### STUDIES ON INFECTIOUS BURSAL DISEASE VIRUS

DISSERTATION

### Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Shamaila Ashraf, M.Sc., M.Phil.

\* \* \* \* \*

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Dissertation Committee:

Professor Y.M.Saif, Adviser

Professor D. J. Jackwood

Professor T. Morishita

Assistant Professor J. Lejeune

Approved by

Adviser Graduate Program in Veterinary Preventive Medicine

### ABSTRACT

Infectious bursal disease virus (IBDV) causes an acute and contagious disease in young chickens from 3-6 weeks of age. Two serotypes of the virus are recognized of which serotype 1 viruses are pathogenic to chickens and are classified into classic, variant, and serotype 2 viruses are nonpathogenic. The disease is controlled by vaccination. In the first part of the study interactions between a mild and a pathogenic strain of IBDV in specific pathogen free (SPF) chickens were studied. Chickens were inoculated with the Bursine-2 vaccine followed by the pathogenic STC strain at various time intervals. Persistence of virus strains was monitored by the reverse transcriptase polymerase chain/restriction fragment length polymorphism (RT-PCR/RFLP), bursa/body weight ratios and histopathological lesion scores. The mild strain interfered with the replication of the pathogenic strain.

Currently available ELISA kits were evaluated for their ability to detect antibodies elicited by serotype 1 and serotype 2 viruses. Virus neutralization (VN) test differentiates between antibodies elicited by the two serotypes as well as subtypes of serotype 1 viruses. SPF chickens were inoculated with either serotype 1 STC or serotype 2 OH strain. Sera from these chickens and naturally exposed chickens were tested by five commercial ELISA kits and the VN. The ELISA kits detected antibodies to both serotypes of the virus. Therefore, while determining the antibody profiles of the flocks, the presence of serotype 2 antibodies should be taken into account.

Simple and quick diagnostic assays are needed for developing control strategies against IBDV. A differential RT-PCR assay was developed. Two primer sets were designed. Primer set one targeted the segment A of the virus and specifically amplified serotype 2 strains. Primer set 2 targeted the segment B of the virus and amplified the vv strains. These primer sets were validated with 26 different strains maintained in our laboratory. The primer set 2 was also tested with 20 suspected vv field isolates. All except three samples tested positive with primer set 2. The Taiwan strains appeared genetically similar to the classic viruses upon sequencing.

Dedicated to my Parents Sheikh Mohammad Ashraf and Mrs. Shahida Ashraf

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# VITA

Oct 3, 1975		Born - Rawalpindi, Pakistan.
1994		B.Sc. University of Punjab. Pakistan. Major: Botany, Zoology, Chemistry.
1997		M.Sc. Quaid-e-Azam University. Biochemistry and Molecular Biology Islamabad, Pakistan.
1999		M.Phil. Quaid-e-Azam University. Islamabad, Pakistan Biochemistry and Molecular Biology
2000 – presen	nt	Graduate Research Associate, Food Animal Health Research Program, Ohio Agricultural Research and Development Center (OARDC), The Ohio State University, Wooster, Ohio. U.S.A.

# **PUBLICATIONS**

### **Research Publications**

- 1. Interference between mild and pathogenic strains of Infectious bursal disease virus in chickens. S. Ashraf, G. Abdul-Alim, M.Q. Al Natour and Y. M .Saif. Av Dis.49:99-103. 2005.
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### FIELD OF STUDY

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### **CHAPTER 1**

### LITERATURE REVIEW INFECTIOUS BURSAL DISEASE VIRUS

### **1.1 Introduction**

Infectious bursal disease virus (IBDV) is the causative agent of infectious bursal disease (IBD) that affects young chickens about 3-6 weeks of age. It is a highly contagious and acute viral disease that is characterized by destruction of lymphoid cells in the bursa of fabricius. Ever since the disease was recognized some 40 years ago, it continues to pose a threat to the commercial poultry industry. The economic impacts of the disease are manifold including losses due to morbidity and mortality, immunosuppression in the surviving chickens since IBDV infection exacerbates infections with other disease agents, reduction in the chicken's ability to respond to vaccination and risk of introduction to exotic places from importing infected poultry products. The economic impact of the disease is influenced by pathogenicity of the strain of virus, susceptibility and breed of flock, other prevalent pathogens and environmental and management practices.

The causative agent is a bisegmented, double stranded RNA virus that belongs to the family Birnavirideae. Two distinct serotypes have been recognized. Pathogenic strains are grouped in serotype 1 viruses while serotype 2 strains are non-pathogenic. Initially

recognized strains were named Classical strains. Mutations in the RNA genome of the virus have resulted in the appearance of variant strains in the US and very virulent strains all over the world.

Losses due to the classical IBDV reach up to 50% morbidity and less than 3% mortality in broilers and up to 20% mortality in commercial Leghorn pullets (211). Losses due to very virulent strains of the virus in Europe have reached approximately 30-40% mortality in broilers and 50-70% in commercial layers (37,44,317).

IBDV infection also lowers the egg production, leads to deterioration of egg shell and internal egg quality. IBDV infection results in immunosuppression which is marked by higher viral respiratory infections and increased mortality due to airsacculitis and colisepticemia during the end of the growing period in broilers (211).

Significant progress has been made in recent years that led to better understanding of structure, morphology and molecular biology of this virus. Specific and sensitive diagnostic tools are now available and effective vaccines have been prepared for combating the disease.

#### 1.1.1 History

In 1957, Albert S. Cosgrove recognized a syndrome, on a broiler farm near Gumboro, Delaware. It was named "Gumboro disease" after the geographic location of the first recorded outbreak. The disease was characterized by ruffled feathers, watery diarrhea, trembling, severe prostration, dehydration, hemorrhages in the leg and thigh muscles, increased mucus in the intestine and enlargement of the bursa of fabricius. The syndrome was characterized by 10% flock morbidity and mortality ranging from 1- 10% occurring throughout the Delmarva region (171).

Initially, Variant infectious bronchitis virus (Gray strain) was suspected to be the causative agent since the kidney lesions induced by the Gray virus and those seen in avian nephrosis were similar, hence the syndrome was given the name avian nephrosis-nephritis (331). The concurrent occurrence of two diseases, lack of SPF eggs and limited ability of the diagnostic tools to isolate the virus, led to this conclusion. Subsequent studies indicated that birds immune to Gray virus could still be infected with the Infectious bursal disease (IBD) virus and would develop changes in the cloacal bursa like IBD (191).

Within three years of its initial recognition at the East coast, the disease spread to all 13 southwestern poultry producing states (1). None of the conventional treatments like antibiotics, vitamin supplements, molasses or different management practices altered the course of the disease (171) and different management practices had variable or insignificant effect on the severity of the disease (241,242). It was also noticed that the disease transmitted from farm to farm through leftover feed from the affected farms (65,66).

Winterfield isolated the agent in embryonating eggs and noticed that it was difficult to maintain on serial passage. The isolate was called infectious bursal agent (IBA) and regarded as the true cause of IBD. Edgar in 1961 named the syndrome "Infectious bursal disease"(IBD) instead of Gumboro disease while Gray virus was identified as an isolate of infectious bronchitis virus with nephrotoxic tendencies (191). Planned infection with the disease agent was proposed as a control measure on the premises where IBD was already established.

Hitchner studied the clinical manifestations of the infectious bursal agent (IBA) and differentiated them from infectious bronchitis virus (IBV) (110). Cho and Edgar characterized IBA in experimentally infected chickens and observed gross changes in the bursa and population shifts in circulating blood cells over the course of infection (41).

Benton and Edgar concluded that the etiologic agent was a virus, relatively resistant to extreme temperature and pH and a wide range of chemical treatments (18,41). By 1967 Moulthrop and Wills were able to adapt an isolate to chicken embryos (289).

In 1970, Cho suggested that IBD infection was equivalent to "biological bursectomy"(40). In 1972 Allan *et al* (7) reported that IBDV infection at an early age had immunosuppressive effects. Several studies reported significant immunosuppression following exposure to IBDV and Newcastle disease virus (ND), or Marek's disease, or E.*coli* or S. typhimurium (7,40,78,79,335). These observations greatly emphasized the need for the development of control measures.

By 1976 the structural and growth characteristics became available in detail and it was concluded that the virus could not be placed into any previously recognized group and should be placed in a new taxanomic category (227). Classic strains like Edgar, 2512 and Irwin Moulthrop (IM) were isolated from the United States in the early 60's and caused 30-60% mortality in chickens causing hemorrhagic inflammation and B cell depletion (182,291). The vaccine strain, Winterfield 2512, was isolated by Winterfield in 1965 and was modified for vaccine production (328). A mild strain adapted to chicken embryos was used to produce the first licensed vaccine "Bursa Vac"(289).

The existence of the second serotype was reported in 1980 (199) and were found to be non-pathogenic to chickens. Variant viruses like Delaware and GLS were isolated in the 80's from vaccinated farms in Delmarva poultry producing area. These viruses did not cause mortality or trigger inflammation. These strains were able to break through the maternal immunity against standard or classic strains (262).

At the beginning of the 1980's most commercial broiler farms were not vaccinated against IBD in many European countries, since it was believed that maternal immunity from the vaccinated chickens would protect against IBD infection. The very virulent IBDV (vvIBDV) first appeared in the Netherlands in the 80's and rapidly spread all over the world including Central Europe, South Eastern Europe (348) Japan (181,232) Russia (265,276) the Middle East, South America (56) Dominican Republic (55) and Asia (33,36). Australia, New Zealand, Canada and US are still free from the vvIBDVs. These viruses were able to breakthrough the immunity provided by the maternal antibodies and cause more than 70% mortality and in addition to the bursa of fabricius, it caused lesions in the thymus and bone marrow (29).

#### 1.1.2 Etiology

The etiological agent of the disease is Infectious bursal disease virus (IBDV) belonging to the family Birnaviridae of the genus Avibirnavirus. The genus name Birnavirus was proposed to describe viruses with 2 segments of double stranded RNA. Other viruses included in this group are Infectious pancreatic necrotic virus (IPNV) of fish, Tellina virus, oyster virus, blotched snakehead virus (BSVN) (52) and crab virus of bivalve mollusks belonging to Aquabirnavirus while Drosophila X virus belongs to genus Entomobirnavirus. All of these contain two segments of double stranded RNA surrounded by a single protein capsid of icosahedral symmetry (58).

#### **1.1.3 Types and subtypes**

Two distinct serotypes of the virus have been recognized. Serotype 1 viruses are pathogenic to chickens while serotype 2 viruses are nonpathogenic to chickens and have been isolated from chickens and turkeys. Serotype 1 viruses can be further categorized into 4 groups on the basis of their pathogenicity: Classical strains, variants, attenuated strains and very virulent strains (179). Different pathotypes of the virus and the cell culture adapted strains differ markedly in virulence. Three criteria currently being used for the characterization of IBDV strains include antigenicity, genetic relatedness and pathogenicity.

Classical IBDV has traditionally affected poultry worldwide since the first reported incident from Gumboro. Classical strains cause bursal inflammation and severe lymphoid necrosis in infected chicken, resulting in immunodeficiency and moderate mortality from 20-30% in specific pathogen free (SPF) chicken (179).

Variant strains appeared in the US in 1983. These strains were antigenically different from classic strains and caused a rapid and severe bursal atrophy (313) and in contrast to classical strains produced no clinical signs of illness. Antigenic variants have been recognized by their ability to escape cross-neutralization by antiserum against the classical strains (179).

Attenuated strains have been generated by adapting the classical and variants strains to chicken embryo fibroblasts (CEF) or other cell lines (179). Since they are not pathogenic they have been used as live vaccines.

Emergence of the very virulent strains during the 1980's in Europe, Japan and China resulted in dramatic losses to the poultry industry. Very virulent strains have been

characterized by severe clinical signs and high mortality ranging from 60-100%. Very virulent strains can breakthrough the immunity provided by the maternal antibodies. The vvIBDVs produce similar signs as of the classical strains and the same incubation period of 4 days but the acute phase is more severe and more generalized in the affected flocks (314).

Serotype 2 viruses are apathogenic and do not cause any mortality or bursal lesions in specific pathogenic strains and isolated from chickens and turkeys. The examples of these strains include OH, MO, KM, SW and GK15. Serotype 2 strains do not produce any clinical disease in the chickens. However, OH strain of serotype 2 IBDV is known to cause mortality and gross lesions in embryonating eggs (4) but did not produce any lesions in chickens. Both serotypes co-exist in chickens and turkey flocks in the USA (134) and antibodies to both serotypes are prevalent in the field.

#### **1.1.4 Prevalence**

Classical IBDV have traditionally affected poultry worldwide ever since the first outbreak of disease was reported from Delaware, Maryland and Virginia (Delmarva) region. Cosgrove was the first to report this disease in 1962 (46). By 1970, the disease had been reported from Canada (117), Mexico (186), Europe (94,168), Africa (237), the Middle east (68) and Asia (204).

The virus is resistant to chemical agents and hard to eradicate from poultry houses. The infection is endemic in nature and birds are constantly exposed to the virus. Breeder flocks are vaccinated against the virus to provide maternal immunity to the off-springs so all chicken flocks are seropositive for the virus (191).

Antigenically different strains called variants were isolated from Delmarva region in USA in 1983 (262). Snyder *et al.* (293) observed a distinct geographic distribution pattern of the virus. In isolated and less dense broiler growing areas such as ME, NE, WA, OR, OK, CA, OH and FL, classic strains of IBDV were predominant. Eastern areas such as MS, AL, GA, VA, NC and Delmarva area had variant strains predominantly. Other areas such as AR, PA, TN and IN appeared to be more in a transitional stage with respect to their IBDV populations. Once a given IBDV type is introduced, it establishes itself in a house and becomes prevalent (293).

Very virulent IBDV strains were identified from Europe in 1987 and from there these strains spread to South Africa, Asia and the Middle East and are now distributed worldwide (55). These strains have recently been isolated from Central and South America (55,56). There is no evidence of vvIBDVs in the United States, Australia and New Zealand. Most European countries have reported the isolation of vv IBDV, however some Scandinavian countries are only sporadically affected (51,226).

### 1.1.5 Structure

The IBDV is a non-enveloped virus with a diameter of 60 nm and a density of 1.336 g/ml in CsCl (150). Capsid of the virion consists of a single layer of 32 capsomers arranged in a 5:3:2 symmetry (108). Density equilibrium in CsCl is the common method of purifying IBDV except for the Australian 002/73 and IM strain which are unstable in CsCl gradient(10).

The IBDV particle has a sedimentation co-efficient of 460S in sucrose gradient (57,307). Various densities reported for the IBDV particle range from are 1.33 to 1.29g/ml (26,108,227). A single virus preparation has been reported to have as many as 6 bands

(212). Usually the buoyant density of 1.33 g/ml corresponds to the typical IBDV particle in cesium chloride (CsCl). Bands obtained at 1.32 g/ml had double stranded RNA and represent incomplete virus particles and those at 1.31 g/ml had incomplete virus particles without RNA while 1.29g/ml had irregular shape, poor assembly and an unusual amount of dsRNA. The bands showed similar SDS-PAGE profile except the band obtained at 1.29g/ml. Particles of 1.29 g/ml were obtained when the virus was grown in CEF as opposed to bursa of fabricius. Incomplete virus particles are not normally obtained from bursa (150, 210). Formation of incomplete virus particles can be one of the reasons for the loss of pathogenicity for chickens when IBDV is passaged repeatedly in CEF cells. (150, 210).

The three dimensional structure of IBDV virion has been determined by electron cryomicroscopy. The outer and inner surfaces of the capsid are made of trimeric subunits (26). Virus architecture is based on a T=13 lattice (239). Capsid is 9 nm thick and non-spherical in shape since the subunits close to the 5 fold symmetry axes are at a larger radius than those close to 2-3 fold axes. The VP2 forms the external trimeric subunits and protrude out of the shell forming a honeycomb surface. The VP3 forms the inner Y-shaped trimers that are packed closely to form a continuous shell and are connected to VP1. VP4 formed the rim around each 5 fold axis on the inner surface of the capsid (26).This model suggests 780 copies of VP2, 600 copies of VP3, 60 copies of VP4 and is in accordance with the observed composition of 51% VP2, 40% of VP3, 6% VP 4 and 3% VP1(58).

#### **1.1.6 Physiochemical properties**

The virus is non-enveloped and quite resistant to physical and chemical agents. Due to the stability and hardiness of the virus, it persists in poultry premises even after thorough cleaning and disinfection. The virus is inactivated at a pH of 12.0 but not at pH 2.0 (18). Benton (18) reported that IBDV survived a temperature of 37C for 90 min and 56C for 5 hours. A marked reduction in infectivity of the virus was observed after treatment with 0.5% Formalin for 6 hours. The virus remained unaffected by ether, choloroform, phenol, thiomaesal, Staphene and Hyamine 2389 treatments. The virus survived treatments with various concentrations of three disinfectants (an iodine complex, a phenolic derivative and a quaternary ammonium compound) for a period of 2 minutes at 23C, only the iodine complex had any deleterious effects.

Cho and Edgar (41) reported that the virus was inactivated by exposure for 1 hour to 1% formalin, 1% cresol and 1% phenol. It remained stable at 60C for 90 min and was still infectious at room temperature for approximately 25°C for 21 days. Petek (244) observed that IBDV was more resistant then Reovirus to heat, ultraviolet irradiation and photodynamic inactivation.

The hardiness of the virus makes it difficult to eradicate it from poultry houses after outbreaks of IBD (6). Heat resistance of IBDV is an important factor to be considered in trade of poultry due to extensive international trade of processed and partially processed poultry meat.

Alexander and Chettle (6) constructed the heat inactivation curves of classical IBDV at 70C, 75C and 80C. These biphasic multiple kinetic curves showed an initial rapid drop in

infectivity followed by a more gradual decline. In the second phase it took 18.8 min at 70C, 11.4 min at 75C and 3.0 min at 80C for reducing the infectivity by 1 log 10. Landgraff (167) showed that IBDV survived at 60C but did not survive at 70C for 30 min and 0.5% chloramines killed the virus after 10 min. Invert soaps with 0.05% sodium hydroxide either inactivated or had a strong inhibitory effect on the virus (279).

To investigate heat labiality of the virus, it was subjected to 37, 65, 71,74,77,82 or 100C for 1 min. Virus titer declined greatly at temperatures above 65C, 71 and 100 at rates of >90, 99 and 99.99% respectively. Similar viral reduction curves were obtained after heating for 6 min at 71 or 74 min (195). Minimum heating patterns for 1 log 10 reduction in titer ranged from 1 min at 65C (195) to 18.8 min at 70C (6).

The virus is unusually resistant to inactivation by cooking so there is a risk of introduction to the backyard flocks through uncooked chicken meat products since viable virus might be present in meat from apparently healthy chickens. Drumsticks and chicken patties experimentally injected with  $10^7$ - $10^9$  TCID<sub>50</sub> of virus and cooked to internal temperature of 71 and 75C respectively in hot oil or steam in a flame grill still contained the infectious virus (195). Although this data provide guidelines, careful consideration is required while extrapolating it to natural conditions due to artificially high doses used in these experiments.

IBDV was completely dissociated into subunits at a high hydrostatic pressure of 240 MPa at 0C as revealed by the change in intrinsic fluorescence spectrum and light scattering. Electron microscopy showed that virus morphology had an obvious change after pressure treatment. Elevating pressure also destroyed the infectivity of the virus while retaining its immunogenicity(306).

#### **1.1.7 Biochemistry**

The genome consists of two pieces of high Mr dsRNA that sediments at 14S components in sucrose gradients (150,215,307). The molecular weights of the two segments are 2.2 x 10<sup>6</sup> and 2.5 x 10<sup>6</sup> (213). It was reported that the two segments migrated similarly when co-electrophoresed. The RNA segments from serotype 2 viruses migrated similarly, but differed from serotype 1 viruses when co-electrophoresed (17,136).

Bouyant density of 1.62 g/ml in cesium sulphate, melting point of 95.5 C in the presence of RNase, pairing of Adenine and Uracil and Thymine and cytosine, precipitation from 4M but not from 2M LiCl, green staining with acridine orange, all point towards a dsRNA genome (150). Both genome segments contain 94 x 10<sup>3</sup> 5' genome-linked protein (VPg). There are no poly(A) tracts at the 3' ends of the RNA segments. The defective particles banding at 1.30 g/cm<sup>3</sup> appear to have a truncated A segment.

The virion contains five polypeptides including VP1 (94 x 10  $^{3}$ ) an RNA dependent RNA polymerase as well as VPg; pre-VP2 (62 x 10  $^{3}$ ) and VP2 (54 x 10  $^{3}$ ), the major capsid and type specific antigen VP3 (30 x10  $^{3}$ ) and VP 4 (29 x10  $^{3}$ ) a component of the virion. Negatively charged non-structural protein (16.5 x10  $^{3}$ ) VP5 is encoded by an ORF preceding to segment A encoded polypeptide. Although VP 1 can guanylylate itself, no guanylyl transferase activity has been detected and viral RNA made in the cells retains its 5' VPg. Also, the viral RNA is not capped at the 5'end (252). No N-linked glycosylation of any of the virion proteins has been detected (252).

#### **1.2 Viral proteins**

Five proteins have been identified in IBDV by SDS-PAGE analysis and they are generally referred to as VP1 (90Kd), VP2 (40 Kd), VP3 (35 Kd), VP4 (28 Kd) and VP5 (21 Kd) (57,116,218). The VP2 is the most abundant polypeptide and makes up more than 50% of the virion protein (57). The VP3 is the second most abundant protein and makes up 40% of the virion protein. Both VP2 and VP3 are responsible for the structural integrity of the virion. The VP4 and the VP1 are minor proteins of the virion accounting for 6% and 3% respectively.

There are two open reading frames on segment A which encodes a polypeptide 110kd in the form N-VPX-VP4-VP3–C by the action of VP4. The VP4 cleaves this polypeptide between VPX and VP4 and between VP4 and VP3 (149). Similar precursor proteins have been demonstrated for VP3 and VP4 of an Australian isolate (002/75) (11,115). The small ORF (435 bp) on segment A encodes VP5 while the genome segment B encodes VP1.

