possesses any protective abilities against naturally occurring viruses. In the few bursa of Fabricius encountered with a lesion score of +2, genomic material for IBDV could not be detected in any of those blocks, this is likely due to low viral load or because these lesions were due to stressors other than IBDV; with the exception of this case where there was enough viral load in the block to extract genomic material. It was identified as a Variant E.

From the large number of samples submitted for identification, most of them had more than 95% similarity to reference strains, example: case 290 was 100% identical in nucleic acid to STC and Bursavac G603™, case 1042-1 was most similar to Edgar IBDV strains, cases 340, 366 and 373 were most similar to AL-1 strain of Variant E.

The inability to identify all strains present in multivalent vaccines was attributed to the nature of the RT-PCR reaction which amplifies the virus with higher logarithmic concentration than the other viruses that have lower concentration. So when a sequencing
reaction is run and analyzed, only the nucleotides that are most abundant are identified. This was noticed when sequencing a bivalent vaccine, SVS-510™, where only the predominant variant portion of the vaccine was identified and not the classic portion. To overcome this, the seeds of that vaccine were isolated and sequenced independently. This variant portion of this bivalent vaccine was identified in figure 1 and 2 as SVS-510 (Variant portion) while the classical portion was identified as SVS-510 (Standard portion).

The research potentials of this technology have allowed quick profiling of IBDV strains that are causing lesions in the field. This is a diagnostic tool that can assist veterinarians in choosing which vaccines to use based on the nucleic acid sequence of the hypervariable region of VP2. This in turn may allow the identification of potentially new vaccine strains.

As with all molecular techniques that are used to predict the relative similarities and differences between IBDV strains, determining the actual antigenic differences among viruses requires testing *in-vivo* (14).

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Acknowledgment:

The authors would like to thank Dr. Stanley Kleven and Dr. John Glisson for reviewing this manuscript. Histopathological screening for samples submitted after August 2004 was done by Dr. Frederick Hoerr.
Figure Legend

Figure 3.1: Consensus nucleic acid phylogram of common IBDV vaccines and strains, in addition to some field strains identified from paraffin embedded tissues deduced from 1000 trees by Neighbor-Joining method. Numbers at the forks indicate the number of times (as a percentage) the groups consisting of the sequences which are to the right of that fork occurred after the generation of 1000 trees. P-distance bar in bottom of figure. Arrows indicate field cases identified in our laboratory which are labeled by case number. Other isolates are identified with GeneBank accession numbers and trade name. Samples without accession numbers were also extracted and sequenced in our laboratory. Only 213 nucleotides were sequenced in case 087 A.

Figure 3.2: Consensus amino acid phylogram of common IBDV vaccines and strains, in addition to some field strains identified from paraffin embedded tissues deduced from 1000 trees by Neighbor-Joining method. Numbers at the forks indicate the number of times (as a percentage) the groups consisting of the sequences which are to the right of that fork occurred after the generation of 1000 trees. P-distance bar in bottom of figure. Arrows indicate field cases identified in our laboratory which are labeled by case number. Other isolates are identified with GeneBank accession numbers and trade name. Samples without accession numbers were also extracted and sequenced in our laboratory. Only 213 amino acids were deduced in isolate 087 A.
Fig 3.1
Table 3.1: Number of IBDV strains isolated and identified from 2003-2005 according to geographical origin of the paraffin embedded tissues

<table>
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<td>62</td>
<td>9</td>
<td>15</td>
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*Unique: Any virus that did not group with common strains of IBDV or possessed different genomic characteristics.

§Strains that could not be sequenced: The sequence data had too many unknown nucleotides (N’s) that when aligned with other viruses, it was unidentifiable but melting curve analysis as well as histopathological examination confirmed it was an IBDV
Table 3.2: Distribution of IBDV strains in the United States of America from 2003-2005

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<th>Variant E</th>
<th>Unique*</th>
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<tr>
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*Unique: Any virus that did not group with common strains of IBDV or possessed different genomic characteristics.
Strains that could not be sequenced: The sequence data had too many unknown nucleotides (N’s) that when aligned with other viruses, it was unidentifiable but melting curve analysis as well as histopathological examination confirmed it was an IBDV
Table 3.3: Distribution of IBDV strains in the world during 2003-2005

<table>
<thead>
<tr>
<th>Location</th>
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<th>Variant A</th>
<th>Variant E</th>
<th>Unique</th>
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<td>Argentina</td>
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</tr>
<tr>
<td>Dominican Republic</td>
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<tr>
<td>El-Salvador</td>
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<td></td>
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<tr>
<td>Guatemala</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>4</td>
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<td></td>
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<td>Peru</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Venezuela</td>
<td>2*</td>
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<td>China</td>
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<tr>
<td>Japan</td>
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</table>

* vvlIBDV
Chapter IV

Detection of infectious bursal disease virus from formalin-fixed paraffin-embedded tissue by immunohistochemistry and real-time reverse transcription-polymerase chain reaction\(^1\)

\(^{1}\)Mohamed M. Hamoud, Pedro Villegas and Susan M. Williams
Submitted to Journal of Veterinary Diagnostic Investigations
Abstract

Formalin fixed paraffin embedded tissues blocks are used routinely to diagnose the economically important immunosuppressive infectious bursal disease virus (IBDV) in chickens. Immunohistochemical detection of viruses in tissue blocks has been done with varying results between laboratories. Extraction of IBDV RNA from tissue blocks allows IBDV strain identification at a molecular level. This allows correlation between virus identity and histological lesions present in the tissue. Experimentally RT-PCR detectable IBDV RNA could always be extracted from tissue blocks with acute +3 or higher histological lesion scores. However, many blocks from diagnostic field cases did not yield detectable IBDV RNA, in spite of having severe IBDV histological lesion scores. The reason for this can be the effect different formalin fixation conditions have on RNA detection from tissue blocks. To study the effect of various fixation parameters on RNA extraction and immunohistochemical detection of IBDV, bursas with high viral loads and maximum histological lesion score of 4 for IBDV were fixed in formalin under various conditions (different pH levels, temperatures, concentrations of formalin, and fixation duration). Only tissues fixed in formalin with a pH of 7.0, concentration of 5 or 10% formaldehyde, storage temperature of 25°C or less, and kept for up to 2 weeks in formalin yielded detectable IBDV RNA upon extraction. No RNA could be detected from tissues fixed under extreme temperature, pH or formalin concentrations. Optimal fixation conditions for IHC detection of IBDV were 10% formalin concentration, pH 7.0 and temperature of 4°C, where maximum intensity of immunostaining was observed.
**Key words:** Infectious Bursal Disease, Chicken, Immunohistochemistry, RNA extraction, Paraffin embedded tissue, formalin fixation.
Introduction

Classical infectious bursal disease (IBD), or Gumboro disease, is an acute, highly contagious viral disease of young birds characterized by severe necrosis, hypoplasia, or atrophy in the bursa of Fabricius, mainly observed at 3–6 weeks of age. IBD has worldwide distribution, and the effects of the disease are economically significant to the commercial poultry industry. Highly virulent strains cause increased mortality rates in chickens. Survivors of classical IBD are immunosuppressed, which is also the outcome of IBDV infection with antigenic variant strains. Variant IBDV differ from the classical IBDV strains in that they usually do not cause mortality and can infect chicken younger than 3 weeks of age. Variant viruses cause immunosuppression by viral induced apoptosis of lymphocytes in the bursa. Immunocompromised chickens have an increased susceptibility to other pathogens, such as Newcastle disease virus, infectious bronchitis virus, infectious laryngotracheitis virus, Salmonella spp, or to other opportunistic agents such as Escherichia coli. Furthermore, immunosuppressed birds may not respond properly to vaccination and may suffer severe or persistent postvaccinal reactions.

Infectious bursal disease virus (IBDV) is a member of the Birnaviridae family with a genome consisting of two segments of double-stranded RNA. IBDV is a non-enveloped icosahedral virus with a diameter of 55-60 nm. The smaller segment of RNA encodes viral protein (VP) 1, which is the RNA polymerase of the virus. The large segment encodes a polyprotein which is processed into three structural proteins, VP2, VP3, and VP4. The large segment also encodes the structural protein VP5 in another reading frame. Of these different genes, VP2 gene encodes for the major antigenic
protein and its hypervariable region is what confers variability among different IBDV strains.

The severity of IBDV infection depends on the virulence of the virus, age of birds, and level of circulating maternal antibodies. Cytotoxic T cells may exacerbate virus-induced cellular destruction by lysing cells expressing viral antigens. T cells may also promote the production of pro-inflammatory factors, such as nitric oxide, increasing tissue destruction\textsuperscript{18}. Outbreaks with high mortality rates caused by very virulent IBDV (vvIBDV) strains have been reported in Europe, Asia and Latin America.\textsuperscript{21} Subclinical and immunosuppressive forms of the disease are prevalent in the United States, where reduction of body weight and lack of uniformity may be produced in mild outbreaks.\textsuperscript{13}

Real-time RT-PCR (rt-RT-PCR) has been used to previously to detect IBDV in tissues of infected chickens that were either frozen or stored in phenol.\textsuperscript{13,14} IBD viral RNA has also been detected by rt-RT-PCR from formalin-fixed, paraffin-embedded bursas.\textsuperscript{17} Using this technique, formalin fixed paraffin embedded bursa of Fabricius from chickens experimentally infected with IBDV, and with histological lesion scores of +3 or more always yielded rt-RT-PCR detectable IBDV RNA. However, diagnostic field cases did not always yield rt-RT-PCR detectable RNA. Some samples (225 blocks over a 3-year period) that had acute +4 histological lesion scores were negative for IBDV with rt-RT-PCR. This finding occurred more often in diagnostic samples submitted from outside the United States than in those originating from the United States (paper to be published in Avian Diseases). The reason why some infected samples tested negative by rt-RT-PCR has yet to be determined. It was recommended that these samples should be fixed in 10 % buffered formalin and kept at room temperature, but pH analysis of formalin and H&E
artifacts indicated that some of the samples were fixed in more concentrated formalin, formalin with variable pH levels or subjected to high temperatures during formalin fixation. These observations suggested that formalin fixation conditions might have an effect on rt-RT-PCR detectable RNA from tissue blocks.

Previous research has proved that formalin fixation of tissues is suitable for immunohistochemistry because formalin is a relatively mild fixative for preservation of antigenic reactivity. However, formalin fixation can impair or block the antigenic reactivity of certain proteins. This impairment may be due to extensive cross-linking of proteins by formaldehyde, where amino groups become blocked and antibodies to those amino groups do not participate in IHC staining.

The objective of this study was to experimentally simulate field conditions in terms of storage time, temperature, pH of unbuffered formalin, and different concentrations of buffered formalin to test their effect on IBDV RNA extraction from paraffin embedded tissue for use in rt-RT-PCR and immunohistochemical staining.

Materials and Methods

Birds and experimental design: A total of 95 specific pathogen free (SPF) chickens were hatched from fertilized eggs for this experiment. All birds were housed in Horsfall-Bauer isolation units in 19 groups of five and maintained under positive pressure. Food and water were provided ad libitum. At 3 weeks of age, the chickens were inoculated with IBDV Blen vaccine which contains the 2512 strain. Inoculation routes of intranasal, oral and intramuscular administration (1x 10^4.0 EID 50/route) were given simultaneously to each infected bird to ensure a high viral load in infected tissue. Five uninfected birds
were maintained as uninfected controls. Bursal tissue was collected 5 days post inoculation and subjected to different fixation protocols as described in the experimental design shown in Table 1.

**Histopathology and immunohistochemistry.** A 37% formaldehyde solution was diluted in either neutral phosphate buffered saline (PBS), distilled water or was used undiluted for formalin fixation. The pH was adjusted with either 10% sodium hydroxide or 5% hydrochloric acid. Paraffin-embedded tissues were sectioned, mounted, stained with hematoxylin and eosin (H&E) by using an automated stainer, and examined for lesions by light microscopy. All sections were assigned a lesion severity score. For all samples, a lesion score of 1 represented no lesions; 2 represented mild reduction in overall follicle size; 3 represented moderate reduction in follicle size; and 4 represented either necrosis or follicle atrophy. All procedures for immunohistochemistry (IHC) were done at room temperature. Five-micron thick tissue sections were cut from paraffin-embedded samples and mounted on charged glass slides. Paraffin was melted from slides (10 min at 65°C) and removed by immersion in Hemo-De three times (5 min each). Slides were then air dried. Antigen retrieval was done by incubating slides with 10% proteinase K for 5 min at room temperature. IHC staining was performed with an automated stainer using a commercial kit according to the manufacturer’s recommendations. The primary antibody used was a mouse monoclonal antibody specific to and cross-reactive among all IBDV strains. The secondary antibody was a peroxidase labeled polymer conjugated to anti-mouse and anti-rabbit immunoglobulins. After IHC staining, sections were counterstained with hematoxylin, cover slipped, and examined by light microscopy. The degree of IBDV infection in each section was scored as follows: - = no infection;
+ = minimal infection (<10% of cells present); ++ = moderate infection (>10% and <30% of cells present); and +++ = intense infection (>30% of cells present).  

**RNA extraction and quantification.** RNA was extracted from formalin-fixed, paraffin-embedded bursas and examined for IBDV nucleic acid by rt-RT-PCR.  

Sections totaling 50 µm in thickness were cut from each formalin-fixed, paraffin-embedded tissue block, deparaffinized in Hemo-De™, washed with 100% ethanol, and digested with 25 µg/ml proteinase K™ for 1 hr at 50°C. RNA was extracted using Trizol™ according to the manufacturer’s recommendations, resuspended in 25 µl of 90% dimethyl sulfoxide, and frozen at -80°C until assayed. Extracted RNA was quantified using eppendorf biophotometer³ and 50-2000 µl UVette¹.  

**Real-time RT-PCR.** The RNA extracted from tissue blocks was denatured at 95°C for 5 min and kept on ice. A rt-RT-PCR was performed using reagents from the LightCycler RNA Amplification SYBR Green I Kit™ and the LightCycler machine™ according to manufacturer’s recommendations. The primers (B5 5’ TCTTGGGTATGTGAGGCTTG and B4 3’ GGATGTGATTGGCTGGGTTA)¹⁷ were designed to amplify a 400-base pair (bp) segment of the hypervariable region of the IBDV VP2 gene (GenBank accession number DQ355819). Briefly, RT was performed at 55°C for 30 min, followed by denaturation at 95°C for 30 sec. Fifty-five PCR cycles were performed, consisting of denaturation (95°C for 1 sec), hybridization (55°C for 10 sec), and extension (72°C for 13 sec). A melting curve analysis was done with an initial denaturation at 95°C (for 1 sec.). DNA melting was accomplished with an initial temperature of 65°C for 10 sec and a gradual temperature increase of 0.1°C/sec until 95°C was reached. After which, there was a cooling segment and a gradual temperature decrease of 2°C/sec till the holding
temperature 20°C was reached. The melting temperature of the expected 400-bp amplicon was between 82°C and 84°C. This estimated melting temperature was used to confirm the presence of IBDV specific products obtained using real-time RT-PCR. A no template control (DEPC-water) and a positive IBDV control was used in each run. Electrophoresis of real-time RT-PCR products were run on 1.5% agarose gel and visualized by ethidium bromide staining, to correlate between melting curve peaks and size of amplified product. This was done initially to verify the specificity of the melting curve peaks seen in the rt-RT-PCR reaction printouts.

Results

Histology and Immunohistochemistry. Out of 90 infected birds, 89 bursas had lesion scores of 4, the remaining bursa had a lesion score of 3. All 5 chickens from the uninfected negative control group had lesion scores of 1. All of the IBDV uninfected negative control slides were negative using immunohistochemistry. All 5 IBDV infected positive control slides demonstrated strong immunoreactivity for IBDV. This demonstrated the specificity of the IHC technique used (Fig. 1, Table 2).

Histological evaluation of 5 bursas from each treatment group demonstrated that for IBDV infected bursas fixed in different concentrations of formalin, the optimal formalin concentration for formalin fixed tissues used for IHC staining was 10% formalin (Table 2). The use of 5% formalin for formalin fixation produced a light nonspecific background staining of erythrocytes and fibroblasts. IBDV antigen was not detected in slides that came from paraffin embedded tissues fixed in 20% or 37% formalin (Table 2). One area in one of the 5 samples fixed in 37% formalin had lymphoid cells with positive staining. Light staining of apoptotic bodies, epithelial cytoplasm and vacuoles were
present and was interpreted as non-specific background staining. Hematoxylin and eosin staining showed severe lymphoid depletion, atrophy in follicles, and increase in interfollicular stroma. Artifacts seen in tissues fixed with 20% or 37% formalin concentrations included granularity of interplical mucin, and mucin in epithelial cysts. Tissues fixed in 37% formalin had diffuse eosinophilic staining.

Tissues fixed in 10% buffered formalin at different fixation temperatures showed variable IHC staining. Fixation at 4°C yielded the best staining. The number of IHC staining positive cells decreased as the fixation temperature increased (Table 2). H&E artefacts observed in tissues stored at 50°C included streaming and smudging of nuclei, loss of follicular architecture and very plump fibroblasts in stroma of atrophied plica.

The pH of formalin used for fixation had the most variable effects on IHC staining. Optimal staining was observed when infected bursal tissue was fixed at pH 7.0. At pH 2.0 minimal IHC staining of apoptotic bodies within the center of the follicles and very rare moderate staining of mononuclear cells was observed in all 5 bursas of this group. At pH 5.0 there was a complete loss of staining. All tissues fixed in formalin at pH 9.0 and 13.0 exhibited non-specific staining of apoptotic bodies and cellular debris. H&E staining artifacts at pH 2.0 included tinctorial variations of epithelial cells, while at pH 5.0 there was cell shrinkage. At pH 9.0 there was streaming of epithelial nuclei and at pH 13.0 there were epithelial shrinkage and compressed fibroblasts.

**IBDV RNA extraction and real time RT-PCR.** The amount of extracted RNA extracted from tissue blocks ranged from 159.92 – 1194.66 µg/ml. Specificity of rt-RT-PCR melting curve analysis was verified by running amplicons on a 1.5% agarose gel stained with ethidium bromide (Fig. 2). Detectable IBDV RNA was extracted from all 5
bursas stored in pH 7 as well as 1 of the 5 bursas stored in pH 13,0. All tissues fixed pH 2.0, pH 5.0 and pH 9.0 had a histopathological lesion score of 4 but none yielded detectable IBDV RNA that was identifiable by rt-RT-PCR melting curve. (Table 3). Real-time RT-PCR melting curve identifiable RNA was extracted from bursas stored at temperatures 4°C and 25°C but not from 50°C (Table 3). Real time RT-PCR detectable IBDV RNA was extracted from bursas of Fabricius stored in 5% or 10% buffered formalin, but not from those tissues stored in 20% buffered formalin or 37% unbuffered formalin (Table 3). Storage time for up to 2 weeks had no effect on IBDV RNA extraction and identification, as melting curve peaks specific for IBDV were present at all fixation times studied. However, storage times longer than 24 hours increased the melting peak by 1°C; and after 2 weeks it was increased by 2°C. (Fig. 3).

Discussion

Formalin fixation is a common method for tissue preservation in preparation for histological evaluation of tissues. For immunohistochemistry, small (10 X 10 X 3 mm) tissue fragments fixed promptly in neutral buffered formalin for 6-24 hours generally had adequate cytological preservation and immunolocalization, with minimal antigen masking. Variations in fixation times or conditions cause the majority of false negatives in immunohistochemistry. It was reported that lack of consistency in formalin fixation protocols among laboratories, influences the outcome of staining in immunohistochemistry. Formaldehyde fixes tissue by reacting primarily with basic amino acids to form cross-linked “methylene bridges”. Formalin fixation conditions have a direct effect on number of methylene bridges. The number of methylene bridges and
which amino groups are cross-linked in the tissue are important variables in the outcome of immunohistochemical staining, and could also be an important factor in the ability to extract rt-RT-PCR detectable RNA from tissue blocks.

Tissues fixed in 20% or 37% formalin undergo shrinkage\textsuperscript{10} and may suffer from alteration of epitope conformation. The IBDV epitopes detected by the monoclonal antibodies used in this study are conformationally dependent;\textsuperscript{5} thus fixation conditions such as formaldehyde concentration or pH that may cause changes in epitope conformation rendering them undetectable by these monoclonal antibodies. Maximum tissue fixation, i.e. cross-linking, occurs in the pH range of 4 to 5.5\textsuperscript{9}, therefore, the presence of too many cross-links or cross-links of certain amino groups may cause antigen masking.\textsuperscript{20} This may be the reason why there was minimal or no immunostaining in tissues fixed in pH $\leq 5.0$. Inconclusive IHC staining at pH $\geq 9.0$ may also be due to tissue shrinkage or alteration of reactive epitopes.

Immunohistochemical staining was best in tissues fixed at 4°C. Increased temperature reduced specific IHC staining, but did not eliminate it. The reduction in staining may be due to tissue shrinkage at temperatures higher than 4°C.\textsuperscript{8} This result is in agreement with Key’s findings; however the mechanism for this effect is unknown.\textsuperscript{12}

Real-time RT-PCR melting curve analysis of infected and non-infected controls validated the RNA extraction procedure. Real-time RT-PCR on known IBDV genomic material was performed as a positive rt-RT-PCR control and on DEPC-water as a negative rt-RT-PCR control. Extracting genomic RNA for identification of IBDV from bursas of Fabricius stored at acidic pH (pH 5.0 and pH 2.0) was unsuccessful in all 10 tissue blocks. Previous research which found DNA damage in cells at low pH,\textsuperscript{11} may
explain the lack of extracted genomic RNA in this experiment. Unbuffered formalin oxidizes to formic acid and an acidic environment causes degradation of nucleic acids because the β-glycosidic bonds in the purine bases are hydrolyzed at pH 4.\(^4\) Loss of some amino group cross-links occurs at low pH and may result in loss of RNA from the fixed tissue.\(^3\) Extraction of genomic RNA at a basic pH (pH 9.0, and 13.0) was also unsuccessful and the reason for this is unknown.

The thermodynamic stability of a nucleic acid duplex strongly depends on the denaturation temperature of the duplex at atmospheric pressure.\(^6\) Denaturation of genomic RNA may have occurred when tissues were formalin fixed at 50°C for 5 days and might explain the absence of rt-RT-PCR detectable RNA from these blocks.

At formalin concentrations of 20% or 37 %, the extraction of RNA from tissue blocks yielded no rt-RT-PCR detectable RNA. This may be due to excessive crosslinking of amino groups resulting from the acidic nature of formalin at higher concentrations; indeed, maximum tissue fixation, i.e. cross-linking, occurs in the pH range of 4.0 to 5.5,\(^9\) thus hindering genomic RNA extraction.