Earlier researchers noticed that the combined molecular weight of the virion proteins exceeds the coding capacity of the viral genome. They investigated this and ruled out the possibility of each genome segment encoding a polyprotein via a monocistronic mRNA. Tryptic peptides of VP-X and VP2 were identical whereas the peptide map of the other proteins differed greatly. This observation led them to propose that VPX is the precursor of VP2. In addition two dimensional peptide maps of other structural proteins were unique which eliminates VP1 as the precursor of other polypeptides (17).

Different researchers have reported various molecular weights of the viral proteins that have resulted from different methodologies used in different laboratories and variable cleavage of the precursor protein (17,57,150,307). However, genuine differences do exist between the two serotypes as shown by the SDS-PAGE. The structural protein of classic and variant viruses had minor molecular weight differences among themselves but the differences were distinct when compared to the serotype 2 viruses. In addition, the bursa derived viruses were different then the cell culture propagated virus in molecular weights and proportions of the viral protein (310).

The VP2 carries major neutralizing epitopes suggesting that it is at least partly exposed on the outer surface of the capsid (26). The VP3 carries a very basic carboxy terminal which is likely to interact with a packaged RNA and is therefore expected to be on the inside of the capsid (115).

The VP2 contains the antigenic region responsible for the production of neutralizing antibodies and is highly conformation dependent (12,17). Neutralizing monoclonal antibodies against VP2 can be used to differentiate the serotypes and strains (17,75). The VP2 is also responsible for antigenic variation (29,198,290,311) tissue culture adaptation (179) and virulence (29,340).

The VP2 has been shown to be an apoptotic inducer in a variety of mammalian cell lines. However, the effect is counteracted by co-expression of the proto-oncogene bcl -2 (80). Recently VP2 and VP5 were shown to be involved in induction of apoptosis in chicken B-lymphocyte cell line RP9 and chicken embryo fibroblast cells (344).

Two biological functions of the neutralization epitopes of cell culture adapted IBDV were described by the use of monoclonal antibodies. The VP2 consists of neutralizing epitopes that are involved in post-adsorptive events while VP3 in spite of its low neutralization titer prevents the initial virus attachment process (251).

The VP3 carries the group specific antigen since monoclonal antibodies against it can react with both serotype 1 and 2 viruses. Basic C- terminal region of VP3 is involved in the packaging or stabilizing the RNA genome within the interior of the viral capsid (116). The VP4 is the viral protease (115,144) and contributes to processing of precursor polypeptide (12,63,144). The VP4 is not found in the virions but forms tubules in both the cytoplasm and nucleus of infected cells (92). It belongs to the U43 family according to classification of MEROPS website (178). Data on VP4 of infectious pancreatic necrosis virus (IPNV) and sequence analysis of IBDV and IPNV suggests the novelty of the VP4 and showed that it shared properties with prokaryotic leader peptidases and other bacterial peptidases. The IBDV VP4 utilizes a serine lysine catalytic dyad (Ser –652 and Lys 692) (178,217). These two residues are essential for the polyprotein processing since their removal completely abolished the polyprotein processing.

Extensive homology exists at the nucleotide and amino acid level between N-terminal and C-terminal polypeptide but very little homology is observed between the internal VP4 of IBDV and IPNV. N-terminal sequencing and site directed mutagenesis identified two cleavage sites at pVP2- VP4 (511LAA513) and VP4 –VP3 (754MAA756) junctions along with additional cleavage sites in C- terminal region of pVP2 (VPX). VP4 cleaves multiple (The/Ala) – X – Ala\* Ala motifs (178). The VPX to VP2 conversion involves cleavage of pVP2 near its C-terminus (12).

In addition, VP4 of Birnaviruses were shown to be species specific, since they did not cleave heterologous substrates (178). Actual cleavage sites used by VP4 at the C-terminus of the outer capsid protein VP2 have also been determined (178,263). Final VPX –VP2 processing is associated with the final maturation or release steps of the virus

(154) and correct scaffolding of the VP3(211). An interaction between VP1, the polymerase and VP3, the inner capsid protein plays a major role in efficient encapsidation (183,300).

The VP5 is encoded by a second ORF immediately preceding and partially overlapping the 110kd polypeptide gene (218). It is a membrane-associated protein involved in viral release (183). The VP5 is a highly basic, cysteine rich and conserved among all serotypes of IBDV strains (344). The VP5 is not essential for viral replication (220) as VP5 recombinant IBDV did not produce lesions *in vivo* (343) and protected chickens against a virulent challenge with strain F52/70 (184,222,343). The VP5 is a class II transmembrane protein. Lombardo *et al* (184) observed that expression of VP5 in different cell systems caused severe cytotoxic effects resulting in cell lysis and proposed that VP5 acts as a death protein and functions in viral release.

The VP1 is encoded by segment B and is RNA dependent RNA polymerase, the minor internal component of the virion. It has the polymerase and capping enzyme activities (116,295,296). Birnavirus VP1 forms a distinct subgroup of RNA dependent RNA polymerase lacking a GDD motif (280). This protein is synthesized *in vitro* in small quantities and incorporated into virions without apparent changes in relative molecular weight (203). In mature virus, it is tightly bound to both ends of the genome and circularizes them (214). Thus, the VP1 is present in the virion as a free polypeptide as well as a genome linked protein called VPg (214). Similar VPg proteins have been identified in the Polio- virus and adenovirus and play a role in genome replication at the initiation level (82). It is involved in the efficiency of viral replication and modulates the virulence *in vivo* (182).

#### **1.3 Genome Organization**

The genome of IBDV is composed of two segments of double stranded RNA, hence the name Birnavirus (58,215,227). The longer segment A is 3.2 Kb in length while the shorter B segment is 2.8 Kb. Segment A is 3261 base pair (bp) in serotype 1 and 3264 in serotype 2 while segment B is 2827 bp (221). Both segments A and B of various strains of IBDV have been completely sequenced by various laboratories.

There are two open reading frames (ORF) on segment A, positioned in different reading frames. The plus strand of segment A has a long monocistronic ORF of 3039 b.p (314). A short ORF of 438 bp precedes and partially overlaps the 5' end of the first ORF (152) and encodes nonstructural protein VP5 (218). This nonstructural protein is present only in the IBDV-infected cells (218,220) and is not required for viral replication but plays a role in pathogenesis (343). Segment B has only one ORF and encodes VP1.

The larger ORF of segment A (3036 b.p) encodes a 110 kd polyprotein precursor, in the order NH2 – VP2-VP4-VP3 – COOH (12) (1012 amino acids) that is cleaved by auto-proteolysis into viral protein VP2 (40kd), VP3 (35 kd)and VP4(24 kd) (116, 213). The processing of three polypeptides occurs stepwise and VP2 can be found as a precursor protein (VP2a 45-50 kd) and a cleaved product (VP2b, 40-42 kd) (316).

Site directed mutagenesis identified two processing sites for VPX – VP4 and VP4 - VP3 precursors 511LAA513 and 754MAA756 respectively. These sites are quite conserved in serotypes 1 and 2. Another site was detected in a 19 amino acid stretch located upstream of 511LAA513 for processing of VPX- VP4.

Specifity of cleavage is thought to be dictated by the conserved AA dipeptide (263). Dibasic residues position at Arg (452), Arg (453), Lys (722) and Arg (723) were suspected VP2 – VP4 and VP4 – VP3 cleavage sites respectively.

Genome segment B encodes the VP1 (208, 214, 266, 296, 313), an RNA dependent RNA polymerase. This 90 kd multi functional protein has the polymerase and capping enzyme activities (149). This protein is responsible for the synthesis of mRNA and replication of the genome (296). More recently it has been shown to have an effect on the efficiency of viral replication and it also modulates the virulence of the virus *in vivo* (182).

The VP2 and VP3 are the major structural proteins of the virus. VP2 contains the antigenic regions responsible for the induction of neutralizing antibodies and for serotype specificity (12, 17, 75). VP3 contains the group specific antigens and elicits neutralizing antibodies (17). Epitopes which do not induce neutralizing antibodies and are common to both serotypes also located on VP2 (17, 238).

Deletion experiments of cDNA fragment of genome segment A have shown that VP4 is involved in the processing of the precursor polyprotein and acts as a virus-encoded protease (152). The additional sequences at the 5'end of the plus strand contains a smaller ORF of at- least 435 b.p that predicts a 145 amino acid protein (152). Both genome segments contain a genome-linked protein (VPg). There are no poly (A) tracts at the 3'end of the RNA segments (252).

There is a highly variable region within the VP2 gene where most of the amino acid changes between antigenically different IBDVs are clustered. The region between amino acids 206 and 350 is extremely hydrophobic and contains the major neutralization site of the virus. Any amino acid change here would result in the antigenic variation of the virus.

Hydrophilicity profile of this region showed the presence of two hydrophilic peaks at each terminus, the larger peak A 210-225 and smaller peak B 312-324 (14, 61). Major differences in the reactivity of neutralizing monoclonal antibodies (Mabs) result from amino acid changes in peaks A and B (71, 266).

A serine rich hepta-peptide region (S-W-S-A-S-G-S) is located after the second hydrophilic region and is conserved in pathogenic strains; less virulent strains have fewer serine residues (29). Serotype 2 OH strain lacked the conserved S-W- S- A-S –G-S motif suggesting that this region might play a role in the pathogenicity or serotype specificity of the virus (311).

Replacement of the VP2 gene of vvIBDV strain D6948 with the corresponding gene of attenuated strain CEF 94 didn't abolish the pathogenicity of the virus in chicken, confirming that VP2 isn't the sole determinant of virulence (23, 25). It was shown by *in vitro* and *in vivo* analysis of reassorted IBDVs that virulence factors are located on the B-segment as well (22) thus confirming the multigeneic nature of virulence.

An important difference between virulent and vvIBDV VP5 was that vvIBDV VP5 has an N-terminal extension of four amino acids. In addition, two unique amino acids 49R and 137W were found in all known vvIBDV VP5 sequences. The hydrophilic peaks and internal sequences of VP3 are not conserved between serotype 1 and 2 viruses (223). Yamaguchi *et al* (340) reported that amino acids residues at position 279 and 284 in VP2 region might play a role in the virulence of the virus and VP1, VP3, VP4 and non-coding regions might be involved in the pathogenicity (30).

Initial sequencing studies showed five amino acid sites 222 (P $\rightarrow$ A), 256 (V  $\rightarrow$ I), 279 {(N $\rightarrow$ I)}, 294(I), 299(N $\rightarrow$ S) to be unique for all vvIBDV strains as compared to the
classical strains (30,33,36,181,340,347), except for the early Ivorian strain. These positions were considered a putative molecular marker for the vvIBDV strains. All variant viruses have a Gln  $\rightarrow$ Lys substitution at position 249 (311). A change of the third serine to arginine in the heptapeptide was a marker of low pathogenic strains (30). However, it is not known whether these amino acid mutations effect the virulence of the virus or are merely evolutionary markers (69).

The Acc I – Spe I restriction area encompassing 14 nucleotide positions is conserved in most vv IBDVs, differentiating them from all other IBDVs and two variable positions allowing for the distinction of two very virulent groups (29). Sequence analysis revealed that Brazilian strains were closely related to vvIBDVs described in Europe and Asia (118).

The *BspM I* restriction site representing amino acid 222 (P $\rightarrow$ A) is responsible for the absence of binding of the neutralizing monoclonal antibodies to vvIBDV strains in antigen capture ELISA (71) and was suggested to be a marker for the vvIBDV. The West African isolate 88180 was unique since it lacked both *BspM I* and *SspI* sites (70).

Using reverse genetics approach specific residues responsible for tissue culture adaptation, virulence and cell tropism have been mapped down to the VP2 (23, 27, 319). Phylogenetic analysis of segment B has shown that vvIBDV strains form a distinct cluster (341) suggesting that these strains have acquired segment B from an unidentified source by genetic reassortment (211).

The 5' and 3' Non-coding regions (NCR) of IBDV segment A and B are similar in size to those of other ds RNA- containing viruses like reovirus (221). The 5'noncoding region (NCR) of both segments has a 32 nucleotide sequence which is conserved between

segments and serotypes. 5' NCR consists of 96 nucleotide before the start codon of VP5 gene in segment A and 111 nucleotides preceeding the start codon of VP1. Inverted terminal repeats are present in both segments. A sequence of 13 nucleotide formed in 5' non-coding region of both segments could possibly be a binding site for chicken 18S r RNA. The 3' NCR regions is highly conserved within segment A or B but is different between segments.

Interestingly, the predicted structure of different NCRs shows a high degree of similarity but that of segment A is different between serotypes (221). Viral and segment specific nucleotide sequences might play an important role in virus replication; recognition of viral DNA or in recognition, sorting or packaging of different segments (221).

Conserved sequences might serve as the signals for viral recognition whereas segment specific inverted terminal repeats and 3' terminal sequences might play a role in the recognition of a particular segment. The VP1 can interact with ends of both segments to form a pan-handle structure (214) which is supposed to protect RNA from degradation by ribonucleases (221). More recently, VP1 has been shown to be involved in the efficiency of viral replication and modulating the virulence *in vivo* (182).

The non-structural protein VP5 has a role as a virulence factor that can induce apoptosis (344). Null mutants of VP5 have reduced virulence and exhibit reduced rates of bursal apoptosis but are replication competent. Terminal nucleotide sequences are important in replicative ability of the virus since they specify signals for RNA replication, transcription and translation (221).

The exchange of non-coding regions between a virulent serotype 1 strain and an avirulent serotype 2 strain did not affect pathogenicity of the virus in chickens, indicating that NCR were not responsible for different pathotypes of IBDV (267).

The analysis of base usage showed that all IBDV genes possess equivalent overall nucleotide distributions, however, the base usage at each codon position indicated that VP5 ORF formed a different cluster from the other genes. Moreover, GC content of IBDV genes and chicken's coding sequence were found to be similar. Dinucleotide frequency of IBDV showed that CpG and TpA were lower and TpG was higher than expected. Dinucleotide frequencies of the VP2 region and the polyprotein indicated that the vvIBDV formed a unique dinucleotide pattern (345).

One milestone of the molecular biology of IBDV was the development of the reverse genetics system. This system allowed understanding the significance of structural elements and /or their biological properties through *in vivo* site-directed mutagenesis. This system enabled researchers to generate VP5 knocked out IBDV strains (220, 343), reassortants (23), inter-serotypic and inter-pathotypic recombinant IBDV strains (21, 23, 24, 267, 268) and generating point mutations induced by exchange of amino acids by site-directed mutagenesis (179, 217, 250, 319).

The specific amino acids involved in cell culture adaptation (Q 253 H; A 284 T) (217, 319), virulence (279 and 284) and cell tropism have been mapped to VP2 by using reverse genetics (27, 340, 347). Boot (23) rescued a very virulent strain after transfection of cDNAs in QM5 cells and suggested that the domain responsible for interacting with the IBDV receptor is located in the hyper variable region of VP2.

By site directed mutagenesis, the variant DEL/E and very virulent UK661 were adapted to tissue culture and the very virulent mutant became attenuated in chickens (217, 319). By using this technology, Islam generated two full-length clones of segment A and B of the Bangladeshi very virulent virus and produced a tissue culture adapted virus by substituting two aa at position  $253(Q \rightarrow H)$  and  $284(A \rightarrow T)$ . The wild type virus and the one mutated at aa 284 did not replicate in chicken embryo fibroblasts.

Loon (319) studied the *in vivo* characteristics and pathogenicity of the mutated amino acids at position 284 and 253. It has been shown that tissue culture adapted virus has reduced pathogenicity as compared to the wild type virus. The reassortant virus replicated poorly in cell culture and did not cause any morbidity and mortality.

Liu (182) noticed that tissue culture adapted GLS virus differed from the bursa derived GLS in two amino acids at positions 87 (Q  $\rightarrow$  R) and 261 (P  $\rightarrow$  L) in segment B, and at positions 253 (Q  $\rightarrow$  H) and 284 (A  $\rightarrow$  T) in segment A. Recombinant viruses between D-78 and tissue culture or bursa derived GLS were generated. Recombinant GLS having B segment of bursa derived GLS had delayed replication kinetics in chicken embryo fibroblasts and Vero cells as compared to the parental strain but grew efficiently in bursa. Hence, they inferred that VP1 is involved in the efficiency of viral replication and *in vivo* modulation of virulence.

#### **1.4 Antigenic Properties**

Infectious bursal disease virus is endemic throughout the world but several different antigenic and pathogenic types exist in specific geographic locations. Two serotypes of IBDV occur in Europe and USA as recognized by the virus neutralization test. These two serotypes are antigenically distinct (199). Serotype 1 viruses are pathogenic to chickens and differ in their virulence (332). They cause lesions in the bursa of fabricius by lymphocytic depletion (268). Whereas serotype 2 viruses are avirulent to chickens and isolated mainly from turkeys (125,153) and chickens.

Serotype 1 viruses can be broadly divided into classic, variant and very virulent IBDVs.

Until 1987, the strains of virus were of low virulence and were controlled by vaccination. Emergence of variant viruses were first reported in USA in 1987. These viruses were reported to undergo an antigenic drift against which the classical IBD vaccines were not protective (129,293).

Six antigenic subtypes of IBDV serotype 1 viruses have been identified by the virus neutralization test (129). Variant viruses occur in the USA and Australia and are different from the classic viruses in terms of pathogenicity and immunogenicity. They overcome the immunity induced by classic serotype 1 viruses and cause rapid bursal atrophy with minimal or no inflammatory response (99, 129, 199).

Vaccination with one serotype 1 subtype didn't insure protection from challenge with another subtype (124, 129, 199) suggesting that variant viruses are antigenically different from classical viruses. Variant viruses present in the USA and Australia are not closely related to each other (264). Significant antigenic differences exist among serotype 1 strains as detected by virus neutralization and led to the grouping of the serotype 1 viruses into 6 subtypes (129). Virus neutralization test proved to be serotype specific and could distinguish between the two serotypes (129, 135, 137, 199).

Serotype 1 induced protection against challenge with variant viruses (123). Serotype 2 viruses are immunologically distinct from serotype 1 viruses since vaccination with serotype 2 (OH) virus did not confer protection against serotype 1. Cross protection

studies indicated that the variant viruses were different from other subtypes of serotype 1 IBDVs. Both serotype 1 and 2 viruses share common group antigens which could be detected by AGPT, Flourescent antibody test and ELISA (38, 130, 135, 137). Capsid proteins VP2 and VP3 contain epitopes that are responsible for group antigenicity (17). The VP<sub>2</sub> carries the serotype specific antigens responsible for the induction of neutralizing protective antibodies (12, 17).

Variation in IBDV antigenicity depends on amino acid changes in peak A and B. Serotype two strain 23/82, North American antigenic variants A, E, GLS-5 and DS 326 neutralization resistance escape mutants all exhibit amino acid changes in the hydrophilic peaks. Only serotype 1 strains show changes in hydrophobic domain. Two smaller hydrophilic areas of VP2 variable domain also influence IBDV antigenicity (71).

Deletion mutagenesis studies with Australian strain 002/73 showed that virus neutralizing monoclonal antibody recognized a discontinuous epitope on VP2 (12). The antigenic region responsible for the production of neutralizing antibody is highly conformation dependent (17, 75) since the antibodies immunoprecipitate VP2 but did not react with the denatured protein in the immunoblot (238). The VP2 carries the epitopes which elicit neutralizing antibodies and distinguish the two strains as well as those which elicit non-neutralizing antibodies and are common to both strains (49). The antigenic region responsible for the production of neutralizing antibody is highly conformation dependent. Monoclonal antibodies have also defined a common sequence dependent antigenic site located at the VP2 (17).

Since protective immunity against IBDV is dependent on the presence of neutralizing antibodies in susceptible birds, precise knowledge of antigenic sites responsible for the induction of neutralizing antibodies is of fundamental importance.

At least three distinct conformation dependent serotype 1 specific virus neutralizing antigenic sites have been identified on VP2 and one linear antigenic site on VP3 (12, 17). Two conformational virus neutralizing antigenic sites were localized in the central region of VP2 consisting of 156 amino acids residues while linear epitope was mapped to C-terminal 105 amino acid residues of VP3. Sequence specific epitope on VP2 are common to both serotypes (17). Of the two non-neutralizing monoclonal antibodies defined epitopes, one epitope is common to both serotypes whereas the second is distinct for serotype 1 and 2. With the help of the monoclonal antibodies against the variant strain DEL-E and GLS, Snyder showed that the presence of six distinct but closely related neutralization epitopes clustered in two or three sets on theVP2.

Very virulent strains are not antigenically different from the classic strains unlike variant strains from USA and Australia (264). It is not clear whether vvIBDV evolved from classical strains or pre-existed in nature with other avian species (121).

Very limited antigenic variation have been reported in the French vv IBDVs (72, 76) Fahey (76) reported two non overlapping epitopes recognized by the virus neutralizing monoclonal antibodies on VP2. Four conformation dependent neutralization epitopes were recognized on VP2 (316). The VP2 sequencing results confirmed that neutralizing epitopes are clustered in the variable domain which is highly hydrophobic (316).

In the vvIBDV strain there is no evidence of antigenic variation from the classical strains (317). A modified epitope has been described with the help of neutralizing monoclonal

antibodies corresponding to a mutation at amino acid position 222 in the first hydrophilic peak of VP2. No antigenic drift has been demonstrated by cross neutralization tests.

Hydrophilic peak B in the VP2 protein is involved in the formation of one or more neutralizing epitopes (139). Several mutations have been observed in the area between amino acid positions 317-323 by the nucleotide sequencing of the region (14, 62) (70, 102,132, 181, 249, 311). In France, some field isolates have shown atypical antigenicity due to the critical amino acid changes in the second hydrophilic peak but these have not replaced the more typical prevalent vvIBDVs (71, 73). A neutralization epitope has been possibly modified in European pathogenic IBDV strains because two monoclonal antibodies bind to the Faragher 52/70 strain, but not the atypical 89 163 isolate in an antigen capture ELISA (73). No genetic or antigenic absolute marker for virulence has yet been described. So the only way to demonstrate virulence is still by the *in vivo* inoculation (69).