Fixation time had no effect on RNA extraction or subsequent identification (Fig.3), but longer fixation times are not recommended due to the higher frequency of non-reproducible sequence alterations. This is demonstrated by the increase in melting peak temperature over time. Formalin may cause cross-linking of cytosine nucleotides on either strand. As a result, in PCR the Taq-DNA polymerase fails to recognize the cytosine and incorporates an adenine in the place of a guanosine, creating an artificial C-T or G-A mutation.\(^{22}\)
In summary, for optimal IBDV RNA extraction and sequence identification, tissues should be fixed in formalin at a pH of 7.0, concentration of 5% or 10% and stored at 4°C to 25°C for 7 days at most. Extremes in temperature, pH or formalin concentration have a negative effect on tissues used for RNA extraction. Longer tissue storage times in formalin may cause inaccuracies in sequencing results. Optimal conditions for fixation of tissues infected with IBDV that will be used for IHC evaluation are 10% formalin concentration, pH 7.0 and temperature of 4°C. For practical purposes, fixation temperature of 4°C is recommended for both procedures. This can be achieved practically by placing formalin jars or whirlpaks in a cooler with a cardboard separating the ice from the samples.

**Acknowledgments:** The authors would like to thank Dr. Stanley Kleven and Linda Purvis for reviewing this manuscript.

**Sources and manufacturers:**

a. Sunrise Farms, Catskill NY

b. IBD-Blen™<sup>a</sup> vaccine, Merial Select, Gainesville GA, U.S.A.

c. Leica AutoStainer XL, Nussloch, Germany

d. Superfrost/Plus; Fisher Scientific Pittsburgh, PA, U.S.A.

e. HemoDe Fisher Scientific, Pittsburgh, PA, U.S.A.

f. Proteinase K: DAKO, Carpinteria, CA, U.S.A.

g. Leica ST 5050, Nussloch, Germany

h. DAKO Envision System; DAKO Carpinteria, CA, U.S.A.

i. Monoclonal antibody #HB9490, ATCC, Manassas, VA 20108 U.S.A.
j. Trizol: Life Technologies, Inc., Gaithersburg, MD, U.S.A.

k. Eppendorf biophotometer, Eppendorf AG, Hamburg, Germany

l. Uvette, Eppendorf AG, Hamburg, Germany

m. ROCHE Molecular Biochemicals, Indianapolis, IN, U.S.A.

References


Figure Legend

**Figure 4.1.** Photomicrograph of Bursa of Fabricius. (IHC technique specificity control for IBDV infected and non-infected tissues). 1a: Non infected tissue stained with IHC. 1b: IBDV infected bursa stained with IHC. All tissues were fixed in 10% buffered formalin at a temperature of 4°C and pH 7.0. IBDV infected controls show brown positive IHC staining (indicated by arrow) of cortical intrafollicular lymphocytes. (100X)

**Figure 4.2:** RT-PCR product of amplified VP2 fragment: lane a, molecular size marker; lane b, amplified VP2 fragment from IBDV control positive; lane c, amplified VP2 fragment from RNA extracted from a tissue block, lane d, negative RT-PCR control (primers with water); lane e, negative extraction control from RNA extracted from an uninfected tissue block

**Figure 4.3.** Real time RT-PCR melting curve analysis for IBDV amplicons extracted from tissues fixed in 10% buffered formalin at 4°C and submitted to different fixation durations (hours). Melting curve peak after 24 hours storage was 83°C, but increased to 84°C after 48-120 hours of storage, and after 2 weeks of storage, to 86°C, indicating changes in nucleotide sequences. RT-control is the reverse transcriptase PCR control for a known IBDV and extraction control is a known paraffin embedded block with extractable IBDV genetic material.
Figure 4.1:

Figure 4.2:
Figure 4.3:

Fluorescence - d(F)/dT

Temperature (°C)

- RT Control
- Extraction control
- 2 weeks
- 120 hours
- 96 hours
- 72 hours
- 48 hours
- 24 hours
- Extraction control
- 2 weeks

Calculated Method: Polynomial
Table 4.1: Fixation conditions for bursal tissues from IBDV infected and control chickens.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Storage conditions</th>
<th>Fixation treatment&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin concentration</td>
<td>4°C for 5 days</td>
<td>5 %</td>
</tr>
<tr>
<td>Formalin temperature</td>
<td>10 % Buffered formalin for 5 days</td>
<td>10 %</td>
</tr>
<tr>
<td>pH</td>
<td>4°C, in 10% Non buffered formalin for 5 days</td>
<td>25°C</td>
</tr>
<tr>
<td>Duration in formalin</td>
<td>4°C, 10 % Buffered formalin</td>
<td>5.0 days</td>
</tr>
<tr>
<td>Infected Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10% Buffered formalin at 4°C</td>
<td>No variables</td>
</tr>
</tbody>
</table>
Table 2: Details of treatment groups, storage conditions, and variables

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Storage Conditions</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Infected Control c</td>
<td>10% Buffered formalin at 4°C</td>
<td>No variables</td>
</tr>
</tbody>
</table>

a Five individual bursas were tested per treatment.

b This group was also used to represent 5 days duration in formalin

c Non infected and infected controls were stored in 10% buffered formalin at 4°C to validate IHC procedure.
Table 4.2: Histopathology and IHC mean staining scores of 5 bursas of Fabricius from chickens infected with IBDV and subjected to different fixation conditions

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Lesion Score $^{a}$</th>
<th>IHC $^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration $^{c}$</td>
<td>5%</td>
<td>4 ± 0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>4 ± 0</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>3.2 ± 0.44</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>37%</td>
<td>4 ± 0</td>
<td>-</td>
</tr>
<tr>
<td>Formalin concentration</td>
<td>4°C</td>
<td>4 ± 0</td>
<td>+++</td>
</tr>
<tr>
<td>temperature $^{d}$</td>
<td>25°C</td>
<td>4 ± 0</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>4 ± 0</td>
<td>+</td>
</tr>
<tr>
<td>pH of formalin</td>
<td>2</td>
<td>4 ± 0</td>
<td>+</td>
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<tr>
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<td>5</td>
<td>4 ± 0</td>
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<td>7</td>
<td>4 ± 0</td>
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<tr>
<td></td>
<td>9</td>
<td>4 ± 0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>4 ± 0</td>
<td>±</td>
</tr>
<tr>
<td>Non infected control</td>
<td>10% Buffered</td>
<td>1 ± 0</td>
<td>-</td>
</tr>
<tr>
<td>formalin at 4°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected control</td>
<td>10% Buffered</td>
<td>4 ± 0</td>
<td>+++</td>
</tr>
<tr>
<td>formalin at 4°C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ Lesion Score: 1 = No lesion, 2 = Mild variation in follicle size, 3= Moderate variation in follicle size, 4 = Either Necrosis or follicle atrophy

$^{b}$ - = No staining; + = Minimal staining, ++ = Moderate Staining, +++ = Severe staining.
± = inconclusive.

c Samples fixed at 4°C and pH 7

d Samples fixed at 10% formalin and pH 7

e Samples fixed at 10% formalin and 4°C
Table 4.3: Effect of fixation conditions on extraction of RNA from bursas of Fabricius from chickens infected with IBDV

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Number of real-time RT-PCR Positive / total number tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin concentration a</td>
<td>5 %</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>10 %</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>20 %</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>37 %</td>
<td>0/5</td>
</tr>
<tr>
<td>Fixation temperature b</td>
<td>4°C c</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>0/5</td>
</tr>
<tr>
<td>pH of formalin c</td>
<td>2</td>
<td>0/5</td>
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<tr>
<td></td>
<td>5</td>
<td>0/5</td>
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<tr>
<td></td>
<td>7</td>
<td>5/5</td>
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<td>9</td>
<td>0/5</td>
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<td></td>
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<td>1/5</td>
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<tr>
<td>Duration in formalin d</td>
<td>1d</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>2d</td>
<td>5/5</td>
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<tr>
<td></td>
<td>3d</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>4d</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>14d</td>
<td>5/5</td>
</tr>
<tr>
<td>Non infected control</td>
<td>10% Buffered formalin at 4°C</td>
<td>0/5</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>Extraction control</td>
<td>10% Buffered formalin at 4°C</td>
<td>5/5</td>
</tr>
</tbody>
</table>

^a Samples fixed at 4°C and pH 7
^b Samples fixed at 10% formalin and pH 7
^c Samples fixed at 10% formalin and 4°C
^d Samples fixed at 4°C % formalin and pH 7
^e This group was also used to represent 5 days duration in formalin
Chapter V
Development of Infectious Bursal Disease (IBD) Viral protein 2 (VP2) and the hypervariable region of VP2 in *Pichia pastoris* for the prevention of IBD in chickens.

Mohamed M. Hamoud, Pedro Villegas
\(^1\)To be submitted to Vaccine.
Abstract

Infectious bursal disease virus (IBDV) causes immunosuppression in chickens and is an infectious disease of global economic importance in poultry. Protection against IBDV is achieved by vaccination with live or inactivated vaccines. Live viruses cause some bursal damage while killed vaccines are costly to produce. Therefore, the goal of this study was to produce a subunit vaccine against IBDV, without actual manipulation of live or killed viral particles, that protects chicken against IBDV infection. The VP2 gene from the Edgar strain of IBDV was obtained from frozen bursas, while that for the hypervariable region of VP2 was obtained from formalin fixed paraffin embedded bursal tissues. The IBDV VP2 gene and the hypervariable region of VP2 were cloned into Pichia pastoris and protein production was confirmed by western blot analysis. Specific pathogen free 1-day old chicks vaccinated with recombinant VP2 protein showed the best protection against 3 week-old challenge with the Edgar strain of IBDV, as there was no morbidity or mortality in the VP2 vaccinated birds compared to up to 90% morbidity and 50% mortality in placebo vaccinated birds. However, recombinant vaccines did not totally protect against viral replication in bursa as confirmed by high histopathological lesion scores and immunohistochemical detection of IBDV in infected tissue. This was probably due to the high viral dose and high virulence of the challenge virus used.
**Introduction:**

Infectious bursal disease virus (IBDV) is the etiological agent of Gumboro disease or infectious bursal disease (IBD). IBDV destroys the B lymphocyte precursors found within the bursa of Fabricius, resulting in bursal atrophy in young chickens [1]. In unprotected flocks, the virus may cause mortality and / or immunosuppression [2]. There are two serotypes of IBDV: 1 and 2. All pathogenic viruses belong to serotype 1, whereas serotype 2 viruses are non-pathogenic for both chickens and turkeys [3],[4]. Very virulent (vv) IBDV strains emerged in Europe in the late eighties, causing up to 60% mortality [5], [30], [6]. Strains of vvIBDV have also spread to other continents [7], [8].

IBDV belongs to the family Birnaviridae (genus Avibirnavirus) which includes viruses with bisegmented dsRNA genomes [9]. Genome segment A has 2 overlapping open reading frames (ORF). The first ORF of segment A encodes a non structural protein, VP5 and is possibly involved in virus release [10], [11]. The other encodes a precursor polyprotein in a large ORF, the product of which is cleaved by autoproteolysis to yield mature VP2 (outer capsid), VP4 (protease), and VP3 (inner capsid) [12]. Genome segment B encodes the virus polymerase, VP1 [13]. VP2 is the major host-protective antigen of IBDV. It contains at least three independent epitopes responsible for the induction of neutralizing antibody [14], thus the antigenic characterization of IBDV has been based mainly on the study of the VP2 gene where some amino acid changes have been shown to be the basis for antigenic variation [15], or have been proposed as putative markers for vvIBDV [16], [17],[18].

Controlling infectious bursal disease (IBD) and its associated immune suppression is critical to the poultry industry. This control is achieved by passive
protection of baby chicks through maternal antibodies that come from breeder hens that have been given conventional live attenuated and/or inactivated IBD vaccines; or by the use of live IBD vaccines in broiler chicks, layers or young pullets to provide active protection against IBDV, or by a combination of both.