#### **1.5 Host reservoirs**

Infectious bursal disease virus (IBDV) is host specific. IBDV has been reported in Ostriches (91), in Baltic ducks and herring Gulls (112), various raptor and passerine species in Japan (234). Anti-IBDV antibodies have been reported from Antarctic penguins (84). The virus has also been documented from the lesser mealworm (*Alphitobius sp.*) fed on contaminated IBDV feed. Experimental inoculation of pheasants, partridges, guinea fowls and quails showed no signs of disease (318). Japanese quails are refractory to IBDV infection (93). They tested negative for virus replication, detection of precipitating or virus neutralizing antibodies and gross as well as microscopic changes in bursa of fabricius associated with infection (324). However, Quail-chicken hybrids can

be infected with IBDV (93). In one study dogs were evaluated as a potential carrier of the virus since viable virus persisted in the feces two days after initial ingestion and maintained its original characteristics (308).

#### **1.6 Pathogenesis**

Pathogenesis is defined as the method used by the virus to cause injury to the host with mortality, disease or immuno-suppression as a consequence (315). The injuries can be evaluated at the level of host, the organ and the cell. IBDV usually infects young chickens between 3-6 weeks of age and causes a clinical disease, while sub-clinically infecting older birds. The outcome of IBDV infection is dependent on the strain and amount of the infecting virus, the age and breed of the birds, route of inoculation and presence or absence of neutralizing antibodies (211).

Sequential studies of tissues from orally infected chickens using immuno-florescence detected the viral antigen in macrophages and lymphoid cells in the cecum at 4 hr PI and in the lymphoid cells of duodenum and jejunum at 5 hr PI (216). The virus reaches the liver at 5 hrs PI and enters the bloodstream from where it is distributed to other organs; the bursal infection is followed by second viremia (191). The virus persists in the bursa of experimentally inoculated SPF chickens till 3 weeks of age but the presence of maternal antibodies in the commercial chicken decreases the duration of its existence in bursa (2). Various studies have shown that the variants and classic viruses exhibit similar pathology but differ from each other with respect to their pathogenicity and immunogenicity (98). Variant viruses (Var A) were reported to induce bursal atrophy with minimal or no immune response in contrast to the classic viruses (IM) which induce a severe

inflammatory response (271). However, it was noticed by the subsequent researchers that variant viruses are not homogenous as a group as thought previously (98). Bursa derived IN virus (variant) caused necrosis and atrophy of the bursa earlier than the bursa derived STC and it was also accompanied by inflammation. The only observed difference between the two was the onset and subsiding of the bursal atrophy. Both viral strains lost their pathogenicity after being passaged 4 times in BGM-70 cells.

Serotype 2 strains do not replicate in lymphoid cells but grow in chicken embryo fibroblasts like the tissue culture adapted serotype 1 strains. Susceptibility of chicken lymphoid cells to the virus does not correlate with the presence of specific binding sites since virus binds to both CEF and lymphoid cells (228,229). The attachment molecule for IBDV is shown to be an N-glycosylated protein (235). The CEF had receptors common to both serotypes and specific ones for each serotype. Receptor sites common to both serotypes were also present on lymphoid cells; however, additional serotype-specific sites were only demonstrated for the apathogenic serotype 2 strains. IBDV infection changes the potassium current properties of chicken embryo fibroblasts (253) resulting in changes in membrane permeability and intracellular homeostasis and contributing to cytolysis and death of the infected cell.

Host systems used to propagate the virus have a profound effect on the pathogenicity of the virus isolates. Significant differences occurred in the pathogenicity and immunogenicity of the virus propagated in BF or in the BGM-70 cells. However, the antigenicity of the viruses propagated in BF or the BGM-70 cells were not significantly different (98,99). Some strains of IBDV can adapt to CEF while others are refractory to grow in it. The SAL strain was adapted and passaged successfully in CEF cells while IN strain was unable to grow in CEF (98,99). The back passage of either IN or SAL in SPF chickens maintained or increased the virulence of both viruses (98,99). Wild viruses from B lymphocytes of BF were reported to be different than those grown in CEF. Differentiating B lymphocytes in the BF provide the optimal micro-environment for highly efficient virus replication; CEF and other cells seem to lack that environment (169).

Replication potential of low passage and high passage in BGM-70; and bursa derived virus in BF of 3 weeks old SFP chicken were compared over a 21 day PI period to find the correlation between virus pathogenicity and replication efficacy in bursa. Virus replication in BF was monitored by virus isolation in SPF chicken embryos, IEM, immunoflorescence (IF), and AC-ELISA. The virus was isolated from bursal tissue from birds inoculated with bursa –derived virus from 3<sup>rd</sup> day PI through 10<sup>th</sup> day PI; the highest virus concentration being 3<sup>rd</sup> day PI. Low passage virus at high dose or high passage virus gave low titer only at 3<sup>rd</sup> day PI. The viral antigen was detected in BF of birds inoculated with bursa-derived virus at 3,5 and 7<sup>th</sup> days PI by IEM and AC-ELISA and until 10 days PI by IF while no viral antigen was detected from birds inoculated with cell culture adapted viruses by embryo inoculation, IEM, IF or AC-ELISA. Viral RNA was detected in bursal homogenate by 21 days PI by RT-PCR and no change in RFLP pattern was reported (3).

Low passage and high passage virus lost their pathogenicity after passage in BGM-70 cells as evident by their lower bursal lesions scores and low levels of neutralizing antibodies at 21 days PI. In contrast, the bursa-derived virus maintained its pathogenicity and caused severe bursal lesions and induced higher VN antibodies at 14 and 21th day PI.

Pathogenesis studies are important to evaluate different propagation systems for strains that can be used for vaccine preparation. Antigenic, pathogenic and immunogenic characteristics of different IBDV strains following propagation in different cells were evaluated in various studies. Adaptation of IBDV to BGM-70 cells decreases its ability to replicate in the BF resulting in lower pathogenicity after being passaged 30-40 times, however, the virus retains its antigenicity and immunogenicity (3,99).

Relative pathogenicity and immunogenicity of IBDV is reduced after propagation in embryos or cell culture. Bursa-derived virus induces the most severe lesions in BF. Embryo –derived virus induces moderate lesions whereas cell culture derived virus fails to produce any gross lesions (255). Virus re-isloation from bursa, spleen and thymus collected from birds inoculated with bursal or embryo derived strains induce a higher neutralizing antibody response than the embryo or cell culture derived strains (255). Cell culture derived strains regain minimal virulence when back passaged through birds (342). Adaptation of IBDV to cell culture is associated with attenuation. Repeated passages at high moi resulted in an attenuated small plaque mutant (212). Recent *in vivo* studies showed that vvIBDV adapted to cell culture by site-directed mutagenesis were partially attenuated for SPF chickens (319) and commercial chickens (250). However, the risk of reversion to wild type prevents their application as live vaccine.

#### **1.6.1** Role of T cells in the immunopathogenesis of IBDV

Appearance of viral antigen in bursa is accompanied by an infiltration of T cells while IgM + cells undergo a precipitous decrease and the immunoglobulin level remains the same (158). Infiltrating T cells were first detected at 1st day post inoculation through flow-cytometry and were shown to persist till 12 weeks (273). The ratio of CD4 and CD8 cells were the same during the 1st seven days PI but CD8 cells became predominant afterwards (158).

IBDV induced bursal T cells have increased surface expression of MHC-II and IL-2 receptors, elevated expression of cytokine genes like IFN- $\gamma$  and IL-6 like factor (273). T cells from the bursa of the recovered bird proliferate when exposed *in vitro* to purified IBDV. While the spleen cells from IBDV exposed – chicken produced nitric oxide stimulating factor when stimulated *in vitro* with purified IBDV. Bursal T cells also suppressed the mitogenic proliferation of the spleen from normal, virus free chicken (275).

T cell immunodeficiency can modulate pathogenicity of the virus since it has been shown that the TX birds have a higher viral burden in the bursa, lower inflammatory lesions in bursa, down regulated IFN- $\gamma$  and IL-2 genes in bursal cells, have a lower incidence of apoptotic bursal cells (273) and undergo a quick follicular recovery then T cell intact birds (275).

### **1.6.2 Effect of IBDV on innate immunity**

IBDV have been shown to modulate the macrophage function by altering the *in vitro* phagocytic activity (162). Macrophages from the infected chicken have upregulated cytokine gene expression and produce increased levels of NO (156).

# **1.6.3** Role of cytokines in the pathogenesis of IBDV

IBDV modulates the T cell functions (275). During the acute phase of the disease septic shock –like symptoms have been observed. In septic shock syndrome, there is an up-

regulation of the cytokine gene resulting in an excessive immune response and increased levels of IFN- $\gamma$  and TNF- $\alpha$  (95). TNF - $\alpha$  is a macrophage produced cytokine involved in inflammation and septic shock. Chicken IFN- $\gamma$  can activate macrophages and enhance their anti-microbial activity (156).

ChIFN- $\gamma$  and TNF- $\alpha$  levels in serum were measured by capture ELISA and cytotoxic bioassay respectively. The increase in the levels of cytokines like ChIFN- $\gamma$  and TNF- $\alpha$  corelated with the acute phase of the disease. Levels of circulating ChIFN- $\gamma$  and TNF- $\alpha$  increased as the disease progresses and were highest in the animals that died of infection. The TNF- $\alpha$  levels lasted longer then the ChIFN- $\gamma$  levels (275).

# **1.7 Immunology**

IBDV is ubiquitous in commercial chickens environment and chickens acquire the infection orally or by inhalation. The virus is transferred from the gut to the other tissues by phagocytic cells like macrophages. In macrophages of the gut associated tissues it could be detected as early as 4 hours after oral inoculation using immunoflorescence (216). The virus then reaches the bursa via the blood where the most extensive virus replication occurs. By 13 hours post inoculation (p.i) most follicles are positive for virus and by 16 hours p.i a second and pronounced viremia occurs accompanied by secondary replication in other organs resulting in disease and death (315).

IgM+ cells are the target organs for the virus. During the acute phase of the disease the bursa undergoes atrophy as the bursal follicles get depleted of B cells. Virus replication causes extensive damage to lymphoid cells in medullary and cortical regions of the follicle. Apotosis of the neighboring B cells augments the destruction of the bursal morphology. By this time an ample amount of viral antigen can be detected in other

lymphoid organs like cecal tonsils and spleen (273, 302). During the acute phase of the disease, there is a reduction in the circulating IgM <sup>+</sup> cells (106, 254) but circulating IgG level remains the same (90, 155). Maternally derived antibodies (MDA) protect chickens against subclinical disease and immunosuppression (88). MDA is known to protect the chickens for 3 weeks of age (170).

T cells are resistant to infection by IBDV (107). During the acute phase of the disease lesions appear in the thymus which are quickly overcome within a few days (273). A dramatic influx of T cells is reported in and around the site of virus replication. The infiltrated T cells could be detected from 1<sup>st</sup> to 12<sup>th</sup> weeks post inoculation although the viral antigen disappears by the 3<sup>rd</sup> week. The IBDV induced cytotoxic T cell limit the spread of the virus by destroying the cells expressing the viral antigen and thus can initiate the recovery process. At the same time IBDV – induced T cells might enhance the viral lesions by producing inflammatory cytokines. T helper cells produce inflammatory cytokines like IFN- $\gamma$  which activates the macrophages to produce nitric oxide (NO) (273). Both humoral and cellular arms of the immune system are compromised during the IBDV infection due to lysis of the B cells and altered antigen-presenting cells.

The IBDV causes a transient inhibition of *in vitro* proliferative activity of T cells to mitogens. The virus stimulates the macrophages to produce T cell cytokine like IFN- $\gamma$  to produce nitric oxide (NO) and other cytokines with anti-proliferative activity. IBD didn't affect natural killer cells levels in chickens (273).The NO production after IBD virus infection exerts antiviral effect since the immune-suppressed chickens that failed to induce NO had more severe disease and higher degree of virus replication, but does not

seem to correlate with the hemorrhagic lesions which result from the reaction of hostfactors and the determinants responsible for virus virulence and virus clearance (248).

The IBDV induced damage to humoral immunity is reversible. Antibody production correlates with the morphologic restoration of the bursal follicles. Mitogenic response of T cells returned to the normal levels. During the course of mitogenic inhibition, T cells of infected chicken also failed to secrete IL-2 upon *in vitro* stimulation (156, 272).

The T cells play a significant role in the pathogenesis of IBDV. Intra bursal T cells and T-cell-mediated responses play significant role in viral clearance and promoting recovery from infection. They defend the host cell by reducing the viral burden but at the same time produce inflammatory cytokines and nitric oxide inducing factor that enhance tissues destruction and also delay the recovery process (275).

Intrabursal T cells were activated by *in vitro* stimulation with IBDV. The activated cells had increased surface expression of chicken MHC class II molecule, Ia and IL-2 receptor CD25. In addition, these cells have an up regulated IFN- $\gamma$  gene (158). Splenocytes exposed to IBDV produced nitric oxide inducing factor (IFN- $\gamma$ ) (275). Intrabursal T cells inhibited the mitogenic response of normal splenocytes by 90%. This bursal T cell – induced mitogen inhibition was found to be dose-dependent and not MHC-restricted (157). In contrast to the bursal T cells, the splenocytes from IBDV exposed chickens did not have suppressive activity. Mitogenic inhibition by bursal T cells is mediated by soluble factors, the nature of which is still unknown (275). Chickens that survive the disease, clear the virus and recover from its pathologic effects (273). It has been shown that the more virulent the virus, the stronger is the suppression of the humoral and cell mediated immunity. Virulent virus also produced a detectable NO production in serum.

Humoral immunity is the primary mechanism of the protective immune response. Infection with IBDV results in the formation of antibodies to the group and serotype specific antigens (137). Field exposure or vaccination results in VN titers higher than 1:1000 (191). But weak responses are obtained in chickens immunized with purified viral polypeptides (77) since viral protein conformation is important in eliciting a high antibody response (12).

Antibody production is stimulated at the primary site of viral replication in gut associated tissue and they can be detected as soon as 3 days PI. These antibodies prevent the spread of the virus to other tissues. Due to the rapid onset of antibodies, the necrotic foci that form in the bursa of fabricius stop expanding and are completely eliminated (15).

### 1.7.1 Target organ

The target organ for pathogenic serotype 1 is the bursa of fabricius (BF). The BF reaches the maximum development between 3-6 weeks of age and at this time chickens are most susceptible to the disease. Infection results in high mortality during the acute course of the disease or in B cell deficiency after recovery from infection (15, 147).

Chickens infected with IBDV immediately after hatching develop a chronic infection with atrophy of BF and B cell depletion (114, 330). Chickens infected with IBDV when older then 12 weeks do not show clinical signs (15). The bursectomized chicken survive the IBDV infections lethal for normal chicken (147).

High concentrations of antigens and high infectivity titers were found in BF of infected chickens, whereas only traces of antigen and low virus titers were detected in the thymus,

spleen (147) and peripheral blood (31,209). *In vitro* infection studies have shown that IBDV replicates in the population of proliferating B cells (209, 284) but not in very immature lymphobalsts (19) or competent B cells (15).

Apathogenic serotype 2 strains do not replicate in lymphoid bursal cells or in other lymphoid cells (229). Treatment of chicken with cyclophosphamide (CY) or surgical bursectomy at 4 weeks of age is known to prevent IBDV infection (147). Bursectomized chickens did not show the disease and had transient lesions in the lymphatic tissues. However, these chickens contained virus and produced antibodies against it. The virus concentration in the bursectomized chickens were about 1000 times lower than the non-bursectomized chickens (147). It appeared that the availability of a large number of bursal cells is an essential factor in the development of IBD (122).

### **1.7.2 Immunosuppression**

Immunosuppression caused by IBDV has a significant economic impact due to widespread nature of the disease in commercial chickens. IBDV infection at an early age compromises the humoral and local immune responses of chickens. Allan made the earliest observation about the immunosuppressive potential of IBDV (7). The extent of the immunosuppressive effect is related to the age at infection. The most pronounced damage results if the infection occurs within the first 2-3 weeks of hatch (7). The birds less than three weeks of age do not exhibit clinical signs but are immunosuppressed (261). Following the ingestion of the virus, lymphoid cells and macrophages in the intestine are infected and carry the virus to the bursa of fabricius (BF) (216). Clinical signs and lesions of IBDV appear shortly thereafter. Chickens infected with IBDV are

more susceptible to various other infections. Chickens exposed to IBDV at 1 day of age had lower antibody responses and were more prone to infection with Newcastle (ND) (78). The infected chicken had a decreased humoral response to vaccines as well (109). Immunosuppression resulted in lower flock performance, more secondary infections, poor feed conversion, less protective response to vaccines and higher rate of carcass condemnation at the processing level (273).

The immunosuppressive effects of IBDV are dependent on the strain of the virus (48,104,197,271). Chicken infected with IBDV at an earlier age succumbed to other infections like inclusion body hepatitis (74), reovirus (205), coccidiosis (9), Marek's disease (269), hemorrhagic-aplastic anemia and gangrenous dermatitis (258), infectious laryngotracheitis (257), infectious bronchitis (243), chicken anemia agent, salmonellosis, *Escherichia coli*, colibacillosis (335) *Mycoplasma synoviae* (89) and *Eimeria tenella* (9, 87).

An enigma surrounding IBDV infections was that although there was immunosuppression against many antigens, the response to IBDV itself remained normal even in one day old susceptible chicken (286). Selective stimulation of the proliferative B cells committed to anti-IBDV antibody production seems to occur (191).

Peripheral blood lymphocytes (PBL) from chickens infected with IBDV have depressed proliferative responses to stimulation with the mitogens concanavalin A(273) or phorbol myristate acetate (200).

#### **1.7.3 Effects of virus on humoral immunity**

IBDV has a predilection for the immature (282) actively dividing B lymphocytes and causes lytic infection of IgM bearing B cells resulting in the decrease in circulating IgM<sup>+</sup> cells. Infected chicken produce less level of antibodies against the antigen (155). Only primary antibody responses are affected. Secondary responses remain unaltered (254,271). IBDV induced humoral deficiency is reversible and overlaps with the restoration of bursal morphology (273).

Chickens infected with IBDV at 1 day of age were found to be completely deficient in serum immunoglobulin G and produced only a monomeric immunoglobulin M (IgM) (126,127). IgG levels varied depending on the age at the time of infection (107). The number of B cells in peripheral blood was reduced after infection with IBDV but T cells were not appreciably affected (107, 284). The adverse effect on antibody responses is due to the damage to the B cells in the bursa and the blood. Since the virus has a predilection for actively dividing B cells as compared to the mature B cells (282).

#### **1.7.4 Effect of virus on cellular immunity**

The extent of which the cellular immune response is affected is not well understood. However, it is known that the effect of IBDV on CMI is transient and less pronounced than the effect on humoral response. Infected chickens show a poor cellular response to certain antigens and show increased susceptibility of disease that are under the control of cellular immune defense (9). The thymic lesions were transient and appeared within the first week of infection peaked at 3-4 days post inoculation (PI) and then subsided. The presence of thymic lesions were not associated with active viral replication of the virus in the thymic cells as shown by the immunoflorescence (IF) and antigen capture ELISA. In addition, T cells from infected chickens during the early stages of virus infection fail to respond optimally to mitogens *in vitro* (43).

Maximum depression in the cellular immunity was shown to occur at 6 weeks postinfection by using the lymphoblast transformation assay. The reason for the delay in this response is not clear considering that the virus persists in the host for approximately 3 weeks. It was speculated that this depression is the over all depression of T-cell function during the virus infection (283). The effect of IBDV on two CMI functions i.e. natural killer cell cytotoxicity and mitogenic response had been studied. It was reported that IBDV had an inconsistent effect on the natural killer cell cytotoxicity but caused a transient early depression of the blastogenic response of spleen cells to phytohemaglutinin (274). *In vitro* mitogen hypo-responsiveness of T cells is mediated by the suppressor cells in the spleens of the infected chicken; the mechanism of reduced *in vivo* cellular immunocompetence are not known (163).

IBDV infected chickens were shown to have a normal natural killer cell levels, mononuclear phagocytic activity and delayed –type hyper sensitivity reaction (90,114). Neither did virus infection alter the normal proportions of CD4 and CD8 subsets of T cells in the circulation and spleen (270).

It was reported that variant A strain of IBD had a significantly higher effect on CMI as compared to the standard Edgar strain when given to 1-day-old chicken which lingered on till 5 weeks. A similar effect was reported in chicken infected at 3 weeks of age (48). Harderian gland in another component of immune system associated with the local immune system of the respiratory tract. IBDV infection of 1-5 day-old chickens produced a dramatic decrease in plasma cell content of the harderian gland that lasted till 7 weeks (59). Broilers infected with IBDV at 3 weeks of age had reduced antibody titers to Brucella abortus (T cell independent antigen) and sheep red blood cells (SRBC, a T cell dependent antigen) in extracts from harderian gland and serum. Decreasing antibody responses to B. abortus were evident at a later time as compared to SRBC antibody response (59).

### 1.7.5 Mechanisms of immunosuppression

Reduction in the number of B cells in the BF due to viral infection is the major cause of immunosuppression. Suppression of B cell function might be caused by damage to helper T cells or other cells involved in generating the immune responses (271). Chickens infected with IBDV have suppressor cells in the spleen, which cause *in vitro* mitogenic hypo responsiveness to concavalin A. These cells prevent normal spleen cells from responding to the mitogen (272). The impairment of T cells and development of suppressor cells (272) was demonstrated *in vitro* by using proliferation tests (42,43,274) or by measuring the cytokine release after mitogen activation of T cells (166).

Besides lymphocyte lysis, apoptosis also plays a role in immunosuppression (236, 302, 322). Apoptosis could occur in a variety of organs (7) like thymus (119) BF and spleen (165,321). CEF and Vero cells infected with IBDV show the biochemical features of apoptosis (304). In addition to causing necrosis, IBDV can also induce apoptosis in avian lymphocytes *in vitro* (322). Viral proteins like VP2 and VP5 have been implicated in the induction of apoptosis (80, 343). Expression of VP243 polyprotein in transiently transfected DT40 B lymphocyte culture suppresses cell growth and proliferative responses to mitogen stimulation indicating that IBDV polyprotein is a mediator of

immunosuppression (245). Apoptotic cells have also been observed in antigen negative bursal cells indicating that immunological mediators like cytokines might be involved in the process (302).

# 1.7.6 Apoptosis

Apoptosis or the programmed cell death plays a major role in the immuno-pathology of IBDV characterized by destruction of cells of BF (16, 31, 147). Only 20% of the lymphoid cells in the BF contain replicating IBDV. The severe damage to the bursa can be ascribed to apoptosis (31, 209). In addition to necrosis, marked atrophy of the BF occurs without eliciting an inflammatory response that is a characteristic sign of the apoptotic process. Replication of the virus in bursa of fabricius results in secondary viremia thus spreading the virus to other tissues.

It has been suggested that early after infection, the cells containing the viral antigen are protected from apoptosis to ensure viral replication. Anti- viral mechanisms kick in and destroy the neighboring cells to prevent the spread of the virus. During the late infection, the infected cells undergo apoptosis thus seeding the virus to other cells. The IBDV infection of a susceptible chicken has been shown to induce apotosis in the bursa as well as thymus (119, 165, 236, 301, 302, 321).

Morphological and biochemical features of apotosis were also observed after *in vitro* infection of IBDV in chicken peripheral blood lymphocytes (322) and chicken embryo fibroblasts (304). Apoptosis occurs in lymphocytes of various organs like thymus (119) bursa and spleen (165). Some researchers believed that apotosis induced by IBDV in cell cultures following *in vitro* infection was an early genetic response of the host cells and was independent of virus replication while others showed that appearance of CPE

coincided with virus replication (304). Jungmann (146) however, showed that proportion of apoptotic cells increased from 5.8 % at 4 hrs post infection (p.i) to 64.5% at 48 hrs p.i. in CE cells after infection with IBDV strain Cu-1 (146). However, treatment of CE cell cultures with UV inactivated IBDV did not induce apotosis.

Whether apotosis is triggered via virus receptor activation is not yet known. Double labeling technique revealed that during the course of early infection maximum number of antigen- expressing cells were not apoptotic. It was only later in the infection that the double-labeled cells appeared. Double labeling technique determined the distribution of both apoptotic cells and cells containing viral antigen in the same section of BF.

Double labeling studies for apoptotic or antigen positive cells revealed that apotosis in bursa occurs both in IBDV positive and IBDV negative cells (302) whereas apoptosis in the thymus occurs in the antigen negative cells only (302). It was concluded that IBDV induced apoptosis indirectly in non-bursal organs. It has been postulated that IBDV impairs the withdrawal of apoptotic cells and therefore results in the increased number of the apoptotic cells (236). Apoptotic cells were located mostly in an area between the cortex and medulla whereas majority of cells positive for viral antigens were found in medulla.

Indirect mechanisms might also be involved in the induction of apoptosis and could have induced apotosis in vivo resulting in rapid depletion of cells in BF. In the infected follicles large number of cells were apoptotic but very few contained the viral antigen (146,302). Interferon production occurs after IBDV infection and is thought to be the major apotosis–inducing factor in the neighboring cells along with TNF- $\alpha$  (146).

Viral protein VP2 and VP5 have been implicated to play a role in apotosis. The VP2 induced apotosis in mammalian cells but not in CE cells (80). The VP5 deletion mutant of IBDV induced less apoptotic cells in infected CE cells and replicated slower than the parental strain (343). A correlation exists between virus replication and apotosis in cells of BF (230).

# **1.8 Replication**

A single replication cycle takes 22-28 hours for IPNV and 6-8 hours for IBDV. The virus binding protein has been found at various types of chicken cells for IBDV (252). The cell receptor recognition site on the virus is not known (191). Replication of IBDV is not restricted by the presence of specific receptor sites.

Strains of both IBDV serotypes bind to lymphoid cells isolated from bursa, thymus or spleen indicating that all these cells are susceptible and have the specific receptor site for the entry of the virus.

The specificity of binding was determined by saturation and competition experiments. The CEF has receptors common to both serotypes and specific ones for each serotype. Receptor sites common to both serotypes were also present on lymphoid cells; however, additional serotype specific sites were demonstrated for apathogenic serotype 2 strain (229). The biochemical steps involved in replication of IBDV or other birnaviruses have not been completely characterized (150). Two factors restricting the study of replication process are that only a low number of cells support virus replication *in vitro* and sensitivity of virus towards Actinomycin D (a selective inhibitor of cellular RNA synthesis) (296).

It has been suggested that cell tropism of the virus determined by receptor-mediated entry step (23,27). After entry into the host cell, the virion RNA dependent RNA polymerase becomes activated and produces two genome length (24 S) mRNA molecules from each of the 14S dsRNA genome segments (252). These mRNA lack a cap and 3' poly A tail and have a VPg attached to their 5' ends (252). Genome –linked proteins have been described indicating that the virus replicates by a strand displacement mechanism (296). The separate strands of the genomic ds RNA can be translated in vitro (10). Replicative intermediates have been described in the infected cells however, there are no reports on the minus strand synthesis (252). The two mRNAs are synthesized in the same relative proportions throughout the replicative cycle (twice as many A as B mRNA molecules) (252). On a plus-strand IBDV segment A cRNA template, minus-strand synthesis occurred in such a way that a covalently linked double-stranded RNA product was generated by a 'copy-back' mechanism. Importantly, enzyme activity was observed only with templates that comprised the 3' non-coding region of plus-strand RNAs transcribed from IBDV segments A and B, indicating template specificity (323).

There are two initiation codons upstream of the polyprotein start codon of segment A. The first at 66 at the start of 12 amino acid polyprotein and second at position 98, potentially initiating the translation of 17K polypeptide. According to Kozak's rules the codon at 66 and initiation codon for polyprotein are good candidates but the one at 98 is a poor candidate for the polyprotein synthesis (14). N-terminus position of VP4 is completely conserved and might be required for the correct activity of the protease. RNA polymerase activity can be demonstrated without any pretreatment of the virus particle showing that transcription and replication occur after the virus had entered the host cells without uncoating (296). The viral nucleic acid replicates by strand displacement reaction. Both ends are circularized by 90 Kd VP1 (296). The activity of viral RNA dependent RNA polymerase requires removal of Ca <sup>++</sup> from the reaction mixture. Optimal polymerase activity was found to be at pH 8.5 at 40C in the presence of Mg <sup>++</sup> ions. Monovalent cations Na<sup>+</sup> and K<sup>+</sup> significantly increase the enzyme activity.

The RNA products synthesized *in vitro* are 24S single stranded RNA (the newly transcribed mRNA), 14 S double stranded RNA (the two genomic double stranded RNA) and large intermediate transcription products containing complexes of RNA (150). It has been reported that the synthesis of the host proteins is not shut off in chicken embryo fibroblasts (CEF) infected with IBDV(15).

Monoclonal antibodies (17/82) (12) and 1/A6 (17) against VP2 show virus neutralizing activity indicating that VP2 may be important in virus adsorption (150). Restriction of IBDV to lymphoid B cells might not be determined by the presence of a specific receptor, but cis–acting elements might play a role in restriction of virus replication in B lymphocytes (268).

Virus particles assemble and accumulate in the cytoplasm. Auto-proteolytic cleavage of polyprotein is the first step governing the IBDV capsid assembly. Although the primary cleavage sites are known, the proteolytic cascade involved in the processing is still not understood. Residues Ser-652 and Lys-692 are important for the protease activity of VP4. The VP4 proteases of birnaviruses are species specific, since they do not cleave heterologous substrates (178).

The VP4 accumulates in the nucleus and the cytoplasm. The mechanism of viral release is unknown (252). The VP5 and N-terminus of VP2 upto 647 nucleotides were not responsible for the different pathotypes of IBDV serotypes 1 and 2 as shown by the production of chimeric viruses.

Birna viruses are cytolytic viruses but the exact mechanism of viral release is not known yet (184). Viral protein expression or the formation and accumulation of virus particles is thought to induce changes in membrane permeability eventually leading to cell lysis. Expression of single viral proteins have been demonstrated in several viruses e.g. 2B protein of poliovirus, NSP4 of rotavirus and E3-11.6 K of adenovirus to induce lysis of the cell (82). The VP5 caused severe cytotoxic effects resulting in the cell lysis and it was proposed that VP5 acts as a death protein and helps in viral release (184).

### **1.9 Viral Interference**

Viral interference is defined as the ability of a virus to inhibit the replication of another virus on co-infection. This phenomenon has been extensively studied by several researchers for many DNA and RNA virus families including Picornavirus, Paramyxovirus, Reo-virus, Bunya virus and Birnavirus but the exact mechanism of interference remains obscure (326). The process of interference sheds light on the process of viral replication and the outcome of these experiments can be applied towards the treatment, therapy or prevention of viral infections.

The phenomenon of interference has been described in three different situations:

 serial passage of viruses at high m.o.i leading to the accumulation of defective interfering (DI) particles (173);

- 2. mixed infections of wild type viruses with certain temperature sensitive (ts) mutants (34,45);
- 3. coinfection of cells with different wild type virus isolates (260).

Interference by the defective interfering (DI) particles has been well documented. Serial undiluted passages of IBDV in chicken embryo cells result in the formation of a small plaque mutant that interferes with the replication of the wild type virus. The yield of wild type virus is reduced by 78% at an equal multiplicity of infection. These defective interfering particles have low density, are avirulent for chickens and contain unprocessed structural proteins (212). It has been proposed that the DI genome competes with the wild type virus for the polymerase or the nucleocapsid protein therefore reducing its replication (173). Combined infections of IBDV with Reovirus in SPF chickens indicated that infections with IBDV before infection with reovirus led to longer persistence of reovirus in some tissues (207).

Influenza virus has a small plaque mutant that interferes with the replication of the wild type virus. This attenuated cold-adapted strain A/AA/6/60 is the vaccine candidate and is known to be interference dominant. It suppresses clinical disease in ferrets when given simultaneously with a virulent strain of Influenza A virus (299,327). Genetic analysis of these strains has indicated that element(s) responsible for the interference properties are located at the segment 7 (240). Such candidates expand the prophylactic use of the vaccine since they provide protection by interfering directly with the replication of the virus and provide protection till the development of antibodies. Thus, there is a possibility of use of this virus as an antiviral agent against Influenza. The ability to interfere with the wild type virus is a desirable trait for live, attenuated vaccine.

During mixed infections the viruses with mutant phenotypes like temperature sensitive (ts), host range, non-sense or small–plaque mutants restrict the growth of the wild type virus. It has been reported that during co infection, these conditional mutants or growth attenuated mutants reduce the yield of the wild type virus up to 99% (326).

The mutants that are dominant over wild type virus arise either spontaneously or by mutagenesis and have a ts phenotype. In most cases, some gene expression from ts mutant is required to interfere with the growth of wild type virus and it occurs only at non-permissive temperature (326). In some cases, however, it occurs both at permissive and non-permissive temperatures. The examples of ts mutants among RNA viruses include polio- virus type 1 (45), foot and mouth disease virus (247) and reovirus type 3(34) and among DNA viruses herpes simplex virus 1 and 2 (145).

The mechanism of interference by the conditional mutants is still unknown. The difference between the action of conditional and DI particle is that DI particles can interfere in the absence of gene expression while conditional mutants cannot. Conditional mutants and the DI particles have some common properties like both can be generated by serial undiluted passage, or by persistent infections and amplified by co infection with wild type viruses (326).

It has been observed during persistent infections that the parental virus is gradually replaced by the mutant progeny, which is of reduced virulence or is temperature sensitive. These mutants have the capability of interfering with the growth of the parental wild type virus (346). Therefore, persistent infections may be established to obtain viruses capable of interfering with the growth of wild type viruses and with less ability to shut off the host systems (326).

Many theories have been proposed to explain the mechanism of interference. These include the "Rotten Apple hypothesis" which proposes that a defective polypeptide enters the multimeric protein complex and inactivates all the components of that complex. Direct competition hypothesis states that two viruses compete for some limiting factor of either the host or viral origin. The roadblock hypothesis speculates that during direct competition, the mutant irreversibly sequesters the limiting factor. The attractive genome hypothesis states that mutant genome has more attractive binding sites or has more sites than the wild type virus (326).

One of the mechanisms by which one virus restricts the growth of another virus is by the production of cytokines. The sharp rise in IFN –gamma mediated by the acute hepatitis A infection plays a central role in the suppression of chronic hepatitis B. A sharp increase in the gamma-interferon levels occurs at the time of hepatitis infection just before the rise in the serum transaminase activity. This is followed by a decrease in hepatitis B DNA and the hepatitis B antigen falls below the detection limit of the assay (320).

Interference can play a significant role in vaccination with live viruses. Live poliovirus vaccines interfere with the implantation of wild type virus in the intestinal tract thereby inhibiting it and later on stimulating the antibody production. The evidence for this phenomenon is provided by field trials where live vaccine displaces the wild enterovirus (325). In an event of the outbreak, administration of this vaccine would provide immediate protection and block the further spread of the virus.

On the other hand, there is an inhibition of oral polio virus vaccine by other enteroviruses resulting in reduced shedding of the vaccine virus and inability to mount an antibody response (325). Although, interference with the replication of a wild type strain is a

highly desirable trait for any live vaccine, there is no evidence for the occurrence of interference phenomenon for many diseases like measles, mumps and rubella (326). There are no reports on the interference exerted by the IBDV vaccine virus on the wild type IBD. Such studies in addition to being intrinsically interesting would enhance the understanding of the replication of the virus and might expand the prophylactic use of the vaccine as an antiviral agent.

# **1.9.1 Interferon induction by IBDV**

Production of interferon is a non-immune response of animal cells to stimulation by viruses or other inducers. Double stranded viruses are potent inducers of interferon. Earlier researchers reported the presence of an "interfering factor" in the tissues of chickens infected with IBDV (278).

Two isolates of IBDV were used to investigate the relationship of virus multiplication and interferon induction in CEF. Interferon production was assayed by plaque reduction method. The results indicated that the kinetics of virus multiplication is closely associated with the interferon synthesis. The interferon was detected between 4-12 hours PI. The strain having a higher titer induced a faster induction of interferon. The production of progeny virus preceded interferon synthesis by 2-8 hours PI. Maximum interferon titers were obtained by the 18-24 hours PI (85). The SPF chickens at 1 and 4 weeks of age were inoculated with a pathogenic and an attenuated strain of IBDV to study the induction of interferon in serum and tissues. It was observed that the pathogenic IBDV stimulated a greater and longer lasting response in the tissues than the attenuated isolate, independent of route or age of inoculation. In addition, the chickens infected with a pathogenic strain had a wider distribution of interferon in the tissues. Interferon was detected from serum, kidneys, lung, thymus, spleen and bursa following infection with the pathogenic strain. The attenuated virus induced interferon production only in the bursa. Interferon production in tissues correlated to the titer of the virus in the tissues.

Also, the serum interferon response was higher after infection with the pathogenic as compared to the attenuated virus. Serum interferon levels peaked at 2- 3.5 days PI. It is the time when viremic stage of IBDV has been reported to occur. The pathogenic and attenuated viruses stimulated similar IBDV neutralizing activity that occurred after peak serum interferon activity (86). The induction of interferon might be a desirable characteristic of a vaccine virus since it could afford early local protection from the damaging effects of infection with the pathogenic strains of IBDV (86).

It has been reported that chicken IFN- $\gamma$  level increased before the appearance of the first clinical signs. During the acute phase of IBD infection an increase in the levels of circulating cytokines like chicken IFN- $\gamma$  and TNF- $\alpha$  occurred as demonstrated by the capture ELISA and cytotoxic bioassay respectively. The increased levels correlated with the progression of the disease and were highest in the animals that died of co infection. TNF- $\alpha$  responses lasted longer than the IFN- $\gamma$  responses (275).

It was recently reported that transient immunosuppression caused by IBDV could be accurately measured by assessing the chicken's ability to respond to inactivated NDV with enhanced IFN- $\alpha$  and IFN- $\gamma$  gene expression. A competitive nucleic acid hybridization microtiter plate assay was developed for chicken IFN- $\alpha$  and chicken IFN- $\gamma$ mRNA. The levels of both cytokines rose from undetectable levels to reach a peak by 4 h, remained high for about 3 days, and fell to undetectable levels by day 5 (231). It is interesting to note that a highly developed Th-1 cytokine system exists in chickens. However, no avian homolog of Th-2 cytokine (IL-4, IL-5, IL-10, and IL-11) has been molecularly identified to date. This suggests that either birds lack these cytokines or that cloning of chicken cytokine cDNAs is done by using the cells that mainly express Th-1 cytokine gene. The second explanation is most favorable since chickens possess an IL-10 R gene. However, biological activities of Th-2 type cytokines have not been demonstrated in chicken cell culture. So it could be speculated that the classic Th-2 type cytokines do not exist in birds and the cytokines that moderate Th-2 characteristics, like IL-6, IL-18 and TGF- $\beta$  might down regulate pro-inflammatory responses thus indirectly favoring Th-2 type immune responses (297).

#### **1.10** Laboratory Host systems

Earlier researchers found it difficult to isolate or serially transfer the virus using chicken embryos. By using the allantoic sac route of inoculation a general trend observed was that all embryos died on the first passage and when the material from 1<sup>st</sup> passage was used to inoculate eggs, 30% died while no embryo mortality occurred on the third passage (168). The reasons for these difficulties was that the virus titer in the allantoic fluid was low in the first passage and embryonated eggs from the flock recovered from the disease were resistant to growth of the virus. Allantoic fluid route was shown to be the least desirable, yielding embryo-infective- dose-50% (EID 50) virus titers of 1.5-2.0 log 10 lower than those of eggs inoculated by the CAM route while yolk sac gave intermediate titers (111). Inoculation of 10-day-old embryonating eggs by CAM route resulted in embryo mortality from days 3-5 post-inoculation.

IBDV has been adapted to grow *in vitro*, with or without cytopathic effect (CPE) in Macrophages and lymphocytes (31), the QT 35 cells (a continuous fibroblast cell line of Japanese Quail origin) (47), bursal lymphoid cells, chicken embryo kidney (CEK) and chicken embryo fibroblasts (CEF) and chicken embryo bursal cells (190,201).

Several mammalian cell lines also support replication such as Rabbit kidney (RK-13), African green monkey kidney (Vero), Buffalo green monkey kidney (BGM-70), and Ovine kidney (OK), Rhesus monkey kidney (MA –104) cells (131,190,244). Transformed avian cell lines TLT-1 (32) LSCC-NP1(120), HD – 11 (20), LSCC-BK3 and LSCC- CU 10 (derived from lymphoid leucosis tumor) (339). The IBDV strains can multiply in some of the established lymphoblastoid cell lines derived from lymphoid leucosis tumors, all of which contain B-cell characteristics. Marek's disease virus–derived lymphoid tumor cell lines of chicken (MSB-1) and turkeys (RP-14) support viral replication (164).

Chicken blood lymphoblasts and monocytes are susceptible to IBDV and after 6 hours of infection 32.5% of bursal cells, 13% of monocytes and 2.5% of separated blood lymphoblasts reacted positively by immunocytology whereas PHA-M stimulated small lymphocytes and unstimulated lymphoid blood cells were totally resistant to the virus (31). The use of non – avian continuous cell lines for the isolation and propagation of IBDV has several advantages over the use of cells of avian origin. Continuous cell lines are easier to handle and are free from vertically transmitted extraneous viruses (99). Attempts to induce clinical disease in ducks with strain J and FK-78 were unsuccessful. Virus multiplied in duck eggs and duck embryo fibroblasts cells but not in duck kidney cells (338).

The IBDV replicated in a cultured chicken avian–leukosis–virus–induced lymphoblastoid cell line (LSCC-CU10) consisting of B lymphocytes but was unable to grow in chicken Marek's disease virus- induced lymphoblastoid cell line consisting of T lymphocytes. The virus attaches chicken embryo kidney cells maximally 75 minutes after inoculation (190).

The cytopathic effects (CPE) of virus in BGM-70 cells and CEF were found to be similar (131). The multiplication cycle in chicken embryo cells is 10-36 hours and the latent period was 4-6 hours (15,136,227). The latent period for Vero cells ranged from 12- 18 hours and longer multiplication cycles of about 48 hrs has been reported (131,151). Higher yields and more extensive maturation phase was observed in Vero than in CEF cultures (151). IBDV specific polypeptides can be detected in chicken bursal lymphoid cells after 90 min of infection *in vitro* while mature virus peptides can be detected after 6 hours of infection (210).

Isolation of IBDV from field cases proved to be very difficult. McFerran (199) reported difficulty in isolation and serial propagation of the virus in cell cultures of chicken embryo origin. BGM-70 cells have been used successfully for the isolation of the virus from the bursas of naturally infected cells and CPE is seen after 2 to 3 blind passages (191). Serial passage of the undiluted virus in chicken embryo cells resulted in the formation of small plaque virus that interfered with the standard virus in mixed infections. The small plaque virus had a low yield, was avirulent for chicken and generated a large number of low-density defective particles. The small plaque size remained constant during subsequent infections carried out at low multiplicities. The defective particle had either lost the large segment of ds RNA or had no RNA and
showed an aberrant protein composition. At an equal multiplicity, wild – type virus yield was reduced by 78% by co infection with small plaque mutant that had been purified away from the defective particles. Defective particles were also able to interfere with the wild type virus (212).

Jackwood (131) compared three mammalian cell lines (MA-104, Vero and BGM-70) for their ability to support several strains of IBDV. The viruses replicated in all three but the CPE was most pronounced in BGM-70 cells. It was later reported that LSCC-BK3 cells were superior to BGM-70 cells and CEF in an infectivity assay (309).

In contrast to classic and variant viruses, the highly virulent viruses could not be propagated in cell culture (5). Attempts have been made to identify the amino acids that might be crucial for cell culture adaptation by using the site-directed mutagenesis (179,217) but these amino acids might vary from strain to strain (191). Adaptation of the virus to BGM-70 cells resulted in a significant reduction in the ability of the virus to replicate in the bursa of fabricius (3).