The IBDV VP2, expressed in yeast [19], [20], the baculovirus system [21], [22], [23], [24], recombinant NDV vaccines [25] or via transgenic Arabidopsis thaliana plants [26] have been studied for the use as subunit vaccines. An advantage of this technology is that a vaccine based on VP2 alone should allow monitoring of the field situation by the discrimination between antibody induced by vaccine (anti-VP2 only) and that induced by infection (anti-VP2 and VP3) [27]. Genetically attenuated viruses generated by the use of reverse genetics can also be used for the generation of new vaccines, although interference of maternal antibodies would still exist. Therefore, as recombinant proteins are less sensitive to neutralization by anti-IBDV maternal antibodies, recombinant viral vaccines expressing the VP2 protein, such as fowl pox virus [28], herpesvirus of turkey (HVT) [29], [30], or fowl adenovirus [31] might be able to mount an active immune response.

Pichia pastoris is a facultative methylotrophic yeast which utilizes methanol. The methanol metabolic pathway of Pichia pastoris involves a unique set of enzymes. In the first step of this pathway methanol is oxidized to generate formaldehyde and hydrogen peroxide which is then decomposed to water and molecular oxygen by catalase. The oxidation is carried out by two alcohol oxidase genes AOX1 and AOX2 [32]. AOX1 is the more active alcohol oxidase and may reach as much as 30% of the total protein in the cell when cultured under growth-limiting rates of methanol. This gene’s promoter is
utilized for expression of heterologous genes. The expression of heterologous proteins in *Pichia pastoris* is fast, simple and inexpensive. Strong aerobic growth allows culturing at high cell densities. High levels of foreign protein expression have been shown for this vector [33].

The objective of this study was to produce a recombinant protein or polypeptide from IBDV genomic material extracted from formalin fixed paraffin embedded tissue blocks without actual manipulation of live or killed virus particles. Proof of principal of this technique could provide use of archival tissue as a valuable source of genomic material for production of proteins or actual viruses using reverse genetics. Here we are aiming for protein production. Another objective was to produce an efficient recombinant vaccine in a system that allows mass production.

2. Materials and methods

2.1. Isolation of the virus

The Edgar strain of IBDV was isolated and characterized in our laboratory. The virus was passed in specific pathogen free chicks several times. The bursa of Fabricius (bursa) was collected from clinically ill birds then ground and homogenized. The cells were frozen and thawed three times and centrifuged at 12,000 × *g*, 4 °C for 10 min. The supernatant was collected and viral particles were pelleted by centrifugation at 96,400 × *g* for 2 h. The pellet was resuspended in Tris- ethylenediaminetetraacetic acid buffer and kept at -20 °C until use.

2.2. Isolation of IBDV RNA

The Edgar IBD virus was incubated for 3 h in a solution containing 0.01M Tris, 0.01M NaCl, 0.01M EDTA, 0.5% SDS (w/v) and 50 μg/ml proteinase K. IBDV RNA
was separated by electrophoresis on a 1.0% agarose gel. The two RNA fragments of the virus, with sizes of 3.4 and 2.9 kb, were visualized by ethidium bromide staining. The large fragment was agarose gel purified and extracted by use of gel extraction kit (QIAquick, Qiagen Sciences, Valencia, CA). This was the source of genomic material for VP2.

RNA was extracted from formalin-fixed, paraffin-embedded bursas and examined for IBDV nucleic acid by real-time RT-PCR [34]. Sections totalling 50 µm in thickness were cut from each formalin-fixed, paraffin embedded tissue block, deparaffinized in HemoDe (Fisher Scientific, Pittsburgh, PA), washed with 100% ethanol, and digested with 25 µg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 1 hr at 50 °C. RNA was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s recommendations, diluted in 25 µl of 90% dimethyl sulfoxide, and frozen at -80 °C until assayed by RT-PCR. This was the source of genomic material for the hypervariable region of VP2 called partial VP2 (pVP2).

2.3. Cloning of VP2 and pVP2 in TOP10 cells and GS-115 Pichia pastoris cells.

Synthetic oligonucleotides (Invitrogen, Carlsbad, CA) corresponding to the 5’ and 3’ conserved ends of VP2 which included restriction sites EcoR1 and Not1, respectively, as well as an ATG start codon on 3’ end and a factor XA cleavage site on the 5’ end were used for the production of cDNA by RT-PCR. Template dsRNA (5 µl) was heated at 97 °C for 5 min and immediately transferred to ice. RT-PCR master mix was then prepared as follows: 1 µl (10 µM) of sense and anti-sense primers, 25 µl of 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM MgSO₄), and 2 µl SuperScript® III/ Platinum® Taq mix were added to 16 µl autoclaved distilled water. The template
RNA and RT-PCR mixture were gently mixed to a final volume of 50 µl. The synthesis of cDNA was done at 55 °C for 30 min. The PCR scheme was 40 cycles of 94 °C for 15 sec, 55 °C for 30 sec and 68 °C for 100 sec followed by a final extension cycle of 68 °C for 5 min. The amplified segment was separated on a 1.2% agarose gel and the appropriate band was excised and purified.

The 2 fragments, corresponding to VP2 and pVP2 of the Edgar strain of IBDV were cleaved by EcoR1 and Not1 restriction enzymes that were introduced by the oligonucleotides during synthesis. The isolated fragments were mixed with 1 µl pCR 2.1 vector and according to manufacturer’s recommendation were cloned into TOP10 Escherechia coli cells. After 24 hour growth on Luria-Bertani (LB) plates containing kanamycin (50µg/ml) white colonies were picked up and grown in 5 ml of LB kanamycin broth (50 µg/ml) at 37 °C in a shaker incubator set at 225 rpm. Minipreps were performed using Qiaprep® Spin kit (Qiagen Sciences, Valencia, CA), and cloned inserts were double digested with EcoR1 and Not1 and visualized on an ethidium bromide gel. Inserts were then ligated to pPICZ A plasmids provided in EasySelect™ Pichia expression kit (Invitrogen, Carlsbad, CA) which had also been digested with the EcoR1 and Not1 restriction enzymes. The ligated DNA construct was transformed into Escherechia coli TOP10 cells and white colonies that grew on low salt LB plates containing zeocin 25 µg/ml were isolated. Plasmid DNA was extracted and analyzed for the presence of VP2 or pVP2 using restriction enzymes. Several cDNA clones corresponding to VP2 were isolated and sequenced. DNA sequences were determined by the dideoxy chain termination method. A plasmid harbouring VP2 or pVP2 with the correct sequence was cloned into P. pastoris after point mutations were corrected using a site directed
mutagenesis kit (Stratagene, La Jolla, CA). Pichia transformation was accomplished by electroporation of GS115 *Pichia pastoris* cells pre-treated with 0.1 M lithium acetate and 10mM dithiothreitol [35] using a Gene Pulser Xcell total Electroporation System (Bio-Rad, Hercules, CA) according manufacturer’s settings for yeast (*Saccharomyces cerevisiae*). Briefly, following characterization, the plasmid was linearized to promote integration into the AOX1 locus, and introduced into GS115 cells; the *P. pastoris* host strain, by electroporation transformation.

2.4. Screening for transformed colonies expressing VP2 and pVP2

Putative multi-copy recombinants were selected by plating the transformation mix on increasing concentrations of Zeocin. Transformed cells were grown on YPDS plates containing 100, 200, 500, 1000 and 2000 μg/ml Zeocin and incubated at 30 °C for 2 days. Colonies that grew in presence of higher concentrations of zeocin were selected and then checked for methanol utilization phenotype by replicate plating of colonies on Minimal Dextrose with histidine (MDH) agar plates, Minimal Methanol with histidine (MMH) agar plates. Strains GS115 Albumin (MutS) and GS115/pPICZ/lacZ (Mut+) were also plated on MMH and MDH plates as controls for MutS and Mut+ growth. Further verification of transformed Mut+ colonies was carried out by PCR analysis of *Pichia* integrants (VP2 and pVP2). To do this we developed a simple genomic DNA isolation protocol, where 5-10 colonies were re-suspended in 50 μl of autoclaved distilled water and subjected to one freeze thaw cycle by placing the PCR tube containing the colony in liquid Nitrogen for one minute followed by incubation at 37 °C for 5 min. and then use of the Qiaprep® Spin miniprep kit according to manufacturer’s recommendation (Qiagen, Valencia CA).
Positive colonies were grown in 100 ml minimal glycerol medium in 500 ml baffled flasks to an OD of 1.5 (usually 1 day). At this point, VP2 production was induced by adding 500 µl methanol every day. The optimal period of induction was determined by collecting samples every day after induction started. Following induction, cells were broken by vortex with glass beads (500 µm), centrifuged, and the supernatant contained the soluble VP2.

2.5. Western blot analysis

Protein extraction and Western blot analyses were performed using standard procedures. The membranes were immunoblotted with primary monoclonal antibodies against IBDV HB9490 (ATCC, Manassas, VA) at 1:500 dilution and secondary antibody horseradish peroxidase-conjugated goat anti-mouse IgG at a 1:8000 dilution (Jackson Lab, West Grove, PA).

2.6. Biofermentation

The fermentor was a New Brunswick Bioflo 3000 fermentor (New Brunswick Scientific Co., Inc. Edison, NJ) with a working volume of 5.0 L (total volume 7.5 L). The fermentation process was scaled up from shaker flasks to 5.0 L fermentation in three stages. The main objectives were: (a) achieving a high mass of active yeast cells at the end of the glycerol-fed-batch stage, (b) adapting to methanol as an inducer as well as a metabolic and anabolic carbon source, and (c) efficiently preserving the VP2 protein accumulated during the final stages of the fermentation. For that purpose 1mM phenylmethanesulfonyl fluoride (PMSF) (Sigma, St. Louis, MO) was added to breaking buffer when breaking the yeast cells with acid-washed glass beads (Sigma, St. Louis.
MO). This step was done according to EasySelect™ *Pichia* Expression Kit Version G 122701 25-0172 (Invitrogen, Valencia, CA).

2.7. Vaccine and challenge study:

*Studies in isolation units*

Two hundred and seventy specific pathogen free (SPF) light breed chicks were grown in Horsfal-Bauer isolation units from 1 day of age. They were divided into 54 groups of 5 birds each. Each treatment consisted of 2 groups (replicates) of five birds each. Experimental design is shown in Table 1. Vaccines used were prepared with or without complete Freund’s adjuvant (Sigma, St. Louis, MO). Vaccines were administered by injecting 0.1 ml of vaccine per bird intra-muscularly at one day of age with the exception of the 2512 vaccinated group that was vaccinated orally with $1 \times 10^{3.5}$ EID$_{50}$ at one day of age. Maternal antibody level at 1-day of age was determined by ELISA. Birds were challenged at 3 weeks of age with oral inoculation of either 0.1 ml breaking buffer (diluent), 0.1 ml containing $10^3$ EID$_{50}$ Edgar or 0.1 ml containing $1.2 \times 10^{4.5}$ EID$_{50}$ of 2512 strains of IBDV, or not challenged at all for non-vaccinated non-challenged controls. Surviving birds were euthanized one week post challenge.

2.8. Evaluation of vaccine protection against challenge

2.8.1 Sample collection and processing

After vaccinating the birds and placing them in isolation units, daily morbidity and mortality was recorded. One week post challenge birds were examined, weighed, bled, euthanatized by cervical dislocation, and necropsied. Bursa, spleen, and thymus were collected from each bird, weighed, and a portion of each was fixed immediately by immersion in 10% neutral buffered formalin for 24 hr. Tissues were then processed and
embedded in paraffin using routine histologic techniques. Relative organ weights were obtained using the formula [relative organ weight = (organ weight/body weight) × 100] [36].

2.8.2. ELISA

Blood was collected from the wing vein at 2, and 3 weeks post vaccination and 1 week post challenge. An IBD antibody test kit (IDEXX, FlockChek IBD, Westbrook, Me) was used to determine antibody titres according to manufacturer’s instruction.

2.8.3 Histopathology.

Paraffin-embedded tissues were sectioned, mounted, stained with hematoxylin and eosin (H&E) by using an automated stainer (Leica AutoStainer XL, Nussloch, Germany), and examined for lesions by light microscopy. All sections were assigned a lesion severity score. For all tissues, a lesion score of 1 represented no lesions, 2 represented mild variation in follicle size, 3 represented moderate variation in size of follicles, and 4 represented either necrosis or follicle atrophy [34].

2.8.4 Immunohistochemistry (IHC)

All procedures were done at room temperature. Tissue sections were cut (5 μm) from paraffin-embedded samples and mounted on charged glass slides (Superfrost/Plus; Fisher Scientific Pittsburgh, PA, U.S.A.). Paraffin was melted from slides (10 min at 65 °C) and removed by immersion in Hemo-De three times (5 min each). Slides were then air dried and antigen retrieval performed by digestion with 10% proteinase K (DAKO, Carpinteria, CA, U.S.A.) for 5 min to expose antigenic target sites. IHC staining was performed with an automated stainer (Leica ST 5050, Nussloch, Germany) with a nonbiotin peroxidase kit (DAKO Envision System; DAKO Carpinteria, CA, U.S.A.)
according to the manufacturer’s recommendations. The primary antibody used was a mouse monoclonal antibody specific to and cross-reactive for all IBDVs (ATCC no. HB9490). The secondary antibody was a peroxidase labelled polymer conjugated to anti-mouse and anti-rabbit immunoglobulins. After IHC staining, sections were counterstained with hematoxylin, air dried, cover slipped, and examined by light microscopy. Intensity of IBDV staining in each section was scored as follows: - = no staining, + = minimal staining (<10% of cells present), ++ = moderate staining (>10% and <30% of cells present), and +++ = extensive staining (>30% of cells present) [34].

2.8.5 IBDV RNA evaluation

2.8.5.1 RNA extraction.

RNA was extracted from formalin-fixed, paraffin-embedded bursas and examined for IBDV nucleic acid by real-time RT-PCR (Pantin-Jackwood and Brown, 2003). Sections totalling 50 µm in thickness were cut from each formalin-fixed, paraffin embedded tissue block, deparaffinized in HemoDe (Fisher Scientific, Pittsburgh, PA, U.S.A.), washed with 100% ethanol, and digested with 25 µg/ml proteinase K (Sigma Chemical Co.) for 1 hr at 50 °C. RNA was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) according to the manufacturer’s recommendations, diluted in 25 µl of 90% dimethyl sulfoxide, and frozen at -80 °C until assayed [34].

2.8.5.2 Real time RT-PCR.

Extracted RNA was denatured at 95 °C for 5 min and kept on ice. An RT-PCR was performed using reagents from the Light Cycler RNA Amplification SYBR Green I Kit (ROCHE Molecular Biochemicals, Indianapolis, IN, U.S.A.). The primers used were
designed to amplify a 400-base pair (bp) segment of the IBDV genome shared by all
strains, which flanks a hypervariable region of the VP2 gene. Amplification and detection
of specific products was also performed using a Light Cycler (ROCHE Molecular
Biochemicals, Indianapolis, IN, U.S.A.) according to the manufacturer’s
recommendations. Briefly, RT was done at 55 °C for 30 min, followed by denaturation at
95 °C for 30 sec. Fifty five PCR cycles were performed, consisting of denaturation
(95 °C for 1 sec), hybridization (55 °C for 10 sec), and extension (72 °C for 13 sec). A
melting curve analysis was done with an initial denaturation at 95 °C. DNA melting was
accomplished with an initial temperature of 65 °C for 10 sec and a gradual temperature
increase of 0.1 °C/sec until 95 °C was reached. The melting temperature of the expected
400-bp amplicon was between 82 °C and 84 °C. This estimated melting temperature was
used to confirm the identity of IBDV specific products obtained using real-time RT-PCR
[34].

2.8.6 Lymphoblastogenesis assay

Lymphocytes used for lymphoblastogenesis assay were isolated from peripheral
blood using Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO) and prepared for plating
according to procedure described by Hassan et al. [37] using cold RPMI 1640 medium
and 10% foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). The viability of the
lymphocytes was determined by staining with a 0.4% solution of trypan blue. The
concentration of viable lymphocytes was adjusted to 1 x 10⁷ cells/ml by adding RPMI
1640 medium with 10% FBS as required. Tests were performed in 96-well flat-bottomed
tissue culture plates. To each of four wells in the first row, 150 µl RPMI containing 10%
FBS was added (media control). To each of four wells in the second row, 50 µl RPMI
containing 10% FBS and 100 µl lymphocyte suspension (1 x 10⁶ cells) was added (cell
control). To each of four wells in the third row, 100 µl lymphocyte suspension and 50 µl of 10 mg/ml Con A (Sigma, St Louis, MI, USA) was added (stimulated cells #1). To each of four wells in the fourth row, 100 µl lymphocyte suspension and 50 µl of 5 mg/ml PWM (Sigma) was added (stimulated cells #2). Finally, to each of four wells in the fifth row, 100 µl lymphocyte suspension and 50 µl of 10 mg/ml IBDV antigen was added. The plates were covered and incubated at 37 °C in a humidified 5% CO2 atmosphere. The evaluation of lymphocyte blastogenesis by dye reduction was carried out according to the method described by Gogal et al. [38]. After 16-18 hr of culture, 20 µl of the Alamar blue dye was added to each well of one plate and was returned to the incubator. Twenty-four hours after the dye was added, the absorbance at 570 nm and 600 nm was measured with a microplate reader. The dye when added is in an oxidized (blue colour) form that is reduced (red colour) as cells proliferate. The 570 nm absorbance measures the reduced form and the 600 nm measures the oxidized form. Because there is some degree of overlap between the two absorbencies, it is necessary to subtract the 600 nm absorbance from the 570 nm absorbance to obtain the true absorbance (specific absorbance), which reflects the specific level of proliferation. The specific absorbance of unstimulated cells (in media alone) was subtracted from the specific absorbance of cells incubated with the mitogens to yield a Δ-specific absorbance.

The lymphocyte stimulation index was expressed as: Stimulation index (SI) = (C2 – C1) / (C3 – C1), where C1 = concentration of spontaneously reduced Alamar blue in the medium, C2 = concentration of reduced Alamar blue in the medium of stimulated cells, and C3 = concentration of reduced Alamar Blue in the medium of unstimulated control cells [39].
2.9 Statistical analysis

The body weight gain, relative bursal, spleen and thymus weights were analysed using analysis of variance and comparisons with a control using Dunnet’s method by use of JMP 4.0.2 (SAS Institute, Cary, NC).

3. Results
3.1. Cloning of pVP2 and VP2 in yeast

RNA was isolated from an Edgar strain of IBDV. Oligonucleotides corresponding to both ends of VP2 were designed and synthesized. The open reading frame of VP2 and the hypervariable region of VP2 were amplified by RT-PCR followed by PCR (Fig. 1). The 1452 bp and the 510 bp fragments were cloned in *E. coli* and following amplification of the plasmid, transfected into GS 115 *Pichia pastoris* cells.

3.2. Screening for transformed colonies

Transformation of *Pichia pastoris* without lithium acetate and dithiothreitol was very poor. Only after adding these two chemicals to GS-115 cells prior to transformation, were there enough transformed colonies generated for evaluation of transformation efficacy. The ability of the transformed yeast to grow in presence of zeocin; along with growth on both MMH and MDH plates followed by performing PCR on yeast genomic material for the presence of VP2 and pVP2 inserts allowed selection of which colonies to choose for expression.

3.3. Expression of pVP2 and VP2

VP2 and pVP2 were produced in yeast cells following induction with methanol. Cells were disrupted by acid-washed glass beads and centrifuged (10,000 rpm, 10 min,
and 4 °C). The protein was found in the supernatant and analyzed by Western blot. A band with the molecular weight of VP2 and another of the molecular weight pVP2 were seen when the membrane was immunoblotted with primary monoclonal antibodies against IBDV HB9490 at 1:500 dilution and secondary antibody horseradish peroxidase-conjugated goat anti-mouse IgG at a 1:8000 dilution (Fig. 2).

3.4 Immunization studies – efficacy of VP2 and pVP2 to protect against challenge with Edgar and 2512 strains of IBDV.

3.4.1. Morbidity and mortality:

Birds vaccinated wild type yeast, diluent or the hypervariable region peptide started showing depression, ruffled feathers, and trembling 2 days post challenge with the Edgar IBDV. Severe prostration and death of morbid birds started on day 3 post challenge (Table 2). Birds challenged with diluent or the 2512 strain of IBDV showed no symptoms of disease and looked apparently healthy. Birds vaccinated with VP2 and challenged with the Edgar IBDV were also apparently healthy and showed no signs of sickness and no mortality was observed (Table 2).

3.4.2. Body and organ relative weights and lesions:

No significant body weight differences were seen between control and experimental groups for body weight gain (Table 3). There was a significant decrease in relative bursa of Fabricius weights among the Edgar challenged groups compared to the 2512 vaccinated and non vaccinated non infected controls. Among the birds challenged with the Edgar strain of IBDV, the VP2 vaccinated birds had the highest bursal relative weights (Table 3). All birds challenged with the Edgar strain of IBDV suffered from +4 severe subacute bursitis with severe lymphocytic depletion with some exceptions in the
VP2 and pVP2 vaccinated groups where some birds had acute lesions instead as indicated by presence of neutrophils in the medulla of the bursal follicles (Table 4). Birds vaccinated with Pichia control or diluent that died 3-4 days post challenge with Edgar IBDV had severe bursal haemorrhage (Fig 3). Dead birds in the group vaccinated with the hypervariable region of VP2 showed oedematous swelling of bursas but had no haemorrhages. Birds that died on 5th day post challenge with the Edgar IBDV had no haemorrhages and their bursas appeared to be of normal size. The 2512 strain used for vaccination and challenge was very mild as the bursas showed very mild or no bursitis and had relative weights comparable to the non- vaccinated non-infected controls (data not shown).

The thymus relative weights of birds challenged with the Edgar strain of IBDV, regardless of vaccine given, were significantly lower in groups not challenged with this strain of virus, with the exception of the VP2 with no adjuvant vaccinated group. The VP2 with the adjuvant vaccinated group had significantly higher relative thymus weights compared to the other Edgar IBDV challenged groups; but was significantly less than non-vaccinated non infected control group (Table 3). This was confirmed by the microscopic observation that some areas of the thymus had moderate to severe depletion of thymic lymphocytes (Fig. 4).

There was spleenomegaly in birds vaccinated with VP2 or VP2 and challenged with the Edgar IBD virus (Table 3). In the enlarged spleens, microscopically there were more lymphocytes and swollen macrophages around the sheathed arteries. There was also more red blood cells seen in the splenic red pulp that seen in spleens of the non-infected non-vaccinated control group.
3.4.2 ELISA

Some of the extra SPF chicks that were purchased were sacrificed and serologically tested for IBDV. They were seronegative at 1-day of age for antibodies to IBDV as determined by ELISA. All birds had no detectable antibody titres at 2 weeks post vaccination. At 3 weeks post vaccination, no antibody titres were found in the groups vaccinated with Pichia controls, the hypervariable region of VP2, diluent, or non-vaccinated. The only groups at 3 weeks post vaccination with detectable antibody titres were those vaccinated with VP2 and they had antibody levels ranging from 59 to 250 for birds given the vaccine without adjuvant and 113 to 455 for birds given the vaccine with adjuvant, while the group vaccinated with the live 2512 virus had titres ranging from 684 to 1013. ELISA GMT antibody titres for 4-week old birds, one week post challenge are presented in Table 5. The low antibody titres, seen one week post challenge with the Edgar IBDV strain; in the VP2 vaccinated group compared to the Pichia control and diluent vaccinated groups, suggest that there were antibodies at time of challenge that neutralized some of the challenge Edgar IBDV, opposed to the Pichia pastoris and the diluent control vaccine groups that had higher titres one week post challenge suggesting that there were no antibodies to neutralize the challenge virus.