#### **1.10.1 Persistence of IBDV in organs**

The bursa and spleen were reported to have substantially higher concentration of the virus as compared to any other tissue. The bursa harvested from chickens at 72 hours PI yielded high virus titers followed by spleen and kidneys. No virus was detected beyond 10 days PI. IBDV was reported to persist in the chicken for a few days but the lesions could be seen for at least 10 weeks, the longest interval evaluated in that study (330).

The IBDV was re-isolated most consistently from the bursa and less frequently from thymus, liver, kidneys, lung and spleen. No virus was isolated from the pancreas (193). The virus was not detected beyond 11 days PI in commercial chickens when inoculated at

1,7 or 14 days of age. In birds inoculated at 21 days of age, IBDV was re-isolated till 8 days only. The precipitating antigen was detected only in the bursa and only at 3rd, 4th and 5th day PI but was not detected in any organ of chicken infected at 21 days of age (193).

Chickens were inoculated with an attenuated cell culture adapted virus at one day of age, the virus could be detected in cell culture inoculated with homogenate of BF, spleen, thymus, liver, kidney and lung for up to 14 days PI in one experiment and 10 days PI in another. No virus was detected after the levels of neutralizing antibodies became significantly high. Also, the virus was not detected in tissues from birds given the virus at 3 weeks and low virus neutralizing antibody titers were detected, indicating an age resistance to CEF virus or insufficient dose (285,287).

When SPF chickens were inoculated with IBDV at 3 wk of age, the viral RNA was detected by RT/PCR till 21 days PI, but attempts to isolate infectious virus from bursal homogenates failed. Infectious virus was detected by embryo inoculation up to 7 days PI in the bursa of SPF chickens inoculated at 2 or 3 wk of age, whereas the viral RNA was detected by RT/PCR for up to 28 days PI. In SPF chickens inoculated at 1 day of age, the bursa-derived virus or its RNA was detected at 7 and 14 days PI when inoculated at a high dose  $10^4$  EID<sub>50</sub>/bird or at a low dose  $10^{2.5}$  EID<sub>50</sub>/bird.

In commercial 1-day-old broiler chickens, the bursa-derived virus was detected at 7 and 14 days PI when inoculated at a high dose 10<sup>4</sup> EID50/bird, whereas the virus was detected only at 14 days PI when inoculated at a low dose 10<sup>2.5</sup> EID50/bird. In SPF and commercial chickens, vaccinated with a modified live IBDV vaccine, the virus is known

to persist in the bursa of SPF chickens till 3weeks but maternal antibodies in the commercial chickens rapidly eliminate it from the bursa since no live vaccine virus nor its RNA was detected in commercial broilers vaccinated at 1 day or 2 wk of age (2).

#### 1.11 Pathology

# 1.11.1 Gross Lesions

The tissue distribution and severity of lesions is dependent on the subtype and pathogenicity of the virus (259,303). Infected birds are dehydrated and have darkened discoloration of pectoral muscles. Hemorrhages occur in thigh and pectoral muscles and are also reported from the mucosa at the proventriculus-ventriculus junction and on the serosal surface and plica of the bursa (97). There is increased mucus in the intestine and renal changes are observed in diseased birds that is due to dehydration (191). The kidneys, tubules and ureters are so distended and filled with urates that they appear white (46).

The cloacal bursa is the target organ for the replication of IBDV and hence the most severely affected. Characteristic pattern of bursal changes observed during the course of infection differ for the classic and variant viruses (259). During the infection with classic viruses, the bursa increases transiently in size accompanied with inflammation. After the inflammation subsides, rapid bursal atrophy occurs. On the other hand, infections with some variant viruses like VA isolate are not accompanied by inflammation while other variants like IN produce inflammation (98,271). Variant viruses result in a rapid atrophy of the bursa (259).

In a detailed study using the Edgar strain of the virus, Cheville recorded the bursal weights for 12 days post inoculation. The bursa began to increase in size and weight due to edema and hyperemia on 3 days PI and by day 4 it doubled in size. By day 5, the bursa returned to its normal weight and from day 8 it atrophied further and became one- third its original weight.

By day 2 or 3 post-infection, the bursa had a gelatinous yellowish transudate covering the serosal surface. Longitudinal striations became prominent and the color changed from white to creamy. The transudate disappeared as the bursa returned to its normal size and the organs turned gray during the period of atrophy (191).

Extensive hemorrhages could be seen on the entire bursa. Pathologic changes in the spleen and thymus were less prominent than those of the bursa (46,119) The spleen might be slightly enlarged and usually had small gray foci uniformly dispersed on the surface (191). Lesions in these organs were noticed at the same time as the changes occurred in the bursa and resolved within 1 or 2 days of appearance (103).

The very virulent infections are characterized by severe clinical signs, high mortality, a sharp death curve followed by rapid recovery. The vvIBDV strains have the same clinical signs and incubation period of 4 days as classical viruses but the acute phase is exacerbated (314). The vvIBDV strains cause more severe lesions in the cecal tonsils, thymus, spleen and bone marrow and a greater decrease in thymic weight index as compared to the moderately pathogenic strains but bursal lesions are similar. It has been shown that the pathogenicity of field strains of IBDV correlate with lesion production in non-bursal lymphoid organs. The results also suggest that pathogenicity of IBDV may be associated with virus antigen distribution in non-bursal lymphoid organs (303).

### **1.11.2 Histopathology**

Histopathologic lesions occur in the bursa, spleen, thymus, harderian gland and cecal tonsils. The first signs of infection occur in the bursa and it is the most severely affected organ. Degeneration and necrosis of individual lymphocytes in the medullary region of the bursa occur as early as 1day post infection. Lymphocyte degeneration is accompanied by nuclear pyknosis and formation of lipid droplets in the cytoplasm (39). Degenerating lymphocytes are surrounded by macrophages. Lymphocytes are soon replaced by heterophils, pyknotic debris, and hyperplastic reticuloendothelial cells.

By 3 or 4 days post infection, all lymphocytes have been affected. At this point of time the bursal weight increases due to edema, hyperemia, and accumulation of heterophils. As the inflammatory reaction subsides, cystic cavities appear in the medullary region of the bursal follicles. Necrosis and phagocytosis of the of heterophils take place and fibroplasia occurs in the inter-follicular connective tissue (39,103,191). Proliferation of the bursal epithelial layer occurs producing glandular structures of columnar epithelial cells containing globules of mucin. Follicular regeneration and repopulation of follicles with the lymphocytes occur but healthy follicles are not formed during the observed time span of 18 days (103). Variant A isolate of IBDV cause extensive lesions in the bursa but lacks an inflammatory response (271).

The spleen shows hyperplasia of the reticuloendothelial cells around the adenoid sheath arteries during the early stages of infection. Lymphoid necrosis occurs in the periarteriolar lymphoid sheath by 3 days post infection. The spleen recovers shortly without any sustainable damage to the germinal follicles (39,191).

Changes in thymus and cecal tonsils appear shortly after infection and include areas of lymphoid necrosis and hyperplasia of the reticular and epithelial components in the medullary region of thymic follicles (39). The damage is less extensive than in the bursa and is quickly repaired by 12 days post infection (39) Variant virus A is reported to cause milder lesions in the thymus than a standard (IM) strain (271).

The harderian gland is reported to be severely affected by virus in 1 day old chickens (298). Normally, the gland is populated with plasma cells as the chicken ages but the infection prevents this infiltration. Harderian gland of the chickens infected at 1 day of age has 5-10 fold fewer plasma cells than those of uninfected chickens from 1-7 weeks of age (59). However, lymphoid follicles and heterophil populations in the harderian gland are not affected by IBDV infection, nor could necrotic or degenerative changes be found in the acini or excretory ducts.

In contrast, the broilers infected at 3 weeks of age have a 51 % reduction in plasma cell content at 5-14 days post infection. (60). Plasma cell numbers reduction was temporary and levels became normal after 14 days. Histologic lesions appearing in the kidneys were nonspecific and resulted from dehydration (103). The liver had some slight perivascular infiltration of monocytes (246).

#### 1.11.3 Ultrastructural changes

Sequential morphologic changes in surface epithelium of the bursa were observed by scanning electron microscope after oral inoculation of 1-day-old chickens with virus. A reduction in number and size of microvilli occurred at 48 hours post inoculation. There was a gradual loss of button follicles at the surface and by 72 hours PI most have involuted. By 96 hours PI many epithelial cells showed erosion. The surface was intact by day 9 PI, but follicles were involuted, leaving deep pits (224).

### 1.12 Diagnosis

Acute clinical outbreaks of classical IBDV in susceptible chicken flocks (3-6 week old broiler and replacement pullet flocks) are characterized by a sudden onset, high morbidity, spiking mortality curves and a rapid recovery time of about 5-7 days from clinical signs. Grossly visible changes are observed in the cloacal bursa that includes changes in color and size of the bursa during the course of infection.

Infections of young chickens or of those having the maternal antibodies are diagnosed by the histopathological examination of the bursa (191). Variant viruses differ from the standard viruses in that they cause less mortality and produce splenomegaly and liver necrosis of the embryos (191).

### **1.12.1 Virus Detection**

The cloacal bursa and spleen are used for the isolation of the virus (111,191). The virus can be found in other organs such as the thymus, liver and bone marrow but in significantly low quantities than in the bursa (39,301). The inoculum for virus isolation is

prepared by homogenizing the tissue sample in antibiotic containing buffer that is centrifuged to remove larger tissue particles and is used for inoculating embryonated eggs and tissue culture (191).

The chorioallantoic route of inoculation in 9-11 days old embryos is the most sensitive route for the isolation of the virus. Classic viruses usually kill the embryos in 3-5 days and produce lesions of vascular congestion and subcutaneous hemorrhages in the embryos (111,170). Variant viruses however, do not kill the embryos but cause embryo stunting, discoloration, splenomegaly and hepatic necrosis (191).

Primary cell cultures of chicken embryo fibroblasts (CEF), bursa (CEB) and kidney (CEK) have been used to propagate the virus. McFerran (199) reported that 3 out of 7 chicken isolates failed to grow in chicken embryo fibroblast (CEF) cells but propagated well in embryonating eggs. Some strains grow well in embryos but are not readily adapted to grow in CEF or CEK (176).

Cell cultures containing 50% bursal lymphocytes and 50% CEF have been used to isolate and serotype IBD virus successfully (187). The fibroblasts served as a matrix for lymphocytes and infected lymphocytes are detected by immunoflorescence (191).

Immunoflorescence (192) and electron microscopy (201) of the infected cell culture or embryonated eggs are valuable tools for monitoring the growth of IBDV particularly those strains lacking pronounced CPE. It is possible to grow the virus in transformed cell lines (105,128,150,153,190,339). The isolation, antigenic analysis and pathogenicity studies of the viruses isolated from field cases are done to detect the changes in the wild virus population (191). Several different kinds of ELISA procedures have been described for testing IBDV (28,113,148,196,294). The ELISA using a monoclonal antibody enhances the detection and characterization of IBDV (76,175). Monoclonal antibody probes have been used for the detection of IBDV antigen in the bursa and spleen tissues as early as two days PI (174).

A polyclonal antigen capture ELISA was found to be more sensitive than the monoclonal antibody capture ELISA for detecting virus antigen and the titration of the viruses propagated in different host systems (100). Conventional methods for the titration of IBDV rely on SPF embryonated eggs and cell culture and require adaptation to cell culture and therefore, could not be used for strains that do not cause embryo mortalities.

RT-PCR/RFLP analysis has been extensively used for the detection and diagnosis of IBDV strains. There has been considerable improvements over the original protocols by the use of a ssRNA internal control (288) which eliminated the false negative reactions and one tube RT-PCR (177). Initially two enzymes were used for placing the viruses into 6 different molecular groups i.e. *BstN1* and *Mbo1* enzyme (138,140). *BstN1* enzyme targets a single amino acid change at position 222 in the VP2 protein that was important in altering the specificity of a neutralizing epitope. The third enzyme *Ssp1* was used to predict the very virulent phenotype (202).

Other enzymes used were *Taq 1* and *Ssp1* (181) or *Sac 1* and *BspM1* (347). Nucleotide sequencing remains the best test to study the genetic relatedness of segment A sequences (69).

By applying these enzymes the variant viruses from USA and Central America have been placed in molecular groups 1 and 2. Classical strains have been placed in 3 and 4 and 5 and 6 contained included classical and vvIBDV. Molecular group 6 contained viruses from the US and from countries around the world. The virus from outside the US were shown to be *Ssp1* positive (202).

It must be emphasized that grouping of these strains in a molecular group does not indicate genetic relatedness. This grouping is based on the restriction enzyme sites, which results from nucleotide mutations that do not always change the amino acid. RFLP profiles and nucleotide sequence could be used to predict relative differences between IBD strains but *in vivo* testing is essential for detecting the actual antigenic differences (141).

Viruses having the same RT-PCR/RFLP profiles have the probability of being antigenically similar than the viruses in a different molecular group. Positions 222 and 318 are located in Hydrophilic regions A and B and form an antigenically important epitope. Mutations in either one position in region A or B could cause antigenic drift and in both regions could cause an antigenic shift (266). The presence of *NgoM IV* restriction site was tested as a genetic marker for the identification of potentially pathogenic wild type (non-vaccine) strains (142). But the presence of this marker was not unique to wild type strains of the virus although absence of *NgoMIV* site was consistent with some level of attenuation and presence with virulent IBDV strains (141).

A multiplex RT-PCR test capable of differentiating between IBDV virus serotypes (180), a multiplex real time quantitative RT-PCR for detection of RNA levels in blood of the infected chickens using Taq-Man® technology (206) and a quantitative competitive PCR (QC-PCR) (334) test for estimating viral genomic copy number have been described.

### 1.12.2 Serology

Serological tests generally used for the detection of IBDV are ELISA, VN and AGP. The ELISA is the most commonly used test for the detection of antibodies to IBDV. It is economical, simple, quick and tests a large number of samples at the same time and is adaptive to automation to computer software (191). The ELISA allows the quantification of antibodies to IBDV and is therefore used for monitoring the immune status of the chicken flocks (196), to check response to vaccination, natural field exposure and decay of maternal antibody titer (170,191). However, ELISA cannot differentiate between the antibodies specific to the two serotypes (123,191). Therefore, while using ELISA for monitoring the chicken flocks for antibodies to IBDV, careful consideration should be given to the fact that the serotype 2 viruses are widespread in commercial chickens and could result in erroneous impression of antibody levels of the flock(3).

Constant virus - diluting serum VN test was the most common procedure before the use of ELISA (286). The VN is the only serologic test to distinguish the serotypes 1 and 2 of IBDV and also to differentiate the antibodies to different subtypes of IBDV (129,199). At-least six different antigenic subtypes of IBDV serotype 1 viruses have been identified by the *in vitro* cross-neutralization test.

The VN titers accurately reflect the relative protection of chickens to IBDV (123,129,137,199). It is essential to use appropriate indicator viruses in VN tests to avoid artificially low titers due to the existence of several antigenic variants (123). Most

chicken sera have high levels of neutralizing antibodies to a broad spectrum of antigenically diverse viruses due to vaccine and field exposure. However, VN is laborious and time consuming and therefore its use is limited to research applications. Although *in vitro* VN tests can be used for detection of antigenic differences between the virus strains, *in vivo* cross protection studies are essential for determining immunogenicity of the virus and complete evaluation of host response (129).

Another method used to detect antibodies to IBDV is the AGP test. This test has been adapted to the quantitative format (50). It is rapid but insensitive. It does not detect serotypic differences and measures primarily group-specific soluble antigens (191).

There are several commercially available ELISA kits for the detection of antibodies in the serum. Sub-clinical infections and any outbreak of the disease in the large poultry production units could have significant economic impact; therefore the flocks are regularly monitored for any change in the baseline titer.

Several studies have been conducted to evaluate the performance of the commercial ELISA kits for the detection of antibodies. Serum and egg-yolk extracts from broilerbreeder flock were assayed for antibodies against IBDV, IBV, NDV and Reo-virus by a commercial ELISA kit. A high correlation was observed between serum and egg yolk extract titers for all viruses (r = 0.9 for IBDV, 0.84 for IBV, 0.84 for NDV, and 0.91 for RV) suggesting that egg yolks could be used for monitoring the antibody profile of the flock (281).

The use of egg yolks is advantageous over blood since flock biosecurity is not compromised. However, when yolks were assayed by a simple dilution method and titers compared to serum by five different commercial ELISA kits, a poor correlation was observed for the five viruses used (r = 0.35 and 0.85). The yolk titers were found to be lower than the serum titers for IBDV, IBV, NDV and Reo-virus and the correlation varied with the kit. The difference between the yolk and serum titers were non-significant for IBDV when tested KPL (Kirkegaard and Perry Labs.,Gaithersburg, Md.) and for NDV by the IDEXX kit (IDEXX Corp., Portland, Maine). Therefore, it was concluded that the ability to predict the serum titer of a single hen from the mean titer from hen eggs would be inadequate (148).

The reason for contrasting observations regarding correlation between ELISA and VN in these studies might be due to different methods used for processing the yolks in the two experiments. In the former study yolks were extracted with chloroform, homogenized and tested while in the later study they were simply diluted in buffer before testing.

One of the concerns regarding the use of commercial ELISA kits is the variability in the antibody titers among and within the kits. A large variation in the antibody titer of the flock is observed, when multiple samples from the same flock are tested by ELISA. The variation in an ELISA kit (IDEXX Corp., Portland, Maine) for the detection of antibody titers against IBV and IBDV was characterized by assaying the common serum pools having a low (1: 2000), a moderate (1: 4000) and a high (1:8000) titer on different lots of the kit, on different plates, at different days and by different technicians. Significant variation existed for both viruses between separate lots and among test plates within the same lot. Also, the coefficient of variation was higher than the accepted values for immunological assays. High tittered sera against IBDV achieved maximum absorbance and the kit was unable to detect titers higher than 1:8000-1:9000. Varying the length of the substrate (OPD) incubation times and the use of non-standard methods for diluting

the samples significantly affected the mean titer on all combinations. In most cases, the variability in the titers between the days and the technicians was not significant (160). The ELISA results were significantly different among different laboratories and among different days in the same laboratory. The correlations between mean daily titers and laboratory ambient temperatures were small and insignificant. It was thereby concluded that a single determination on each serum sample would not provide a reliable estimate of the antibody titer and the use of standard reference pools of serum to be included in the assays was recommended (161).

A complication for the user while interpreting the results is the variability between the ELISA kits on the market. Five different commercial ELISA kits namely, BioChek standard (BioCheck CV., Gouda, The Netherlands), IDEXX Flock check standard, IDEXX-XR Flock check (IDEXX Corp., Portland, Maine), KPL Proflock and KPL Proflock Plus (Kirkegaard and Perry Labs., Gaithersburg, Md.) were evaluated for the detection of antibodies to serotype 1 of IBDV. The specificity of VN was found to be 100 % while that of ELISA vaired from 63.8- 100%. The ELISA with lowest specificity showed the highest sensitivity at 5 days post inoculation. All ELISA reached a sensitivity of 100 % from 14 to 21 days post inoculation. All ELISA's and VN showed a highly significant correlation for the decrease in the maternally derived antibodies (r = 0.44 – 0.76). A certain correlation was found between VNT and ELISA but it varied from ELISA to ELISA. No significant difference was observed between the antibody levels in breeder sera; egg yolk and the progeny at 1 and 4 days of age. Therefore, it is essential to have an idea of the technical performance of the kits while interpreting the data from these kits (54).

Earlier studies reported an excellent correlation between ELISA and VN titers (28,294,305). Later on it was shown that titers to IBDV variant viruses detected with the commercially available kits do not adequately represent the immune status of the chicken flocks (133), also the kits reacted differently to the monospecific polyclonal antisera against several IBDV strains thereby limiting their ability to assess protection levels against the variant IBDVs (143). Therefore, there was a need to produce kits to better detect the antigenic variants of IBDV.

The ELISA kits currently available in the US include IDEXX (IDEXX Corp., Portland, Maine), Synbiotics (Synbiotics Corp., San Diego, CA) and Affinitech (Affinitech Ltd., Bentonville, AR). Each of them comes with standard positive and negative control sera and the recommended procedures for the calculation of titers. Both IDEXX and Synbiotics have introduced improved versions of their standard kits. The difference between the standard and improved version of kits lies in the nature of the coating antigen. In IDEXX flock check kit (standard), intact viral particles from the classic strain have been used as coating antigen while IDEXX-XR has a mixture of recombinant antigens from the variant strains as the coating antigens. In the standard Proflock kit, tissue culture propagated classic strain antigen has been used as a coating antigen. Improved versions of both kits claim to be more sensitive than the standard versions and capable of detecting antibodies to a wider spectrum of antigens.

The ImmunoComb® Orgenics system is a solid state dot ELISA system for the semi quantitative determination of antibody titer by visual inspection. It can be used to differentiate the susceptible, immunized and field-challenged chickens. The assay is rapid, and the commercial kit is self-contained, field deployable and is user friendly (170).

### 1.12.3 Differential diagnosis

The lesions and symptoms of coccidiosis are very similar to IBD. However, muscular hemorrhages and edema differentiate IBD from coccodiosis. Other diseases that resemble IBD are infectious bronchitis virus, hemorrhagic syndrome, Marek's disease (191).

### **1.13 Management practices**

### **1.13.1 Prevention and Control**

The epidemiology of infection is not extensively studied but it is known that the virus is contagious so contact with the infected birds and contaminated fomites could result in the spread of infection. Rigorous biosecurity measures have to be implemented in order to stop the spread of virus from one flock to the next. The virus is environmentally stable and resistant to many physical and chemical agents. Integrated nature of commercial poultry operations and vectors like lesser mealworm, mosquitoes and rats pose extra problems for the control of this infection. No therapeutic treatment has been found to have an effect on the course of the viral infection (46,191). There are no reports of the use of the antiviral compounds and interferon inducers for the treatment of IBD (191).