3.4.3 Immunohistochemistry and real-time RT-PCR detection of IBDV.

The Edgar IBDV challenged birds were all positive for IBDV when using IHC with variable degrees of staining as shown in Table 5. The bursas of birds vaccinated with VP2 mixed with Freund’s adjuvant and challenged with Edgar strain had the least staining intensities, suggesting low viral load in tissues. This was confirmed by the
inability to detect any IBDV RNA in genomic material extracted from formalin fixed paraffin embedded tissue blocks using real-time RT-PCR (the paraffin blocks used for extraction were the same ones used in making the slides for IHC). The bursas of birds vaccinated with VP2 without Freund’s adjuvant and challenged with Edgar IBDV had slightly more intensity than the previously mentioned group but much less when compared to the *Pichia pastoris* control and the diluent control groups. They also had detectable IBDV RNA in genomic material extracted from the same paraffin embedded tissue blocks (Table 4). The low intensity of IHC staining along with the absence of detectable IBDV RNA by real-time PCR in some of the VP2 vaccinated groups, compared to the very intense staining seen in non-vaccinated Edgar IBDV challenged groups, suggest that some virus neutralization occurred at time of challenge and hence the lower antibody responses detected. The birds vaccinated with the hypervariable region of VP2 and challenged with the Edgar IBDV showed similar IHC staining intensities and real-time RT-PCR positive for IBDV just like the *Pichia pastoris* control and diluent control groups when challenged with a similar virus. This suggests that the hypervariable region of VP2 subunit vaccine did not provided protection against IBDV challenge. *Pichia pastoris* and diluent controls groups that were non-challenged with IBDV viruses had no IHC staining and were negative for real-time RT-PCR detectable IBDV RNA. Immunohistochemistry could not detect IBDV in all of the 2512 IBDV vaccinated or challenged birds with only one exception where mild staining of the bursa was observed in one of the diluent vaccinated 2512 challenged birds. However, real-time RT-PCR could detect the 2512 virus in the diluent control and non vaccinated 2512 challenged groups. The 2512 vaccinated and 2512 challenged group had no real-time RT-PCR
detectable RNA, no IBDV IHC staining, and +1 lesion scores suggesting that the antibodies generated by using the live virus completely neutralized the challenge virus. The VP2 vaccinated group also had no detectable IBDV RNA suggesting that the virus was neutralized at time of challenge (data not shown).

3.4.4. Lymphoblastogenesis assay

The stimulation indices of the different treatments are presented in Table 6. Lymphocytes stimulated with the T-cell specific mitogen (Concanavalin A) were not significantly different from each other with the exception of one group which was vaccinated with the Pichia control and challenged with Edgar, that was significantly higher than the rest. The lymphocytes of birds vaccinated with VP2 and adjuvant as well as those vaccinated with the Pichia control vaccines had a significantly higher response to the B-cell specific pokeweed mitogen, while non-vaccinated 2515 IBDV challenged birds had significantly lower response to the IBDV antigen, when compared to other treatment groups.

Comparing the lymphocyte stimulation indices of birds given the vaccines with or without adjuvant revealed a very slight increase in stimulation indices of the ones that had been given the vaccine with adjuvant although the difference was not significant.

4. Discussion

Infectious bursal disease virus is highly infectious and very resistant to inactivation. Thus, despite strict hygienic measures, vaccination is unavoidable under high infection pressure and it is necessary to protect chickens against infection during the first weeks after hatch. After hatching, chickens are immunized with live vaccines, and because maternal immunity interferes with vaccination with live vaccines, the major problem with active immunization of young maternally immune chicks is determining the
proper time of vaccination. It is well known that less attenuated strains (“hot vaccines”) may cause lesions in the bursa follicles and, thus, immunosuppression even in vaccinated birds. To avoid live vaccine side effects [40], a *Pichia pastoris* expression system expressing IBDV VP2 or the hypervariable region of VP2 has been developed. *Pichia pastoris* as a yeast expression system was chosen because it combines the simplicity of prokaryotic growth requirements, growth potential and ease of manipulation along with the post-translational modifications of eukaryotes to ensure that the expressed protein is functional and has the proper conformation. Using this expression system both VP2 and the hyper variable region of VP2 were expressed. Ethidium bromide gels of RT-PCR amplicons showed that the primers succeeded in amplifying VP2 and pVP2 of Edgar IBDV. Colonies screened for ability to grow in presence of high levels of zeocin, methanol utilization phenotype and presence of genomic inserts were selected for biofermentation. Intra-cellular expression of VP2 and pVP2 of Edgar in *Pichia pastoris* was confirmed by immunoblotting with primary monoclonal antibodies.

Vaccination with recombinant VP2 in combination with Freund’s adjuvant gave best results in terms of protection parameters. Birds vaccinated with recombinant VP2 mixed in Freund’s adjuvant showed no morbidity or mortality. This was also observed the VP2 vaccine without adjuvant, but the difference between the two VP2 vaccinated groups with or without adjuvant could be observed in the intensity of IHC staining and ability to detect IBDV RNA from infected tissue. Low intensity of IHC staining of bursas of Fabricius of the VP2 with adjuvant vaccinated group that was challenged with Edgar IBDV indicates the presence of IBDV in tissue; however, the absence of real-time RT-PCR detectable RNA suggests low viral load in tissue. This low viral load was still able
to cause severe damage to bursal tissue. This can be attributed to the high virulence of the challenge dose, as the Edgar IBDV used for challenge was serially passed in chickens several times prior to use in this experiment. Wild type *Pichia pastoris*, diluent or the hypervariable region of VP2 vaccinated birds had 30 to 90% morbidity and 20 to 60% mortality; indicating the virulence of the Edgar IBDV strain used. Also, that the yeast and breaking buffer used to make the VP2 vaccine did not protect against challenge with Edgar IBDV. Thus, the protection against morbidity and mortality observed in the VP2 vaccinated group is due to vaccination with VP2. This protection maybe due to T-lymphocytes, as indicated by increase in relative thymus weight (Table 3), and that cortical depletion was minimal in the VP2 vaccinated group compared to the other treatment groups that were Edgar IBDV challenged (Fig 4). The effect of IBDV infection on cell-mediated immune competence is transient and less obvious than its effect on humoral responses [41]. Some *in vitro* cellular functions have been found to be compromised in IBDV-infected chickens, with lymphoid cells of chickens exposed to IBDV responding poorly to mitogens [42]. While others reported that maximal suppression of cell-mediated immune responses occurred at 6 weeks post infection [43], in our study, mitogen stimulation of peripheral blood lymphocytes of vaccinated and non vaccinated chickens could not verify the role that T-cells have on IBDV vaccination or challenge, as there were no significant differences between stimulation indices of lymphocytes of different treatments when stimulated with T-cell mitogen (Concanavilin A) with the exception of the Pichia control vaccinated group challenged with the Edgar IBDV. This may be because the *Pichia pastoris* homogenate acts like an adjuvant and stimulated the antigen presenting cells in particularly the macrophages to secrete
cytokines that stimulate the T-cell mediated immune system. The lymphocytes of birds vaccinated with VP2 and adjuvant as well as those vaccinated with the Pichia control vaccines had significantly higher stimulation indices when stimulated with the B-cell specific pokeweed mitogen, compared to other treatments indicating the role of B lymphocytes in protecting against the Edgar IBDV. This could also be due to the previously mentioned probability of innate immune stimulation and the role that macrophages have in enhancing the humoral immune system. The use of a lower infective dose or less virulent virus for challenge in the future might be important to help identify the role of T-lymphocytes in protecting against IBDV infections.

The low geometric mean titres of antibodies against IBDV at 2 and 3 weeks are not unusual when recombinant vaccines are used. Previous researchers have reported that titres of 150 – 400 are common and that these low titres are fully protective against controlled challenge and exposure to IBDV [19]. The antibody titres against IBDV one week post challenge (Table 4) are typical of those seen in IBDV challenge studies [44], as non-vaccinated groups had higher GMT titres, than the vaccinated groups. This may be due to that the protected birds have antibodies that neutralize some of the challenge virus thus leaving the bird with less virus particles available for infection opposed to the unprotected birds that get the whole challenge dose. Generally antibodies of recombinant protein vaccines have lower GMT than that seen with live virus vaccines [19], as live virus vaccines produce polyclonal antibodies that react to both VP2 and VP3 that is present in the IDEXX ELISA plates, whereas the VP2 vaccines produce antibodies only for VP2 and thus get a lower reaction with the IDEXX ELISA plates. Anti-VP3 antibodies do not confer protection against IBDV infection [45]. The low level of anti-
VP2 antibodies or the ability to detect VP3 genomic material by RT-PCR can be used to differentiate between vaccinated and infected flocks.

In summary, recombinant vaccines expressing VP2 and not just the hypervariable region of VP2 are needed to protect against IBDV infections. The use of paraffin embedded tissue as a source of genomic material for protein production in yeast is feasible. However, it highly recommended for conformationally dependant proteins; to fully reconstruct the whole genome before expressing it in yeast expression system. Using these recombinant vaccines will help combat and control this disease in the future, without the side effects seen with live viruses. *Pichia pastoris* expressed antigenic proteins can be mass produced at very high levels; to achieve comparable antigenic levels in inactivated vaccines, sacrificing a lot of SPF chicken embryos will be required. Specific pathogen free embryos might not be available in the future for veterinary use; as shortages of available SPF embryos have been reported. This is due to the high demand for these embryos for the use in production of human therapeutics. A demand that will increase drastically with the emerging of new human disease epidemics or pandemics that threatens the human race.

**References:**


Figure legends:

Fig. 5.1. RT-PCR product of amplified VP2 and pVP2: lane 1, molecular size marker; lane 2, 3, 5 and 6 amplified VP2; lane 4, negative control; lane 8 and 9, amplified pVP2

Fig. 5.2. Western blot analysis of recombinant proteins. Lane 1 Molecular weight marker, lanes 2 and 3 two clones of yeast expressing pVP2, Lanes 4 and 5 two clones of yeast expressing VP2 and lanes 6 and 7 negative controls (yeast transformed with wild-type plasmid). The membrane was immunoblotted with primary monoclonal antibodies against IBDV (ATCC HB9490)

Fig: 5.3. Photomicrographs of bursas from SPF chickens stained with IHC that were vaccinated and challenged with diluent (A); Vaccinated with diluent and challenged with Edgar IBDV (B); Vaccinated with Pichia pastoris control and challenged with Edgar IBDV (C); non vaccinated non infected control (D); pVP2 vaccinated and Edgar IBDV challenged (E) and VP2 vaccinated and Edgar IBDV challenged (F). X100

Fig: 5.4. Photomicrographs of Thymus from SPF chickens stained with H&E were VP2 vaccinated and Edgar IBDV challenged (A); diluent vaccinated and Edgar IBDV challenged (B); Non-vaccinated , non-challenged control (C). X100

Fig: 5.5. Photomicrographs of bursa from SPF chickens stained with H&E that were non-vaccinated , non-challenged control (A). or diluent vaccinated and Edgar IBDV challenged (B); X100
Table 5.1: Experimental design of immunization experiment. Different vaccines were administered at 1-day of age with or without Freund’s adjuvant. Challenge was 3 weeks post vaccination. Experimental replicates had the following treatment groups.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Pichia control</th>
<th>VP2 in pichia</th>
<th>pVP2 in pichia</th>
<th>Diluent</th>
<th>2512</th>
<th>None</th>
<th>none</th>
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<td>No</td>
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<td>Challenge diluent Edgar 2512 diluent Edgar 2512 diluent Edgar 2512 diluent Edgar 2512 diluent Edgar 2512 2512 none</td>
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<td>+: Group contained 5 SPF birds</td>
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<td>-: Not done (as live viruses are administered with adjuvants in commercial poultry).</td>
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</tr>
<tr>
<td>Vaccine</td>
<td>Challenge</td>
<td>Pichia control</td>
<td>VP2 in pichia</td>
<td>pVP2 in pichia</td>
<td>Diluent</td>
<td>2512</td>
<td>none</td>
</tr>
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<tr>
<td></td>
<td></td>
<td>diluent</td>
<td>Edgar</td>
<td>2512</td>
<td>diluent</td>
<td>Edgar</td>
<td>2512</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Morbidity</td>
<td>adjuvant</td>
<td>*</td>
<td>3/10</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Adjuvant</td>
<td></td>
<td>*</td>
<td>5/10</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>Mortality</td>
<td>adjuvant</td>
<td>*</td>
<td>2/10</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
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<td>4/10</td>
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<td></td>
</tr>
</tbody>
</table>

* = 0/10
n/a = not applicable
Table 5.3: Body weight and relative organ weights of 28 day old SPF chickens vaccinated or non-vaccinated against IBDV, one week post challenge. Values ± standard deviations within a column that are followed by the same superscript letter are not significantly different ($P < 0.05$).

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Challenge</th>
<th>Body weight</th>
<th>Bursa relative weight</th>
<th>thymus relative weight</th>
<th>Spleen relative weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2</td>
<td>Edgar</td>
<td>273.03 ± 38.32</td>
<td>0.409 ± 0.23$^b$</td>
<td>0.479 ± 0.15$^a$</td>
<td>0.337 ± 0.05$^c$</td>
</tr>
<tr>
<td>VP2 + adjuvant</td>
<td>Edgar</td>
<td>270.29 ± 22.29</td>
<td>0.282 ± 0.21$^c$</td>
<td>0.396 ± 0.07$^b$</td>
<td>0.303 ± 0.08$^b$</td>
</tr>
<tr>
<td>pVP2</td>
<td>Edgar</td>
<td>259.35 ± 29.02</td>
<td>0.197 ± 0.16$^d$</td>
<td>0.227 ± 0.08$^d$</td>
<td>0.325 ± 0.06$^{bc}$</td>
</tr>
<tr>
<td>pVP2 + adjuvant</td>
<td>Edgar</td>
<td>259.05 ± 25.24</td>
<td>0.177 ± 0.07$^d$</td>
<td>0.262 ± 0.07$^d$</td>
<td>0.315 ± 0.06$^b$</td>
</tr>
<tr>
<td>Pichia</td>
<td>Edgar</td>
<td>259.89 ± 51.89</td>
<td>0.162 ± 0.04$^d$</td>
<td>0.271 ± 0.03$^d$</td>
<td>0.242 ± 0.04$^a$</td>
</tr>
<tr>
<td>Pichia + adjuvant</td>
<td>Edgar</td>
<td>274.70 ± 32.26</td>
<td>0.186 ± 0.11$^d$</td>
<td>0.297 ± 0.05$^c$</td>
<td>0.241 ± 0.08$^a$</td>
</tr>
<tr>
<td>Diluent</td>
<td>Edgar</td>
<td>241.88 ± 57.27</td>
<td>0.230 ± 0.15$^c$</td>
<td>0.306 ± 0.10$^c$</td>
<td>0.299 ± 0.05$^a$</td>
</tr>
<tr>
<td>Diluent + adjuvant</td>
<td>Edgar</td>
<td>264.78 ± 20.24</td>
<td>0.114 ± 0.03$^d$</td>
<td>0.250 ± 0.07$^d$</td>
<td>0.234 ± 0.05$^a$</td>
</tr>
<tr>
<td>Pichia</td>
<td>Diluent</td>
<td>316.60 ± 36.37</td>
<td>0.625 ± 0.22$^a$</td>
<td>0.657 ± 0.15$^a$</td>
<td>0.225 ± 0.06$^a$</td>
</tr>
<tr>
<td>Pichia + adjuvant</td>
<td>Diluent</td>
<td>309.53 ± 40.77</td>
<td>0.545 ± 0.08$^a$</td>
<td>0.541 ± 0.06$^a$</td>
<td>0.225 ± 0.05$^a$</td>
</tr>
<tr>
<td>2512</td>
<td>2512</td>
<td>306.23 ± 41.90</td>
<td>0.423 ± 0.09$^a$</td>
<td>0.595 ± 0.13$^a$</td>
<td>0.180 ± 0.06$^a$</td>
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<tr>
<td>none</td>
<td>2515</td>
<td>271.74 ± 21.60</td>
<td>0.567 ± 0.12$^a$</td>
<td>0.429 ± 0.13$^a$</td>
<td>0.225 ± 0.05$^a$</td>
</tr>
<tr>
<td>none</td>
<td>none</td>
<td>301.90 ± 21.73</td>
<td>0.616 ± 0.09$^a$</td>
<td>0.553 ± 0.12$^a$</td>
<td>0.229 ± 0.04$^a$</td>
</tr>
</tbody>
</table>
Table 5.4: Real-time RT-PCR melting curve detection, histopathological lesion scores and IHC mean staining scores for bursas of birds vaccinated or non vaccinated at 1-day of age and challenged with Edgar or 2512 strains of IBDV at 21 days. Sampling was performed 1 week post challenge. Data for individual bird’s bursa.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Challenge</th>
<th>Vaccine without Freund’s adjuvant</th>
<th>Vaccine with Freund’s adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RT-PCR(^A)</td>
<td>Lesion score(^B)</td>
</tr>
<tr>
<td>VP2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edgar</td>
<td>IBDV</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>4N</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>4N</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>4N</td>
</tr>
<tr>
<td>pVP2</td>
<td></td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Edgar</td>
<td>IBDV</td>
<td>+</td>
<td>4N</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>4N</td>
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<td></td>
<td></td>
<td>+</td>
<td>4N</td>
</tr>
<tr>
<td>Pichia control</td>
<td>Edgar</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>IBDV</td>
<td></td>
<td>+</td>
<td>4</td>
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<tr>
<td></td>
<td></td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>Diluent</td>
<td>Edgar</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>IBDV</td>
<td></td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>Pichia control</td>
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</tr>
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<td>1</td>
</tr>
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</tr>
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<td></td>
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<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>2</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

\(^A\) RT-PCR melting curve detection
\(^B\) Histopathological lesion scores
\(^C\) IHC mean staining scores
<table>
<thead>
<tr>
<th>Diluent</th>
<th>Diluent</th>
<th>2512</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
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<tr>
<td></td>
<td>+</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Diluent</th>
<th>2512</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

<table>
<thead>
<tr>
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<th>none</th>
<th>2512</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

\[A\] - = Negative; + = Positive

\[B\] Lesion Score: 1 = No lesion, 2 = Mild variation in follicle size, 3 = Moderate variation in follicle size, 4 = Either Necrosis or follicle atrophy, N = acute necrosis

\[C\] - = No staining; + = Minimal staining, ++ = Moderate Staining, +++ = Intense staining.
Table 5.5: ELISA GMT at 28 days of age to IBDV following vaccination of 1-day-old SPF chickens

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>IBDV Challenge</th>
<th>Vaccine without Freund’s adjuvant</th>
<th>Vaccine with Freund’s adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2</td>
<td>Edgar</td>
<td>436</td>
<td>576</td>
</tr>
<tr>
<td>pVP2</td>
<td>Edgar</td>
<td>1825</td>
<td>1884</td>
</tr>
<tr>
<td>Pichia control</td>
<td>Edgar</td>
<td>2540</td>
<td>2679</td>
</tr>
<tr>
<td>Diluent vaccine</td>
<td>Edgar</td>
<td>2505</td>
<td>2364</td>
</tr>
<tr>
<td>2512</td>
<td>2512</td>
<td>884</td>
<td>N/A</td>
</tr>
<tr>
<td>None</td>
<td>2512</td>
<td>57</td>
<td>N/A</td>
</tr>
<tr>
<td>Pichia control</td>
<td>Diluent</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>1</td>
<td>N/A</td>
</tr>
</tbody>
</table>

^A Chickens were challenged at 21 days of age with IBDV

N/A = not applicable
Table 5.6: Mean lymphocyte transformation indices of vaccinated and non-vaccinated birds challenged or not challenged with 2512 or Edgar IBDV

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Challenge</th>
<th>Con A</th>
<th>PWM</th>
<th>IBDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2</td>
<td>Edgar</td>
<td>1.13± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.99± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.68 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VP2+adj</td>
<td>Edgar</td>
<td>1.17± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.30± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.71 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pVP2</td>
<td>Edgar</td>
<td>0.99± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.68 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pVP2 +adj</td>
<td>Edgar</td>
<td>1.01 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pichia control</td>
<td>Edgar</td>
<td>1.25 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.27± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.88 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2512</td>
<td>2512</td>
<td>1.03 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.76 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>2512 C</td>
<td>1.19 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.09± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>none</td>
<td>1.02 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent the mean of five chicken per treatment; Con A = Concanavalin A; PWM = pokeweed mitogen. IBDV = Infectious bursal disease virus. Values within a column followed by the same superscript letter are not significantly different ($P < 0.05$).
Fig. 5.1. RT-PCR product of amplified VP2 and pVP2: lane 1, molecular size marker; lane 2, 3, 5 and 6 amplified VP2; lane 4, negative control; lane 8 and 9, amplified pVP2

Fig. 5.2. Western blot analysis of recombinant proteins. Lane 1 Molecular weight marker, two clones of yeast expressing pVP2 lanes 2 and 3, two clones of yeast expressing VP2 and negative control, yeast transformed with wild-type plasmid. The membrane was immunoblotted with primary monoclonal antibodies against IBDV (ATCC HB9490)
Fig 5.3. Photomicrographs of bursa from SPF chickens stained with H&E that were Non-vaccinated, non-challenged control (A); or diluent vaccinated and Edgar IBDV challenged (B); X100
Fig 5.4. Photomicrographs of Thymus from SPF chickens stained with H&E were VP2 vaccinated and Edgar IBDV challenged (A); diluent vaccinated and Edgar IBDV challenged (B); Non-vaccinated, non-challenged control (C). X100
Fig 5.5. Photomicrographs of bursas from SPF chickens stained with IHC that were vaccinated and challenged with diluent (A); Vaccinated with diluent and challenged with Edgar IBDV (B); Vaccinated with *Pichia pastoris* control and challenged with Edgar IBDV (C); non vaccinated non infected control (D); pVP2 vaccinated and Edgar IBDV challenged (E) and VP2 vaccinated and Edgar IBDV challenged (F). X100
CHAPTER VI
DISCUSSION AND CONCLUSIONS

Identification of infectious bursal disease viruses from RNA extracted from paraffin embedded tissue.

Formalin fixed paraffin embedded tissues used as a source of genomic material to rapidly identify field isolates of IBDV by RT-PCR and sequencing allowed a direct correlation between the virus strain present and the lesions produced by this virus, in a timely fashion. This also allowed analysis of foreign strains of IBDV with no importation and biosecurity restriction issues because of the inactivated nature of the antigen. Therefore, the information obtained by this technology will help refine vaccination programs against IBDV, as well as to detect new emerging IBDV strains both domestically and internationally.