### 1.13.2 Vaccination

Before the immunosuppressive effects of IBDV were known, planned infection of birds at an early age was used as a measure to control IBD. Young chickens less than 2 weeks of age did not exhibit clinical signs of IBD. Planned infection was usually done on a farm where disease was already established and the chickens would have maternal antibodies. These infections were accomplished by exposing the chickens to the contaminated litter or by mixing the infected chickens with the susceptible ones (172). This technique lowered IBD mortality but often resulted in immunosuppression and further dissemination of field virus.

Later on when the severe immunosuppressive effects of IBDV were discovered, the practice of intentional exposure of chickens became less desirable. On many farms, due to lack of thorough cleaning the virus persists and provides an early exposure naturally (191). Due to the highly infectious nature of the virus and its hardiness, vaccination is inevitable under high infection pressure and mandatory to protect chickens against infection during the first weeks of age. Thus, immunization of the chickens is the principle method of prevention of IBD in chickens.

A live attenuated vaccine based on a mild strain passaged in eggs was developed in 1968. Early vaccines included the virus isolated by Edgar, Mouthrop-Snedecker and Winterfield. Edgar isolate was a moderately pathogenic bursa-origin virus strain used as a live vaccine (197). The other two isolates were attenuated by serial passage in eggs and became some of the first commercially available vaccines, Bursa Vac® (172) and IBD.Blen<sup>TM</sup> (329) respectively. These vaccines reduced clinical signs but caused significant bursal pathology (329). Lukert (171) attenuated a field isolate by passaging in different cell culture systems and this strain served as the seed virus for many vaccines developed during the 1980's. Tissue culture derived vaccines were not only less pathogenic then the embryo derived vaccines but also less effective in stimulating active immunity in chickens having maternal antibodies (332). It is now known that the pathogenicity of the live vaccines is inversely proportional to their attenuation (170).

Once the immunosuppressive potential of the disease and role played by the maternal immunity became clear, the focus shifted to the effective vaccination of the breeder flock in order to provide maternal antibodies to the progeny. Such parental immunization protects the chickens from earlier infections. Maternal immunity protects the chicken for 1-3 weeks of age. Passive immunity might be extended for 4-5 weeks by immunizing the breeder flocks with oil-adjuvanted vaccines (185). Vaccination of breeders at 8-12 weeks of age with live attenuated virus followed by a booster of inactivated oil-emulsion prior to the onset of lay (20-21) weeks resulted in greater transfer of maternal antibodies to the progeny (67,337). In the sensitized hens, the inactivated vaccine induces a long lasting high titer serum antibody response, which is then transferred to the progeny.

The major problem with active immunization of maternally immune chickens is to determine the proper time of vaccination that allows adequate replication of the vaccine virus at the same time protects young chicken from disease. The time of vaccination varies with the level of maternal antibodies, route of vaccination and virulence of the vaccine virus. For a successful vaccination program factors like environmental stresses, management and flock profiling for the presence of maternal antibodies should be taken into account (191).

The decay of maternal antibodies was reported to be linear. In white leghorn chickens, the half-life of IBDV antibodies were reported to be from 3-8 days (46,287,333). Chickens having medium levels of maternal antibodies were shown to be refractory to infection till 2 weeks, while those having high levels of maternal antibodies were resistant to infection till 4 weeks of age. Chickens having high levels of maternal antibodies of maternal antibodies (GMT of VN =1:3275) at hatch were shown to be protected from IBDV infection till 5.5 weeks of age (8).

Live vaccines available in the US are classified according to their virulence as mild, mild intermediate, intermediate plus or hot. Vaccines containing the Delaware variants, either in combination with classic strains or alone, are also available. Live attenuated vaccines are recommended for use at 1 day-of age. An improvement in the total performance of broilers after one-day of age vaccination has been observed. Combined vaccination with IBDV and Marek's disease vaccine at one-day of age results in less rapid decline in IBDV-specific neutralizing maternal antibodies as compared to vaccination with IBDV and MDV alone (159). In addition, IBDV vaccination at 1 day of age slowed down the accelerated rate of IBDV neutralizing antibody decline caused by vaccination with MDV (159). Highly virulent (hot), intermediate and avirulent strains break through maternal VN antibody titers of 1:500, 1:250 and less then 1:100 respectively (185,287). Intermediate strains vary in their virulence and induce bursal atrophy and immunosuppression in 1- day-old and 3- week- old SPF chickens (188). Chickens might be vaccinated with the avirulent strains of the virus if the maternal antibody titer is less than 1:1000. The vaccine virus replicates in the thymus, spleen and bursa where it persists for 2 weeks (189). After the maternal antibody level wanes out,

there is a primary response to the persisting vaccine virus. A complex vaccine made by mixing an intermediate strain and a vaccine strain with a measured amount of IBDV antibody before injection has been used to immunize day-old chickens in the presence of maternal antibodies. This vaccine induced active immunity and provided some protection against challenge with the standard STC virus challenge (96).

Killed virus vaccines in oil-adjuvant are usually used to boost and prolong immunity in breeder flocks but are not used for inducing a primary response in young chickens. Oil – adjuvant vaccines are most effective in chickens that have been primed with live virus either in the form of a vaccine (336) or field exposure to the virus (191). Oil-adjuvanted vaccine might contain both standard and variant strains of the virus. Maternal antibody profiling of the breeder flock should be done to assess the effectiveness of vaccination and persistence of antibody (191).

Antigen content of inactivated infectious bursal disease vaccines was shown to be a reliable indicator of the protective serological response after vaccination and could be used as a measure of vaccine potency. Neutralizing antibody titer correlated well with the VP2 content of the vaccine. A significant correlation was also observed with the VP3 content and antibody response (233).

A novel concept is *in ovo* vaccination of chickens against multiple viral agents at 18 days of incubation. The immune system in birds develops early during embryogenesis and various immune reactions are induced in the late stage chicken embryos. Compared with post-hatch vaccination, *in ovo* vaccination stimulates both the innate and adaptive immune responses. Due to prenatal immunization, *in ovo* vaccinated chickens have good protection by the time of hatch (225). A single *in ovo* injection of a vaccine containing

serotype 1, 2 and 3 of Marek's disease (MDV), a vaccine strain of serotype 1 of infectious bursal disease and recombinant fowl pox vaccine with HN and F genes of Newcastle disease virus (rFP-NDV) induced protection against virulent MDV, IBDV, Newcastle disease virus, and fowl poxvirus. This vaccine induced specific antibodies against the agents present in the mixture and did not adversely affect the hatchability of the chicken. This technique is labor saving and have a potential to circumvent the effects of maternal antibodies and initiating a primary immune response (83).

A universal vaccination program for IBD could not be offered due to variability in maternal immunity and due to different management and control practices. Vaccination of the broilers might not be needed if high levels of maternal antibodies are achieved and the field challenge is reduced. Chickens have been vaccinated with attenuated and intermediate vaccines from as early as 7 days to 2 or 3 weeks of age. IBDV vaccines could be given to broiler at 1 day of age along-with Marek's disease vaccine.

Breeder replacement chickens are vaccinated with a live vaccine at 10-14 weeks of age. Killed oil-adjuvant vaccines are given at 16-18 weeks of age. If antibody profiling undergoes a major drop than re-vaccination of breeders is required (191).

Recombinant vaccines have been described but none is currently available commercially (13,53,75,101,194,292,312). Attempts to produce VP2 subunit vaccines in *E.coli* were unsuccessful due to improper folding and post–translational modification of the protein (75). Immunization with these expressed proteins resulted in low titer antibodies (76). It also indicates that the VP2 by itself might be insufficient to mount a good immune response. Recently, the genome segment A of the highly infectious bursal disease virus was cloned and amplified in *E.coli* and vaccination with the resultant empty viral

particles were shown to confer protection to challenge (256). Yeast expression system produced more immunogenic recombinant proteins that induced virus neutralizing antibodies that were passed on to the progeny and were shown to be protective against challenge with virulent IBDV (76,194). A number of different recombinant viral expression vectors have been described as potential IBDV vaccines (13,64,81,101,313). A fowl pox recombinant virus expressing VP2 under the control of fowl pox early/late promoter induced virus neutralizing antibodies that protected significantly against challenge but the protection was still less then what complete virus offers (101). A fowl pox vector containing VP2 from the virulent virus strain 52/70 as a  $\beta$ -galactosidase fusion protein provided protection but this vaccine caused bursal pathology (13).

A fowl adenovirus carrying VP2 from the Australian strain 002/73 protected SPF chicken against an intermediate virulent classic strain via IV, IM, IP or sub-cutaneously but not via conjunctival sac (277). Immunogenic IBDV protein expression using baculovirus and recombinant turkey herpes virus vectors have also been described (64,313).

A chimeric virus between classic and variant strains was produced (D78-Chim). This vaccine induced high VN antibodies that were comparable to both classic and variant strains by themselves. D-78 and D78-Chim induced comparable bursal damage in the presence of maternally derived antibodies (MDA) and were able to break through a similar level of MDA (219).

DNA vaccines encoding the polyprotein from segment A have been described. Vaccination with a DNA vaccine expressing variant E polyprotein provided 90% protection against the homologous strain while that expressing a classic STC provided 100% protection against the homologous strain (35). None of these vaccines are currently available in the market for use. The molecularly cloned vaccines produced by site – directed mutagenesis have a great risk of reversion to the wild type that has greatly limited their practical application as potential vaccines in the field (250).

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# **CHAPTER 2**

# INTERFERENCE BETWEEN MILD AND PATHOGENIC STRAINS OF INFECTIOUS BURSAL DISEASE VIRUS IN CHICKENS

## 2.1 SUMMARY

Infectious bursal disease virus (IBDV) is a contagious, immunosuppressive disease of young chickens that is controlled by vaccination. Cross-protection occurs between different strains of the virus due to shared neutralizing epitopes. However, interactions between two antigenically similar strains (a mild and a pathogenic) co-infecting the same host have not been investigated. Groups of specific pathogen free chickens were inoculated with a mild strain followed by a pathogenic strain at 0, 16, 24 or 48 hours post inoculation (PI) with the mild strain. Virus persistence and the predominant strain of the virus were determined by the reverse transcriptase polymerase chain reaction/restriction fragment length polymorphism analysis respectively in bursas at 2, 4, 8, 14 and 21 days PI with the pathogenic strain. Severity of infection was assessed by the bursa/body weight ratios and histopathological lesions scores. The mild virus interfered with

replication of the pathogenic virus. The greatest interference was observed when the pathogenic strain was inoculated 24 hours PI of the mild strain. The interference phenomenon observed might be due to competition for host receptor sites or production of cytokine(s). This interference phenomenon could have practical implications for vaccine usage and protection.

#### **2.2 INTRODUCTION**

Infectious bursal disease (IBD) is an acute and contagious disease of chickens that induces high morbidity and mortality in chickens 3-6 weeks of age (8). The disease in younger chickens is usually sub-clinical and results in immunosuppression with subsequent poor immune response to different infections and vaccines. Therefore, the disease has a significant economic impact (3).

The causative agent is a bisegmented, double stranded RNA virus belonging to the genus Avibirnavirus of the family Birnavirideae. Of the two serotypes recognized, only serotype 1 viruses cause disease while serotype 2 viruses are not pathogenic to chickens (16). Serotype 1 has two major antigenic groups, classic and variant and several subtypes (6, 18). The bursa of fabricius is the target organ where the viral infection results in a marked atrophy due to depletion of lymphocytes. The virus persists longest in bursa of infected chickens as compared to the other organs such as the spleen, kidney and thymus (P. D. Lukert, personal communication).

The virus is very resistant to various physical and chemical agents and is hard to eradicate completely from poultry houses even after thorough cleaning and disinfection (2). Due to its widespread and persistent nature, flocks are highly likely to be exposed to field and vaccine strains of the virus. Hence, it is important to study the interactions between different strains of the virus. Understanding the interactions between viruses could aid in vaccine usage and protection studies.

The availability of molecular techniques such as reverse transcriptase polymerase chain reaction/restriction fragment length polymorphism analysis (RT-PCR/RFLP) makes it possible to identify different strains of the virus. The objective of this study was to investigate the interactions of two strains of the virus during mixed infection in SPF chickens. The two viruses are antigenically similar but vary in pathogenicity.

#### 2.3 MATERIALS AND METHODS

**Chickens and embryonated eggs.** Specific-pathogen free (SPF) eggs were incubated in our facilities at Food animal health research program, Ohio agricultural research and development center, The Ohio state university. The hatched chicks were kept in cages in a disease containment building that had rooms supplied with HEPA- filtered intake and exhaust air. At 2 weeks of age the chickens were wing banded, weighed and transferred to sterile flexible plastic isolators with filtered intake and exhaust air. Birds were provided with food and water *ad libitum*.

**Viruses.** The pathogenic strain used is the STC strain used by the USDA for challenge studies was propagated in 5 two-week old SPF chickens. Birds were euthanized and the bursas were harvested 5 days post inoculation (PI), homogenized and a 10% w/v suspension prepared in Minimal essential media (MEM). The bursal homogenates were titrated in 10 days old SPF embryonated eggs via chorioallantoic membrane (CAM) route as described previously (5). The titer was expressed as mean embryo infective dose EID<sub>50</sub>

per milliliter on the basis of lesions and mortality and was calculated by the method of Reed and Muench (11). The homogenates were aliquoted and stored at -70C for future use. The dose of the virus was adjusted to  $10^2$  EID <sub>50</sub>/chick and administered via the oral route.

The mild virus used is the Bursine-2<sup>®</sup> (Burs) vaccine strain (Fort-dodge, Iowa, USA) which is a live virus vaccine having intermediate virulence (13). It was diluted to a titer of 10  $^{4.9}$ TCID <sub>50</sub>/dose and administered orally.

**RT-PCR/ RFLP analysis.** Bursas obtained from birds in each group at a single time interval were pooled, homogenized and a 10% w/v suspension was prepared in Phosphate buffered saline (PBS) (pH=7.2). The double stranded RNA was extracted using the Trizol reagent (TRIZOL LS Reagent, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The primers targeted a 743 base pair region on the VP2 gene and an internal control (900 base pairs) were used as previously described (17). RT- PCR amplification was carried out with Gene Amp PCR System 9700 (Perkin –Elmer, Foster City, CA). Reagents for RT-PCR (10 X PCR buffer, MgCl<sub>2</sub>, Rnase inhibitor, AMV and Taq polymerase (Promega, Madison, WI) dNTPs (Invitrogen, Carlsbad, CA) were used according to the manufacturer's instructions. The RT-PCR was performed at 42C for 60 min, followed by 95C for 3 min, and 35 cycles of: 95C for 1 min, 53C for 1:30 min, 72C for 1 min. A final elongation step at 72C for 7 min was included. The RT-PCR products were digested with the restriction enzymes *Bst*NI and *Mbo*I as described earlier (7).

**Antibody capture ELISA.** A 1/800 dilution of serum samples and 1/10000 dilution of rabbit anti-chicken IgG conjugate (Sigma, St.Louis, MD, USA) was used for the detection of anti-IBDV antibodies as previously described (4, 19). The cutoff value was

determined by adding 3 x standard deviations to 2.1x mean absorbance value of all negative controls (22). The serum samples obtained during trial 2 and 3 were tested for antibodies by ELISA.

**Histopathology.** Sections of bursal tissues from the inoculated and control chickens were fixed in 10% Prefer<sup>®</sup> fixative solution (Anatech, Ltd., Battle Creek, MI) and stained with hematoxylin and eosin (H&E). Sections of bursa were examined microscopically and photographed by digital camera equipment (LEICA DMIRB, Leica Microsystems Wetzlar, Germany). Bursas were scored according to the system of Rosales *et al.*(12) and assigned a 1 = no lesions, normal; 2 = focal, mild lymphocyte depletion; 3 = multifocal, 1/3 to  $\frac{1}{2}$  of the follicles show lymphocyte depletion; and 4 = diffuse, lymphocyte depletion of all follicles.

**Statistical analysis.** Bursa/body weight ratios were calculated for each bird by the following formula: (Bursa weight/body weight)  $\times$  1000. The geometric mean of bursa/body weight ratios of the inoculated groups at each time interval were compared with the negative control groups for statistical analysis of significance by analysis of variance followed by Fisher's least significant difference test.

**Experimental Design.** Three separate trials were performed to study the interactions of STC with Burs. For trial 1, 125 one-week old chickens; for trial 2, 175 two-week old chickens and for trial 3, 125 two-week old chickens were allotted into groups of 25 chickens each. All chickens received 0.2 ml  $10^2$  EID<sub>50</sub> of STC virus and 0.2 ml of Burs containing  $10^{4.9}$  TCID <sub>50</sub>/dose orally. The schedules of inoculations and sample collection for each trial are shown in Tables 1, 2 and 3.

At different time intervals, five chickens from each group were euthanized and blood was collected. Body and bursa weights were recorded for each chicken. Each bursa was divided in half; one half was used for histopathological analysis, and the other half for RT-PCR analysis. Bursal samples from each group collected at a specific time interval were pooled for RT-PCR analysis, homogenized and kept at –70C for testing. Serum collected from the blood samples was aliquoted and kept at –20C for testing for antibodies with ELISA. Samples that were negative or weakly positive by RT-PCR were inoculated in 10 days old embryonated eggs and tested by RT-PCR/RFLP.

#### 2.4 RESULTS

**RT-PCR/RFLP analysis.** The RT-PCR products (734 bp) and internal controls (900 bp) are shown in Fig.2.1. *Bst*NI generated four fragments 172 bp, 154 bp, 139 bp, 119 bp with STC and three fragments 424 bp, 172 bp and 119 bp with Burs (Fig.2.3). *Mbo*I generated two fragments 362 bp and 229 bp with STC and two fragments of 480 bp and 229 bp with Burs (Fig 2.2).

*Trial 1.* The RT-PCR/RFLP results obtained from Trial 1 are shown in Table 2.1. Burs was detected up to 8 days post inoculation (PI) in the group given Burs alone. The STC strain was detected up to 14 days PI in the group given STC alone, 21 days PI in the group given both viruses simultaneously, and only at day 4 PI in the group given STC 24 hours PI.

*Trial 2.* This trial included additional groups and RT-PCR results are shown in Table.2.2. Geometric mean bursa/body ratios and bursal lesion scores are shown in Table.2.3.

Burs virus was detected by RT-PCR/RFLP analysis up to 8 days PI in the homogenate of the bursal samples collected from the vaccinated chickens with Burs only. These chickens had no significant difference in bursa/body weight ratios throughout the experimental period although mild lymphocyte depletion was detected at 4 days PI but the bursas were histologically normal after that point.

The STC virus was detected in the group given only STC virus up to 21 days PI and the chickens in this group had a significant decrease in bursa/body weight ratios starting at 8 days PI and their bursas had marked lymphocyte depletion. Bursal follicles were depleted of lymphocytes and consequently the bursal lesion scores were highest among all groups. Burs was detected up to 4 days PI and STC up to 21 days PI in the group vaccinated and exposed to STC simultaneously. These chickens had a significant decrease in bursa/body weight ratios starting at 8 days PI and gradually became non-significant by 21 days PI. Bursal lesions scores showed severe to moderate lymphocyte depletion at 14 and 21 days PI respectively.

Burs was detected at 4 and 8 days PI while STC virus or its RNA were detected until 21 days PI in the group given STC 16 hours PI. These chickens had a significant decrease in bursa/body weight ratios starting at 8 days PI. Bursal lesion scores showed moderate to severe lymphocytic depletion towards the end of the experiment.

Viral RNA from both STC and Burs were detected in the group given STC at 24 hours PI up to 8 days PI, however no infectious virus was isolated by embryo inoculation or RT-PCR at 8 days PI. This group had no significant difference in bursa/body weight ratios throughout the experimental period although the bursas had mild lymphocyte depletion at 4 days PI only. Both viral strains could be detected up to 8 days PI when chickens were given STC at 48 hours PI with Burs. The chickens had a significant decrease in bursa/body weight ratios only at 8 days PI and lymphocyte depletion in the bursa throughout the experiment. The negative control group was negative by RT-PCR throughout the experimental period and had normal bursas.

*Trial 3.* The RT-PCR results are shown in Table 2.4. The bursa/body weight ratios and bursal lesion scores are shown in Table 2.5. The general trends in the bursa/body weight ratios and histopathological lesion scores (fig 2.4) were similar to those obtained in trial 2 (Table 2.3).

Burs could be detected up to 14 days PI in the group inoculated with Burs. There was no significant difference in the bursa/body weight ratios in this group throughout the experimental period although moderate cell depletion was observed at 8 days PI. In the group given STC alone, the virus could be detected up to 21 days PI. This group had a significant decrease in bursa/body weight ratios starting at 8 days PI and severe bursal damage throughout the experiment.

The STC virus was detected up to 14 days PI in the group given the STC at 16 hrs following Burs. This group had a significant decrease in bursa/body weight ratios at 8 days PI.

Both Burs and STC could be detected up to 14 days PI in the group where the STC was given at 24 hrs following Burs. This group had a significant decrease in bursa/body weight ratios at 8 and14 days PI and was completely recovered by 21 days PI. The bursa had moderate lymphocyte depletion at 4 days and gradually became normal by 21 days PI. Serum samples collected from the inoculated chickens starting from 8 days PI up to 21days PI had antibodies against IBDV. Pre-immunization serum and serum samples from 2 and 4 days PI had no antibodies in both trials (results not shown).

### 2.5 DISCUSSION

Interactions between the two viral strains in the host influenced the outcome of infection. Results of these experiments indicate that the mild virus has the potential to interfere with the replication of the antigenically similar pathogenic virus. Antibodies against the virus were not detected until 8 days PI and hence were unlikely to have played a role in the early clearance of the virus.

The phenomenon of virus interference or the inhibition of virus growth by another virus has been well recognized (20). Interference *in vitro* has been described in three situations: (1) serial passage of viruses at high multiplicity of infection leading to development of defective-interfering particles, (2) mixed infections of wild type viruses with certain temperature sensitive mutants (23) (3) co-infection of cells with different wild type virus isolates (14).