Fixation conditions can have a tremendous impact on the success of RNA extraction, as properly handled samples generate good results while improperly handled samples can generate average results at best. In chickens experimentally infected with IBDV, all paraffin embedded blocks with bursas having acute +3 or +4 bursitis had extractable genomic material that was positive for IBDV based on melting curve analysis, indicating high sensitivity of the primers used. Most of the 452 blocks analyzed had +3 or +4 acute lesion scores for IBDV, yet only 227 were positive for IBDV extractable genomic material. IBDV genomic extraction rate from samples originating from the U.S.A. was 87%, while that from overseas was only 19%. The effects of different fixation conditions on tissues with higher than normal viral load was evaluated. These
changes in temperature, pH and concentration of formalin were enough to render a positive IBDV formalin fixed paraffin embedded tissue block into a negative one.

Nucleic acid and deduced amino acid analysis of extracted IBDV genomic material has shown that the Variant E strain of IBDV is the predominant strain present in the U.S.A. The few classical strains found in samples from the United States were mainly of vaccine origin. There were no vvIBDV strains found in the U.S.A. Internationally, four vvIBDV strains from both Venezuela and China were identified. Variant A strains were found in both Peru and Venezuela. Variant E strains were identified in El Salvador, Guatemala, Mexico, Peru and Venezuela. Internationally, more classical than variant strains were identified, this is similar to what Lukert and Saif have reported.

Phylogenetic analysis showed that serotype 1 viruses grouped together in several different groups. Previous knowledge of strain origin and identity along with location of viruses on phylogenetic trees was the basis used for grouping these viruses. All variant viruses sequenced were either Variant A or Variant E. Variant E had three different branches.

Several unique virus strains that did not group with the common IBDV strains and fell on branches of their own were identified. Genomic material was isolated and identified from a unique IBD virus strain that was missing 63 nucleotides from its hypervariable region in the VP2 gene, yet the virus was apparently able to replicate in the bursa and cause +4 acute lymphocytic damage. VP2 is important for virus attachment to cell receptors and is the viral protein that is recognized by the chicken’s immune system. Missing these 21 amino acids apparently did not affect the virus’s ability to attach and
enter the susceptible B-lymphocytes. Constant vaccination pressure in the past may have contributed to the emergence of variant strains and vvIBDV. This unique case missing the 21 amino acids may have resulted from random mutations by the virus in an attempt to escape from the immunoglobulins produced in vaccinated chickens; or maybe this virus was always there in the population of IBDV viruses, and constant vaccination against variant IBD viruses suppressed other viruses, giving this virus favorable propagation conditions.

The inability to identify all strains present in multivalent vaccines was attributed to the nature of the RT-PCR reaction which amplifies the virus with higher logarithmic concentration than the other viruses that have lower concentration. So when a sequencing reaction is run and analyzed, only the nucleotides that are most abundant are identified. This was noticed when sequencing a bivalent vaccine, SVS-510™, where only the predominant variant portion of the vaccine was identified and not the classic portion. To overcome this, the seeds of that vaccine were isolated and sequenced independently. This variant portion of this bivalent vaccine was identified in figure 1 and 2 as SVS-510 (Variant portion) while the classical portion was identified as SVS-510 (Standard portion).

To investigate why samples with +4 acute lesion scores of IBDV yielded no detectable RNA, bursas with high vial loads were subjected to different fixation conditions. Extracting genomic RNA for identification of IBDV from bursas of Fabricius stored at acidic pH (pH 5.0 and pH 2.0) was unsuccessful. Previous research which found DNA damage in cells at low pH, may explain the lack of extracted genomic RNA in this experiment. Unbuffered formalin oxidizes to formic acid and an acidic environment
causes degradation of nucleic acids because the $\beta$-glycosidic bonds in the purine bases are hydrolyzed at pH 4. Loss of some amino group cross-links occurs at low pH and may result in loss of RNA from the fixed tissue. Extraction of genomic RNA at a basic pH (pH 9.0, and 13.0) was also unsuccessful and the reason for this is unknown.

The thermodynamic stability of a nucleic acid duplex strongly depends on the denaturation temperature of the duplex at atmospheric pressure. Denaturation of genomic RNA may have occurred when tissues were formalin fixed at 50°C for 5 days and maybe the reason why there were no rt-RT-PCR detectable RNA extracted from these blocks.

At formalin concentrations of 20% or 37%, the extraction of RNA from FFPET blocks yielded no rt-RT-PCR detectable RNA. This may be due to excessive crosslinking of amino groups resulting from the acidic nature of formalin at higher concentrations; indeed, maximum tissue fixation, i.e. cross-linking, occurs in the pH range of 4.0 to 5.5, thus hindering genomic RNA extraction.

Fixation time had no effect on RNA extraction or subsequent identification, but longer fixation times are not recommended due to the higher frequency of non-reproducible sequence alterations. This is demonstrated by the increase in melting peak temperature over time. Formalin may cause cross-linking of cytosine nucleotides on either strand. As a result, in PCR the Taq-DNA polymerase fails to recognize the cytosine and incorporates an adenine in the place of a guanosine, creating an artificial C-T or G-A mutation.

Real-time RT-PCR as diagnostic tool for IBDV is not always available, so another important tool for identifying IBDV is immunohistochemistry. We had the hypothesis that just as fixation conditions have a negative impact on RNA used for
identifying IBDV in formalin fixed paraffin embedded tissues; these same adverse fixation conditions may also impact IHC identification negatively. Over the years formalin fixation of tissues has been used for tissue preservation and for histological evaluation of tissues. Some have shown that small (10 X 10 X 3 mm) tissue fragments fixed promptly in neutral buffered formalin for 6-24 hours generally had adequate cytological preservation and immunolocalization, with minimal antigen masking. Variations in fixation times or conditions cause the majority of false negatives in immunohistochemistry, as lack of consistency in formalin fixation protocols (formalin concentration, pH and fixation time) among laboratories influences the outcome of staining in immunohistochemistry. Formaldehyde fixatives fix tissue by reacting primarily with basic amino acids to form cross-linked “methylene bridges”. Formalin fixation conditions have a direct effect on number of methylene brigdes. The number of methylene bridges and which amino groups are cross-linked in the tissue are important variables in the outcome of immunohistochemical staining, and could also be an important factor on the ability to extract rt-RT-PCR detectable RNA from FFPET.

Tissues fixed in 20% or 37 % formalin undergo shrinkage and may suffer from alteration of epitope conformation. The IBDV epitopes detected by the monoclonal antibodies used in this study are conformationally dependent; thus fixation conditions such as formaldehyde concentration or pH that may cause changes in epitope conformation rendering them undetectable by these monoclonal antibodies. Maximum tissue fixation, i.e. cross-linking, occurs in the pH range of 4 to 5.5, therefore, the presence of too many cross-links or cross-links of certain amino groups may cause antigen masking. This may be the reason why there was minimal or no immunostaining
in tissues fixed in a pH <7.0. Inconclusive IHC staining at pH >7.0 may also be due to tissue shrinkage or alteration of reactive epitopes.

Immunohistochemical staining intensity was highest in tissues fixed at 4 °C. Increased temperature reduced specific IHC staining, but did not eliminate it. The reduction in staining intensity may be due to tissue shrinkage at temperatures higher than 4 °C. This result is in agreement with other researchers findings; however the mechanism for this effect is unknown.

One of the ideas we had when planning for these experiments, was that we could use the genomic material we extracted from infected bursal tissues for strain identification, to produce recombinant proteins that provide protection against IBDV in susceptible flocks. We had the vision of getting field samples from flocks suffering from immunosuppression; identify IBDV histopathologically and molecularly identify the strain causing the problem and provide the grower with a tailor made vaccine to control his IBDV problem. This proof of concept is possible as we were able to identify IBDV strains by sequencing, and the genomic material extracted from paraffin embedded tissue was successfully expressed in *Pichia pastoris* yeast expression system.

Using the information and genomic material gathered from this technology we can use *Pichia pastoris* to express various proteins specific for each strain of IBDV identified; thus, generating a recombinant protein vaccine library that can provide specific protection against a particular IBDV strains. Once a recombinant IBDV VP2 library is established, very specific vaccines can be provided rapidly to prevent economic losses caused by IBDV infections. This rapid screening and identification process allows for processing relatively large number of samples in a very short time. This combined
with the easy of processing, allows for extensive epidemiological survey to be carried out. This helps in finding out what types of virus are present in a certain location at a certain time. This technology has allowed identification of unique viruses, and newly emerging viruses. Depending on how wide spread these newly emerging viruses become; we will be able to determine what vaccines are needed to be produced in order to protect flocks against particular strains of IBDV. Infectious bursal disease virus is highly infectious and very resistant to inactivation. Therefore, despite strict hygienic measures, vaccination is unavoidable under high infection pressure and it is necessary to protect chickens against infection during the first weeks after hatch. After hatching, chickens are immunized with live vaccines, and because maternal immunity interferes with vaccination with live vaccines, the major problem with active immunization of young maternally immune chicks is determining the proper time of vaccination. It is well known that less attenuated strains (“hot vaccines”) may cause lesions in the bursa follicles and, thus, immunosuppression even in vaccinated birds. To avoid live vaccine side effects, a subunit VP2 vaccine was produced in *Pichia pastoris* that is a yeast expression system. It combines the simplicity of prokaryotic growth requirements, growth potential and ease of manipulation along with the post-translational modifications of eukaryotes to ensure that the expressed protein is functional and has the proper conformation. Using this expression system we expressed both VP2 and the hyper variable region of VP2. Using these recombinant vaccines will help combat and control this disease in the future, without the side effects seen with live viruses. *Pichia pastoris* expressed antigenic proteins can be mass produced at very high levels; to achieve comparable antigenic levels in inactivated killed vaccines, sacrificing a lot of SPF chicken embryos will be required.
Specific pathogen free embryos might not be available in the future for veterinary use; as shortages of available SPF embryos have been reported. This is due to the high demand for these embryos for the use in production of human therapeutics. This demand may increase drastically with the emerging of new human disease epidemics or pandemics that threatens the human race.

Extracting genomic material from formalin fixed tissue has been done, unlocking a historical archive. Expressing these extracted genes and using these generated sequences to look back will help identify viruses, their biology and their evolution. Researchers have reconstructed the human influenza virus that caused the 1918 epidemic and found it to be of avian origin. Using this technology to identify IBDV strains can also be done and may give an insight on how the virus is changing over time. Also rapid identification of newly emerging viruses and how predominant they are is important for veterinarians, to allow them more information in controlling a complex disease.

In summary, the research potentials of extracting RNA from formalin fixed paraffin embedded tissue has allowed rapid accurate profiling of IBDV strains that are causing lesions in the field. This is a diagnostic tool that has assisted veterinarians in choosing which vaccines to use based on the nucleic acid sequence of the hypervariable region of VP2 of IBDV viruses present on their farms. Another potential is the identification of potentially new vaccine strains. As with all molecular techniques that are used to predict the relative similarities and differences between IBDV strains, determining the actual antigenic differences among viruses requires testing in-vivo. For optimal IBDV RNA extraction and sequence identification, tissues should be fixed in formalin at a pH of 7.0, concentration of 5% or 10% and stored at 25 °C or lower.
temperature. Extremes in temperature, pH or formalin concentration have a negative
effect on tissues used for RNA extraction. Optimal conditions for fixation of tissues
infected with IBDV that will be used for IHC evaluation are 10% formalin, pH 7.0 and
temperature of 4 °C. For practical purposes, fixation temperature of 4 °C is recommended
for both procedures. Also recombinant vaccines expressing VP2 and not just the
hypervariable region of VP2 are needed to protect against IBDV infections. Use of
paraffin embedded tissue as a source of genomic material for protein production in yeast
is feasible. But it is highly recommended for conformationally dependant proteins to fully
construct the whole genome before expressing in yeast expression system.