These results describe the previously unreported event of *in vivo* viral interference between IBDVs. The RT-PCR/RFLP analysis was found to be a suitable technique for identifying and studying dual viral infections. The RT-PCR/RFLP analysis can successfully differentiate the two strains during concurrent infection. The infection of chickens with the mild strain of IBDV is a self-limiting process as no virus or its RNA could be detected after 14 days PI. Previously it was reported that the virus or its RNA persisted up to 7 and 14 days after vaccination with a live modified vaccine in SPF chickens (1). Several observations can be made from the data. In trial 1 (Table 2.1), it was shown that the mild strain was detected up to 8 days PI while the STC was detected up to14 days PI. During dual infection the mild virus interfered with the pathogenic strain given 24 hrs later and inhibited its replication in the host as evident by the negative RT-PCR indicating a significant interference between the two strains.

This raises a question of whether Burs is capable of competing with STC if it were given a longer time to establish itself in the host. To investigate this further, trial 2 was performed with additional groups subsequently given STC at 16, 24 or 48 hours PI. A different lot of vaccine and two weeks old chickens were used in the following trials due to difficulty in handling small bursas and the need for additional bursal tissue for histopathology.

Although the RT-PCR/RFLP analysis was positive for both viruses at 4, 8 and 14 days PI, results from bursa/body weight ratios and histopathological lesions were similar to the controls indicating that the mild virus interfered with the replication of the pathogenic virus. The most significant interference occurred at 24 hours PI as evident by negative RT-PCR, recovered bursa/body weight and normal bursa in this group by 21 day PI. It was interesting to note that interference did not occur in the groups exposed to both strains simultaneously and STC dominated in these chickens. The variation in RT-PCR might be due the pooling of the bursas from each time interval. Being a very sensitive technique the results would be positive even if 1 out of 5 birds was infected.

Bursa/body weight ratios and histopathological analysis of bursa in both trials showed the same general trends (fig. 2.4). The intermediate vaccine is known to cause some bursal damage by itself (13). The bursas collected at the end of the study had recovered and

were repopulated with lymphocytes. Interference in the replication of pathogenic viruses by the mild viruses might be due to the competition for receptor sites on host tissues (20). Several examples of attenuated virus strains interfering with the replication of wild type viruses are available in the literature including those for reovirus (20), poliovirus (15) and influenza virus (21). Other factors like cytokines might be involved in interfering with the viral replication before the development of the antibodies.

Viral interference in IBDV have been reported before *in vitro* where serial undiluted passages of IBDV in chicken embryo cultured cells generated a small plaque virus that interfered with the standard virus in mixed infections. At an equal virus multiplicity, yield of wild type virus was reduced to 78% during co-infection with small plaque mutant (9). However, this is the first report of *in vivo* interference between a mild and a pathogenic field virus.

Studies on viral interference contribute to the understanding the interactions of viruses and possibly provide a means of improving vaccination strategies. The ability to interfere with wild strains is a desirable trait for any live, attenuated vaccine strain (20).

The ability to reduce the replication of the pathogenic virus during mixed infections imparts an additional benefit to the live virus vaccine and has the potential to greatly expand the prophylactic use of the vaccine virus. This is of particular significance in IBDV whose replication cycle is short (12 hours in chicken embryo cells) (10) as compared to time required for the vaccine to induce protective immunity (2-3 weeks). Therefore, the vaccination could be useful in the face of an outbreak.

Taken together, our findings support the hypotheses that viral interference occurs in live chickens. The most significant interference occurred when infection with the mild and field strains are 24 hours apart.

### 2.6 ACKNOWLEDGEMENTS

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**Fig. 2.1.** RT-PCR of bursal samples collected at various time intervals. M= Marker; 2 = false negative reaction; 3,6,7,11, 12,13 = IC only; negative; 1,4,5,8,9,10 = positive.



**Fig.2.2.** RFLP analysis of RT-PCR products with *Mbo*1 enzyme. M = Marker; 1 = STC; 2 = Burs; 3 = Both; 4 and 6 = Burs; 5 and 7 = STC; 8 and 9 = Both.



**Fig. 2.3.** RFLP analysis of RT-PCR products with *BstN*1 enzyme. M = Marker; 1 = STC; 2 = Burs; 3 = Both; 4 and 6 = Burs; 5 and 7 = STC; 8 and 9 = STC.



**Fig. 2.4.** Histopathology slides of the bursa showing various stages of lymphocyte depletion during infection. Bursal lesion score 0 = Normal bursa packed with lymphocytes; Bursal lesion score 1 = mild, scattered lymphocyte depletion in few follicles; Bursal lesion score 2 = Moderate lymphocyte depletion in 1/3 to 1/2 of the follicles; and Bursal lesion score 4 = severe lymphocyte depletion in all follicles.

Incoulum	<b>Inoculation</b>	Virus(es) present at Days Post Inoculation <sup>A</sup>						
moculum		2	4	8	14	21		
Burs	0 hr	-	Burs	Burs	-	-		
STC	24 hrs	STC	STC	STC	STC	-		
Burs STC	24 hrs 24 hrs	STC	STC	STC	STC	STC		
Burs STC	0 hr 24 hrs	-	STC	-	-	-		
Negative control		-	-	-	-	-		

**Table 2.1.** RT-PCR/ RFLP analysis of pooled bursal samples collected from different groups of SPF chickens at specified time intervals (Trial.1). <sup>A</sup> Bursas from 5 chickens from each group were collected and pooled at the specified time. Burs = Bursine; - = Negative sample.

Inoculum	Inoculation Time	Virus(es) present at Days Post Inoculation <sup>A</sup>						
Time		2	4 8		14	21		
Burs	0 hr	Decus	D	D				
STC	24 hrs	Burs	Burs	Burs	-	-		
D	241	-	STC	STC	STC	STC		
Burs STC	24 hrs 24hrs	STC& Burs	Burs	STC	STC	STC		
Burs STC	0 hr 16 hrs	a -	a STC &Burs	Burs	STC	STC		
Burs STC	0 hr 24 hrs	a -	a STC& Burs	a STC& Burs	a -	a -		
Burs STC	0 hr 48 hrs	ND	STC& Burs	STC& Burs	a -	a -		
Negative control		-	-	-	-	-		

**Table 2.2.** RT-PCR/ RFLP analysis of pooled bursal samples collected from different groups of chicken at specified time intervals (Trial.2). <sup>A</sup> Bursas from 5 chickens from each group were collected and pooled at the specified time Burs = Bursine; - = Negative sample; <sup>a</sup> = These samples were either negative or produced weak bands and were used further for embryo inoculation followed by RT-PCR analysis.

Inoculum	Inoculation		Average bursa/body weight ratios at days post inoculation		5 A	Mean bursal lesion scores at days post inoculation				В	
	time	2	4	8	14	21	2	4	8	14	21
Burs	0 hr	1.383 <sup>a</sup> (0.36)	1.437 <sup>b</sup> (0.23)	$1.205^{ab}$ (0.35)	1.268 <sup>a</sup> (0.35)	$0.63^{ab}$ (0.19)	1	2	1	1	0
STC	24 hrs	1.236 <sup>a</sup> (0.19)	1.45 <sup>6</sup> (0.08)	0.772 ° (0.33)	0.372 <sup>b</sup> (0.117)	0.092 ° ( 0.10)	0	2	4	4	4
Burs STC	24 hrs 24 hrs	1.576 <sup>a</sup> (0.23)	1.699 <sup>a</sup> (0.14)	0.719 <sup>c</sup> (0.42)	0.667 <sup>c</sup> ( 0.48)	0.551 <sup>ab</sup> (0.29)	1	2	2	4	2
Burs STC	0 hr 16 hrs	1.363 <sup>a</sup> (0.20)	1.454 <sup>b</sup> (0.19)	0.671 <sup>c</sup> (0.26)	0.597 <sup>b</sup> (0.26)	0.141 <sup>c</sup> (0.08)	1	1	3	2	4
Burs STC	0 hr 24 hrs	1.545 <sup>a</sup> (0.19)	1.515 <sup>ab</sup> (0.12)	1.328 <sup>ab</sup> (0.17)	1.392 <sup>a</sup> (0.19)	0.730 <sup>a</sup> (0.07)	1	2	0	1	1
Burs STC	0 hr 48 hrs	NA	1.425 <sup>b</sup> (0.21)	1.034 <sup>bc</sup> (0.30)	1.455 <sup>a</sup> (0.10)	0.497 <sup>b</sup> (0.18)	NA	2	2	1	2
Negative co	ontrol	1.457 <sup>a</sup> (0.15)	1.624 <sup>ab</sup> (0.09)	1.538 <sup>a</sup> (0.08)	1.631 <sup>a</sup> ( 0.14)	0.73 <sup>a</sup> ( 0.11)	0	0	0	0	0

**Table 2.3.** Bursa/body weight ratios and lesion scores in SPF chickens following inoculation with Bursine-2 and STC virus at different time intervals (Trial 2). <sup>A</sup> Values are for 4-5 chickens. Geometric mean values within column followed by different superscript letters are significantly different (P<0.05). Values in brackets are the standard deviation. <sup>B</sup> Mean bursal lesion scores: 0= no lesions, 1= mild scattered lymphocyte depletion in few follicles, 2= mild to moderate lymphocyte depletion in 1/3 to 1/2 of the follicles, 3= mild to moderate lymphocyte depletion in all follicles.
Inoculum	Inoculation	Virus(es) present at Days Post Inoculation <sup>A</sup>							
	Time	2	4	8	14	21			
Burs	0 hr	а							
		Burs	Burs	Burs	Burs	-			
STC	24 hrs								
		-	STC	STC	STC	STC			
Burs	0 hr				а	а			
STC	16 hrs	Burs	Burs&	Burs&	Burs&	-			
			STC	STC	STC				
Burs	0 hr				a				
STC	24 hrs	Burs	Burs&	Burs&	Burs&	-			
			STC	STC	STC				
Negative con	ntrol								
		-	-	-	-	-			

**Table 2.4.** RT-PCR/ RFLP analysis of pooled bursal samples collected from different groups of chickens at specified time intervals (Trial.3). <sup>A</sup> Bursas from 5 chickens from each group were collected and pooled at the specified time. Burs = Bursine;  $^-$  = Negative sample; <sup>a</sup> = These samples were either negative or produced weak bands and were used further for embryo inoculation followed by RT-PCR analysis.

Inoculum	Inoculation	A Average bursa/body weight ratios at days post inoculation					B Mean bursal lesion scores at days post inoculation				
	Time	2	4	8	14	21	2	4	8	14	21
Burs	0 hrs	1.652 <sup>a</sup> (0.19)	1.675 <sup>a</sup> (0.24)	1.379 <sup>ab</sup> (0.272)	1.615 <sup>ab</sup> (0.18)	1.619 <sup>a</sup> (0.12)	0	1	3	0	0
STC	24 hrs	1.54 <sup>a</sup> (0.31)	1.827 <sup>a</sup> (0.02)	1.231 <sup>b</sup> (0.24)	0.146 <sup>c</sup> (0.26)	0.351 <sup>c</sup> (0.247)	1	2	4	4	4
Burs STC	0 hrs 16 hrs	1.584 <sup>a</sup> (0.22)	1.775 <sup>a</sup> (0.10)	1.173 <sup>b</sup> (0.31)	0.462 <sup>c</sup> (0.35)	0.93 <sup>bc</sup> (0.63)	1	2	4	4	3
Burs STC	0 hrs 24 hrs	1.705 <sup>a</sup> (0.17)	1.645 <sup>a</sup> (0.20)	0.862 <sup>b</sup> (0.64)	1.386 <sup>b</sup> (0.45)	1.41 <sup>ab</sup> (0.64)	1	3	2	1	0
Negativ	e control	1.583 <sup>a</sup> (0.33)	1.871 <sup>a</sup> (0.06)	1.875 <sup>a</sup> (0.16)	1.9 <sup>a</sup> (0.23)	1.718 <sup>a</sup> (0.04)	0	0	0	0	0

**Table 2.5.** Bursa/body weight ratios and lesion scores in SPF chickens following inoculation with Bursine-2 and STC virus at different time intervals (Trial 3). <sup>A</sup> Values are for 4-5 chickens. Geometric mean values within a column followed by different superscript letters are significantly different (P<0.05). Values in brackets are the standard deviation. <sup>B</sup> Mean bursal lesion scores: 0 = no lesions, 1 = mild scattered lymphocyte depletion in few follicles, 2 = mild to moderate lymphocyte depletion in 1/3 to 1/2 of the follicles, 3 = mild to moderate lymphocyte depletion in >1/2 of the follicles and 4 = severe lymphocyte depletion in all follicles.

# **CHAPTER 3**

# DETECTION OF ANTIBODIES AGAINST SEROTYPES 1 AND 2 INFECTIOUS BURSAL DISEASE VIRUS BY COMMERCIAL ELISA KITS.

# **3.1 SUMMARY**

Two distinct serotypes of Infectious bursal disease virus (IBDV) are recognized in chicken and turkey flocks in the US. Serologic testing of chicken flocks for serotype 1 viruses is routinely performed to monitor disease status and vaccination. Earlier studies indicated that ELISA detects antibodies to both serotypes of the virus while virus neutralization (VN) test is serotype specific. It is useful to evaluate new commercial ELISA kits for their ability to differentiate between antibodies elicited by the two serotypes. Three trials were performed in which chickens were orally inoculated with either a high or a low dose of serotype 1 STC or serotype 2 OH strains of IBDV. Sera collected at 0,7,14 and 21 days from these chickens and antisera procured from naturally infected broilers (n=20) and layers (n=30) flocks were tested with five different commercial ELISA kits and by VN. All ELISA kits detected different level of antibodies elicited against serotype 1 of the virus and moderate and high levels of antibodies against serotype 2 virus. A correlation existed between the ELISA and the VN titers of experimentally infected chickens. All serum samples tested from the naturally infected

layer flocks and 65% of the broiler flocks had antibodies against the OH strain. However, no correlation between the VN titers and ELISA titers was observed for the naturally infected broilers and layers sera by majority of the kits. The results indicated that currently available commercial ELISA kits detect antibodies elicited by the two serotypes of IBDV. Hence, the prevalence of serotype 2 antibodies in the flocks should be considered while determining antibody profiles of the flocks against serotype 1 viruses.

#### **3.2 INTRODUCTION**

Infectious bursal disease is an acute and contagious disease affecting young chickens from 3-6 weeks of age. The disease causes immunosuppression in chickens rendering them vulnerable to a variety of other infections. The causative agent is a double-stranded, bisegmented RNA virus belonging to the family Birnavirideae of the genus Avibirnavirus. Two distinct serotypes of Infectious bursal disease virus (IBDV) have been recognized worldwide. Serotype 1 of IBDV infects and causes clinical disease in chickens while serotype 2 viruses are nonpathogenic to chickens (5) but the infection is common in chickens and turkeys (9). Previous studies in our laboratory have indicated that serotype 2 OH strain causes bursal lesions and mortality in embryos but is nonpathogenic for chickens (1).

The serologic tests commonly used for detecting antibodies elicited by IBDV include the virus neutralization (VN) and enzyme linked immunosorbent assay (ELISA). The VN is the gold standard test and the only serologic test that discriminates between antibodies elicited by the two serotypes and various subtypes of the serotype 1 strains (6).

Due to its ability to process a large number of samples simultaneously, ELISA has been routinely used for detecting antibodies to the pathogenic serotype 1 IBDV. Several commercial ELISA kits are available in the market and are routinely used for monitoring antibody profiles or the disease status of flocks and for determining the proper vaccination timings. The antibody titers to serotype 2 viruses, if present, could give erroneous impression of the antibody level of the flock.

Previous studies with a commercial ELISA kit have indicated that they detected antibodies against both serotypes of the virus (3,8,12). A lot of time has elapsed since these studies and several new kits became available in the market with claims of improved sensitivity and specificity for the variant strains of IBDV. The objective of the current study was to evaluate all the commercially available ELISA kits in the USA in terms of their ability to distinguish between antibodies against serotype 1 and serotype 2 viruses.

#### **3.3 MATERIALS AND METHODS**

**Chickens and embryonated eggs.** Specific- pathogen free (SPF) eggs were incubated at our facilities. The hatching chicks were kept in a disease containment building that had rooms supplied with HEPA-filtered intake and exhaust air. At 2 weeks of age the chickens were transferred to sterile flexible plastic isolators with filtered intake and exhaust air. Birds were provided with food and water *ad libitum*.

**Viruses.** The serotype 1 classic STC and serotype 2 OH strains are maintained in our laboratory. Both cell culture adapted strains were propagated in Buffalo green monkey (BGM-70) cells and embryo adapted strains were propagated in 10-day old embryonated eggs according to the protocols described previously (6).

Virus purification. Tissue culture propagated viruses were purified according to the modified Isopycnic separation method combined with differential centrifugation for IBDV(11,14). Briefly, the cellular debris were removed by low speed centrifugation and the clarified supernatant was centrifuged using a Type 45Ti rotor (Beckman Optima™LE-80K ultracentrifuge) at 73,000xg for 3 hours. The virus pellet was resuspended in TNE buffer (0.01 M Tris-HCl; 0.1 M NaCl and 1m M EDTA pH 7.9) and ultra-sonicated (Biosonic III; Bronwill Scientific, Rochester, N.Y) on ice twice for 30 sec each. The virus suspension was laid on 30% (wt/wt) sucrose cushion and centrifuged at 113,000xg (SW28 Rotor, Beckman, Optima™LE-80K ultracentrifuge) for 3 hours. The resulting pellet was resuspended in 2ml TNE buffer and ultra-sonicated as before. Rate zonal centrifugation was performed by dissolving 4.5ml of the virus suspension in 1.84gm of CsCl, centrifuging at 148,862xg (SW55Ti Rotor, Beckman Optima<sup>™</sup>LE-80K ultracentrifuge) for 18 hours at 4 C. The resulting bands were collected by the side puncture of the tube and the fractions having a density of 1.29 - 1.33 were collected and washed by adding TNE buffer and centrifuged at 147,215xg for 2 hours in SW41Ti Rotor (Beckman Optima<sup>™</sup>LE-80K ultracentrifuge). The resulting pellet was re-suspended in TNE buffer and sonicated twice for 30 sec each on ice. The virus was frozen at -70C till used. The presence of viral particles was confirmed by electron microscopy (Fig 3.1). Purified viruses were titrated in BGM-70 cells and titer was expressed as  $100TCID_{50}/50$ ul.

**Virus Inactivation.** The virus was inactivated by incubation for 2 hours at 37C with 0.2% beta-propiolactone (Sigma Chemicals Co., St.Louis, Missouri). Water-in-oil emulsion vaccines were prepared as described before (4).

**Virus Neutralization test.** The microtiter VN test was conducted with a constant virus diluting serum in BGM-70 cell culture as described previously (7). Briefly, heat inactivated antiserum in serial twofold dilution were incubated with  $100TCID_{50}$  of either the STC or the OH strain for 1 hour and added to a confluent monolayer of BGM-70 cells. Plates were read daily for the appearance of cytopathic effects. Readings were taken 5 days post-inoculation (PI) and titer calculated according to the method of Reed and Muench (10). The STC and OH strain viruses were used as the homologous and the heterologous strains. VN titer was expressed as the reciprocal of the highest serum dilution that neutralized 100 TCID<sub>50</sub> of virus. A geometric mean titer (GMT) was calculated for each group at each time point. The samples negative at the last serum dilution (1:10) were designated as negative.

**ELISA procedure.** Five different types of commercially available IBDV ELISA kits obtained from three manufacturers were used. The kits produced by company A are being referred to as A 1 and A 2, those by company B as B 1 and B 2 and that from the third company as C. The sera were diluted, ELISAs conducted and titers calculated in accordance with the manufacturer's recommendations. For calculation of the GMT, all the negative samples were assigned a value half of the cutoff titer values provided by the manufacturer for each respective kit.

**Experimental design. Trial #1**: Forty, 3-week old SPF chickens were allotted into four groups of 10 birds each. Group 1 birds were orally inoculated each with  $10^5 \text{ TCID}_{50}$  of the STC virus, group 2 birds received each  $10^5 \text{ EID}_{50}$  of the OH virus, group 3 birds received both viruses and group 4 birds served as negative controls (Table 3.1). The chickens were bled at 0, 14 and 21 days PI. Sera were tested by four different commercial ELISA kits for the presence of antibodies against IBDV.

**Trial # 2:** Thirty-five, 2-week old SPF chickens were allotted into five groups of 7 birds each (Table 3.4). Either a high  $(10^{7}\text{TCID}_{50})$  or a low  $(10^{2}\text{TCID}_{50})$  dose of purified STC (passage (P)= 10) or the OH strain (P=12) was given to 7 chickens orally. Because of low antibody titers detected at 14 days PI, the birds were injected with 1 ml of  $10^{5}$  TCID<sub>50</sub> of the respective inactivated oil-emulsion vaccine subcutaneously at four weeks of age. The chickens were bled at 0,7,14 and 21 days PI with the inactivated vaccine.

**Trial #3:** Thirty-eight, 2-week old, SPF chickens were allotted into five groups of 10 birds in each of three groups and four birds in each of the remaining two groups (Table 3.8). Either a high  $(10^{5}\text{EID}_{50})$  or a low  $(10^{2}\text{EID}_{50})$  dose of embryo propagated STC or OH strain was orally inoculated to the chickens. The chickens were bled at 0,7,14 and 21 days PI with the virus.

Serum samples from trials 2 and 3 were tested by the VN test and five commercially available ELISA kits.

**Naturally infected chicken sera:** Sera from naturally exposed broiler chickens (n=20) were procured from Ohio department of agriculture (ODA). The vaccination history of

the flock was not available. Serum samples (n=30) from a layer farm were obtained at 71 weeks of age. The layers had been vaccinated with IBDV at 15, 25 and 35 days of age with Clonevac D-78 vaccine (Intervet, Inc. Millsboro, Delaware, USA). Serum samples were tested by five different commercial ELISA kits and by VN test. Serum samples from the layers and broilers were assayed individually by ELISA and VN and GMT were calculated from these values for each of the group. In addition, equal volumes of the individual sample were pooled within a group from each of the layers and boilers and assayed by ELISA and VN testing. The titers obtained from this treatment would be referred to as pooled sera titers (Table 3.12).

**Statistical analysis.** Optical density (OD) values from each group at a particular time point from each ELISA kit were compared to the negative control values by the Wilcoxon Rank Sum test. The p-values <0.05 were considered significantly different from each other. Mean OD and standard deviation were also calculated for each group. The Spearman's correlation coefficient between ELISA and VN titers was determined by using SAS 9.1 (Statistical analysis software, SAS Institute Inc. Carry, NC, USA).

## **3.4 RESULTS**

All tests were in accordance with the manufacturer's ranges provided for positive and negative control values. Antibody titers increased from 7 days till 21 days in all trials. The negative control group remained negative for the presence of antibodies by all kits and by VN at all time points tested. All samples tested by VN with the heterologous strains tested negative.

Trial 1.

**Response to STC virus.** All of the samples from the chickens inoculated with STC at 14 and 21 days PI tested positive by the kits A 2, B 1 and B 2 except for two serum samples that tested negative by A 1 at 14 and one sample that tested negative at 21 days PI (Table 3.1).

**Response to OH virus.** The results obtained from the chickens inoculated with the OH strain were variable. The A2 kit was the most sensitive detecting 4 out of 10 samples as positive at 14 days PI followed by the B 1, B 2 and A1 kits respectively. By 21 days PI, A2 detected 6 out of 10 samples as positive followed by B1, B2 and A1 detecting 4,3 and 0 samples out of 10 as positive.

**Response to STC and OH viruses.** All the chickens inoculated with both the STC and the OH strain of the virus tested positive by all kits at 14 and 21 days PI.

The GMT from different ELISA kits are shown in Table 3.2. Chickens inoculated with both strains of the virus had the highest antibody titer followed by the group inoculated with the STC virus. The group inoculated with the OH virus had the lower titers as compared to the other two groups. The OD values of sera from the chickens inoculated with the STC, the OH and both strains were significantly different than the negative control groups at all time intervals tested (Table 3.3) indicating that ELISA kits detected antibodies elicited by both serotypes of IBDV.

Trial 2.

**Response to STC virus.** In chickens inoculated with the high and low doses of the STC virus, the greatest variability amongst the ELISA kits for detecting positive samples was observed at 7 days PI. The A 2 kit was the most sensitive kit detecting all samples as positive at 7, 14 and 21 days PI followed by the C kit which detected all 7 samples as positive for the high dose and 6 samples as positive for the low dose at 7 days PI (Table 3.4).

The A1 and B 2 kits detected 5 and 6 out of 7 samples as positive from the high dose and 4 and 6 out of 7 samples positive at the low dose groups respectively. The B 1 kit was the least sensitive for the detection of antibodies against the STC strain. It detected 3 of 7 samples as positive in the high dose and 2 of 7 samples as positive from the low dose group. All samples tested positive by all kits at 14 and 21 days PI for antibodies to high and low dose STC virus. The antibody titers increased gradually for both the high and the low dose groups of the chickens (Table 3.5).

**Response to OH virus.** In the chickens inoculated with the high doses of OH virus, all the ELISA kits showed high numbers of positive samples. Whereas, birds inoculated with the low doses of the OH virus had a low numbers of positive samples with all the kits except for the A2 kit which detected high positive numbers.

The GMT of VN antibodies gradually increased in all groups with the homologous virus (Table 3.5). The OD values of sera from the chickens inoculated with the high and low doses of the STC and the OH were significantly different than the negative control groups

(Table. 3.6). At time intervals where no antibodies were elicited, the OD values were similar to the negative controls. The ELISA titers correlated with the VN titers at 21 days PI in this trial although a weak correlation was observed at 7 and 14 days PI (Table 3.7).

Trial 3.

**Response to STC virus.** All chickens inoculated with high dose of STC virus tested positive at all time intervals by all ELISA kits. The results were variable in the chickens inoculated with low dose of STC virus at 7 days PI but all samples tested positive by all ELISA kits at 14 and 21 days PI and also by VN test with the homologous strain (Table 3.8). Corresponding GMT of the virus-neutralizing antibody with the homologous strain gradually increased after inoculation (Table 3.9).

**Response to OH virus.** No antibodies were detected by any of the kits in the chickens inoculated with the high dose of the OH strain at 7 days PI and the kits detected samples as positive at 14 and 21 days PI with minor differences. Low VN titers were obtained in this group ranging from 20-40. No antibodies were detected by any of the kits in chickens inoculated with the low dose of the OH virus and no neutralizing antibodies were detected at any time interval with the homologous strain.

The OD values of sera from the chickens inoculated with the high and low doses of the STC and the OH were significantly different than the negative control groups at time intervals where an antibody response was generated (Table. 3.10). A good correlation existed between the VN titers and ELISA titers from all the kits in this trial with r-values ranging from 0.4-0.87 (Table 3.11).

**Naturally infected broiler and layer chickens.** The results from the chickens naturally exposed to the virus are shown in Table 3.12. All serum samples from the naturally infected broilers sera tested positive by all the ELISA kits with high titers ranging from 4528 to 17487. VN testing indicated the presence of antibodies against the OH strain of the virus in 13 out of 20 samples. The GMT of virus neutralizing antibodies against the STC strain was 1235 and against the OH strain was 276. There was no correlation between the VN and ELISA titers from any of the kits except A1 against the OH strain and B1 and B2 kit against the STC from the broiler sera (Table 3.13).

All serum samples from the layer flock tested positive for the presence of antibodies against IBDV by all the ELISA kits with high titers (Table 3.12). The VN titer of the OH strain antibodies is lower than the STC in the layers flock. There was no correlation between the VN and ELISA titers of the naturally exposed layers sera against the OH strain and very weak correlation against the STC in some kits (Table 3.13).

## **3.5 DISCUSSION**

In this study, all ELISA kits detected antibodies elicited by both serotypes of IBDV. The passage number of the viruses used in the first trial was not available. Both strains replicated well after oral inoculation in trial 1 and resulted in an antibody response that was detected by all kits at 14 and 21 days PI for the STC strain. Fewer chickens were positive when inoculated with the OH strain as compared to the STC but the antibody response, when present, was detected by all four kits. The OD of all groups inoculated with the virus in this study differed significantly from the negative control groups indicating that ELISA kits detected antibodies to both serotypes of the virus.

In trial 2, the chickens did not respond to the oral inoculum probably due to a high passage (P) number of both strains (OH: P 12; STC: P10) but the same inoculum later injected as an inactivated vaccine elicited a good antibody response. This is in agreement with our previous findings (13). There were some variations in antibody detection for serotype 1 STC strain at 7 days post-vaccination (PV) when the antibody titer was low as compared to the other time intervals. All chickens tested positive for antibodies against the STC strain at 14 and 21 days PV with the vaccine by all ELISA kits when the sera had medium or high antibody titers. The majority of the chickens responded to the high dose of the OH strain of the virus by 14 and 21 days PV but only few chickens responded to the low dose of OH by 21 days PV. The ELISA kits had the greatest variability for detection of the low and medium antibody titer sera against the serotype 2 OH strain but detected the antibodies elicited by the serotype 1 and 2 strains at all time intervals. This variation also reflected in the mean OD values of the groups being statistically similar similar to the negative controls where no antibodies were generated.

In trial 3, a low passage number of the STC virus (P=2) was used for inoculation of chickens resulting in a robust antibody response detected by all the kits. The OH virus from the previous trial (P=12) was propagated once in chicken embryos, titrated and inoculated in chickens. This inoculum when titrated in chicken embryos produced lesions but failed to produce any bursal lesions in the SPF chickens and failed to mount antibody response in chickens at the low dose. This is in agreement with previous findings (1). All the chickens in this trial responded to the high dose of STC strain at all time intervals and low dose at 14 and 21 days PI. The low titer sera had variations in the antibody detection by the ELISA kits. The sera increased in titer from 7 till 21 days PI as seen by all ELISA

and VN test. Chickens receiving the high dose of the OH virus responded by 14 and 21 days PI but the ones receiving the low dose failed to produce an antibody response at all time intervals.

Several ELISA kits detected a low antibody titer indicating good sensitivity of these kits. The number of samples positive by VN and ELISAs matched in most instances. Since serotype 2 viruses form an antigenically distinct group from the serotype 1 viruses, no cross-reaction between the STC and OH strains was observed by the VN.

All the pre-inoculation SPF sera and the sera obtained from the controls throughout the experiment were negative by all kits. The A 2 was the most sensitive kit since it detected the lowest amount of antibodies not detected by any other kit. The B 2 showed the lowest percentage serum samples as positive for antibodies against the OH strain.

All of the naturally infected chickens tested positive for the presence of antibodies by all ELISA kits and by VN against the STC strain. Seven of twenty serum samples from the broiler flock tested negative for the presence of antibodies against OH strain while all the sera from the layers tested positive for antibodies against the OH strain. It was previously reported that antibodies to serotype 1 and 2 were detected in 77 and 47 % of the chicken flocks respectively by VN tests (9). The GMT of each group calculated from the titer values obtained by assaying samples individually, and the titer of sera pooled in equal quantities and assayed subsequently from each of the experimentally and naturally infected chickens was within the acceptable values of one dilution factor.

Previous reports evaluating the different commercial ELISA kits for serotype 1 classical and variant antibodies found some correlation between the VN test and ELISA that varied with the ELISA kit used (2). Similar observations were made in this study. A certain correlation was observed between all the ELISA and the VN titers for the experimentally infected chickens at all time points in trial 2 and 3, although the correlation varied from kit to kit. The ELISA and the VN titers of the naturally infected broiler chickens did not correlate for the A1, A2 and C kit and correlated with the rest of the kits against the STC strain. Only A1 and B2 kits correlated with the OH strain VN titers. The ELISA and VN titers did not correlate with each other for the naturally exposed layers against both strains. The lack of correlation might be due to the presence of antibodies against the two serotypes. The VN titers do not correlate with the ELISA if the virus used for the *in vitro* assay is antigenically different than the one in the field. The layers could have been exposed to more viruses in their lifetime as compared to the broilers.

Hence, we conclude that the currently available ELISA kits detect antibodies elicited by the two serotypes and thus the total antibody detected by the commercial ELISA kits might also contain a fraction of antibodies elicited by serotype 2 viruses.

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**Fig 3.1.** Infectious bursal disease virus particles purified by sucrose and CsCl differential and isopycnic ultracentrifugation.

<b>X</b> 7 <b>*</b>	Deser				
virus	Days PI	A 1	A 2	B 1	B 2
STC <sup>A</sup>	14	8/10 <sup>B</sup>	10/10	10/10	10/10
	21	9/10	10/10	10/10	10/10
ОН	14	1/10	4/10	2/10	2/10
	21	0/10	6/10	4/10	3/10
STCLOU	14	10/10	10/10	10/10	10/10
510+01	14 21	10/10	10/10	10/10	10/10
Neg Control	14	0/10	0/10	0/10	0/10
	21	0/10	0/10	0/10	0/10

**Table 3.1.** Serum samples tested for antibodies against IBDV by commercial ELISA kits (trial. 1). <sup>A</sup> Three week old SPF chickens were orally inoculated with 1ml of either 10 <sup>5</sup> TCID <sub>50</sub> of the STC or 10 <sup>5</sup> EID <sub>50</sub> of the OH or the same dose of both viruses. <sup>B</sup> Positive/No.tested.

		ELISA kits									
Virus	Days PI	A1	A 2	B 1	B 2						
STC	14	1590 <sup>A</sup>	7769	2277	7427						
	21	2146	11458	4024	6530						
ОН	14	314	803	439	343						
	21	275	7356	386	375						
STC	14	2270	8270	2901	9763						
+ OH	21	1951	98948	4056	7876						
Neg Control		275 <sup>B</sup>	499	198	198						

**Table 3.2.** Geometric mean ELISA titers tested by four commercially available ELISA kits (trial .1). Three weeks old SPF chickens were orally inoculated with 1ml of 10<sup>5</sup> TCID <sub>50</sub> of the STC; 10<sup>5</sup> EID <sub>50</sub> of the OH or the same dose of both viruses. <sup>A</sup> ELISA GMT. <sup>B</sup> The value assigned to the negative control are half the value of the cutoff titer provided by the manufacturer.

		ELISA kits									
Virus	Days PI	A1	A 2	<b>B</b> 1	B 2						
STC	14	<sup>A</sup> 0.573 <sup>b</sup> <sup>B</sup> (0.17)	1.757 <sup>b</sup> (0.26)	0.479 <sup>b</sup> (0.17)	1.311 <sup>b</sup> (0.46)						
	21	0.632 <sup>b</sup> (0.18)	2.085 <sup>b</sup> (0.20)	0.699 <sup>b</sup> (0.111)	1.225 <sup>b</sup> (0.52)						
он	14	0.156 <sup>b</sup> (0.08)	0.469 <sup>b</sup> (0.22)	0.106 <sup>b</sup> (0.048)	0.150 <sup>b</sup> (0.146)						
on	21	0.260 <sup>b</sup> (0.275)	0.734 <sup>b</sup> (0.34)	0.128 <sup>b</sup> (0.05)	0.172 <sup>b</sup> (0.15)						
STC+	14	0.603 <sup>b</sup> (0.117)	1.832 <sup>b</sup> (0.25)	0.545 <sup>b</sup> (0.12)	1.614 <sup>b</sup> (0.43)						
OH	21	0.627 <sup>b</sup> (0.13)	1.988 <sup>b</sup> (0.28)	0.712 <sup>b</sup> (0.15)	1.343 <sup>b</sup> (0.38)						
Neg	14	0.072 <sup>a</sup> (0.009)	0.239 <sup>a</sup> (0.03)	0.068 <sup>a</sup> (0.01)	0.062 <sup>a</sup> (0.01)						
Control	21	0.084 <sup>a</sup> (0.01)	0.24 <sup>a</sup> (0.05)	0.082 <sup>a</sup> (0.009)	0.060 <sup>a</sup> (0.01)						

**Table 3.3.** Mean OD and standard deviations of the ELISA values (trial.1). <sup>A</sup> Mean OD values from a group. <sup>B</sup> Standard deviations. OD values from groups that were statistically different from negative controls (p < 0.05) at a particular time point by Wilcoxon rank sum test are denoted by different uppercase superscripted letters.

	Days		ELISA	kits		
Virus and dose	PV <sup>B</sup>	A 1	A 2	B 1	B 2	С
STC <sup>A</sup>	7	5/7 <sup>C</sup>	7/7	3/7	6/7	7/7
10 <sup>7</sup> TCID <sub>50</sub>	14	7/7	7/7	7/7	7/7	7/7
	21	7/7	7/7	7/7	7/7	7/7
STC	7	4/7	7/7	2/7	6/7	6/7
10 <sup>2</sup> TCID <sub>50</sub>	14	7/7	7/7	7/7	7/7	7/7
	21	7/7	7/7	7/7	7/7	7/7
OH	7	4/7	6/7	5/7	4/7	7/7
10 <sup>7</sup> TCID <sub>50</sub>	14	5/7	6/7	5/7	5/7	6/7
	21	5/7	6/7	6/7	5/7	7/7
	7	0/7	4/7	0/7	0/7	1/7
OH	14	0/7	6/7	0/7	0/7	1/7
10 <sup>2</sup> TCID <sub>50</sub>	21	1/7	6/7	1/7	2/7	7/7
Neg cont	7	0/10	0/10	0/10	0/10	0/10
	14	0/10	0/10	0/10	0/10	0/10
	21	0/10	0/10	0/10	0/10	0/10

**Table 3.4.** Serum samples tested for antibodies against IBDV by commercial ELISA kits (trial.2). <sup>A</sup> Two-week old SPF chickens were orally inoculated with 0.2 ml of the STC or the OH strains of IBDV. Two weeks PI each chicken was subcutaneously injected with 1 ml of the same dose of inactivated vaccine from respective strain. Sera collected 7,14 and 21 days PI was tested by five ELISA kits. <sup>B</sup> PV= post vaccination. <sup>C</sup> No. Positive/ No. Tested.

			EI		VN			
Virus and Dose	Days	A 1	A 2	B 1	B 2	С	STC strain	OH strain
STC 10 <sup>5</sup> TCID <sub>50</sub>	7 14 21	826 <sup>A</sup> 3295 3929	6481 13456 15597	554 5110 8568	655 9247 12201	21760 149859 277966	2826 <sup>B</sup> 3712 13227	< 10 < 10 < 10
STC 10 <sup>2</sup> TCID <sub>50</sub>	7 14 21	697 2386 4057	5481 13234 14482	252 3574 7683	1052 5390 10101	14719 72029 188594	287 1281 8901	< 10 < 10 < 10
OH 10 <sup>5</sup> TCID <sub>50</sub>	7 14 21	826 1356 1570	6510 6814 8005	738 1471 1393	1100 2433 2989	9357 20350 26832	197 500 1424	< 10 < 10 < 10
OH 10 <sup>°2</sup> TCID <sub>50</sub>	7 14 21	275 275 309	873 2114 2890	198 198 228	198 198 242	279 263 1358	5 61 655	< 10 < 10 < 10
Neg cont		275 <sup>°</sup>	499	198	198	200		

**Table 3.5.** Geometric mean titers of ELISA and VN (trial.2). Two-week old SPF chickens were orally inoculated with 0.2 ml of the STC or the OH strains of IBDV. Two weeks PI each chicken was subcutaneously injected with 1ml of inactivated vaccine having the same doses as before, from respective strain. <sup>A</sup> GMT from ELISA. <sup>B</sup> GMT from VN. <sup>C</sup> The values assigned to negative controls were half of the cutoff titer values provided by the manufacturer.

Virus	Virus ELISA Kits									
and dose	Days	A1	A2	B1	B2	С				
	7	0.210 <sup>b</sup> (0.080)	0.901 <sup>b</sup> (0.330)	0.125 <sup>b</sup> (0.066)	0.135 <sup>b</sup> (0.063)	0.150 <sup>b</sup> (0.007)				
STC 10 <sup>5</sup> TCID <sub>50</sub>	14	0.870 <sup>b</sup> (0.147)	1.939 <sup>b</sup> (0.263)	0.360 <sup>b</sup> (0.078)	1.225 <sup>b</sup> (0.364)	0.731 <sup>b</sup> (0.191)				
	21	1.102 <sup>b</sup> (0.120)	2.072 <sup>b</sup> (0.115)	0.686 <sup>b</sup> (0.077)	1.695 <sup>b</sup> (0.305)	0.921 <sup>b</sup> (0.212)				
STC	7	0.234 <sup>b</sup> (0.082)	0.729 <sup>b</sup> (0.200)	0.111 <sup>b</sup> (0.032)	0.211 <sup>b</sup> (0.099)	0.210 <sup>b</sup> (0.003)				
10 <sup>2</sup> TCID <sub>50</sub>	14	0.722 <sup>b</sup> (0.284)	1.987 <sup>b</sup> (0.180)	0.290 <sup>b</sup> (0.090)	0.936 <sup>b</sup> (0.560)	0.704 <sup>b</sup> (0.374)				
	21	0.938 <sup>b</sup> (0.305)	2.028 <sup>b</sup> (0.079)	0.555 <sup>b</sup> (0.181)	1.448 <sup>b</sup> (0.695)	0.968 <sup>b</sup> (0.393)				
	7	0.342 <sup>b</sup> (0.214)	1.392 <sup>b</sup> (0.694)	0.144 <sup>b</sup> (0.078)	0.361 <sup>b</sup> (0.326)	0.201 <sup>b</sup> (0.07)				
OH 10 <sup>5</sup> TCID <sub>50</sub>	14	0.510 <sup>b</sup> (0.298)	1.644 <sup>b</sup> (0.787)	0.154 <sup>b</sup> (0.075)	0.654 <sup>b</sup> (0.473)	0.430 <sup>b</sup> (0.251)				
	21	0.522 <sup>b</sup> (0.270)	1.538 <sup>b</sup> (0.626)	0.334 <sup>b</sup> (0.206)	1.158 <sup>b</sup> (0.792)	0.413 <sup>b</sup> (0.189)				
OII	7	0.097 <sup>a</sup> (0.028)	0.268 <sup>a</sup> (0.138)	0.072 <sup>a</sup> (0.043)	0.060 <sup>a</sup> (0.015)	0.145 <sup>a</sup> (0.007)				
10 <sup>2</sup> TCID <sub>50</sub>	14	0.102 <sup>a</sup> (0.013)	0.145 <sup>a</sup> (0.012)	0.052 <sup>a</sup> (0.000)	0.051 <sup>a</sup> (0.011)	0.145 <sup>a</sup> (0.012)				
	21	0.207 <sup>b</sup> (0.192)	0.932 <sup>b</sup> (0.601)	0.081 <sup>a</sup> (0.025)	0.107 <sup>a</sup> (0.048)	0.195 <sup>b</sup> (0.008)				
	7	0.089 <sup>a</sup> (0.009)	0.202 <sup>a</sup> (0.153)	0.055 <sup>a</sup> (0.005)	0.055 <sup>a</sup> (0.009)	0.141 <sup>a</sup> (0.007)				
Neg cont	14	0.091 <sup>a</sup> (0.011)	0.152 <sup>a</sup> (0.024)	0.048 <sup>a</sup> (0.002)	0.053 <sup>a</sup> (0.005)	0.146 <sup>a</sup> (0.009)				
	21	0.093 <sup>a</sup> (0.017)	0.184 <sup>a</sup> (0.125)	0.065 <sup>a</sup> (0.003)	0.078 <sup>a</sup> (0.017)	0.146 <sup>a</sup> (0.009)				

**Table 3.6.** Mean OD and standard deviations of the ELISA values (trial.2). Two-week old SPF chickens were orally inoculated with 0.2 ml of the STC or the OH strains of IBDV. Two weeks PI each chicken was subcutaneously injected with 1ml of inactivated vaccine having the same doses as before, from respective strains. <sup>A</sup> Mean OD values from the individual samples. <sup>B</sup> Standard deviations. OD values from a group that were statistically different from negative controls (p < 0.05) by Wilcoxon rank sum test at a particular time point are denoted by different uppercase superscripted letters.

Days PI	A 1	A 2	B 1	B 2	С
7	0.397 <sup>A</sup>	0.528	0.397	0.501	0.617
	0.036 <sup>B</sup>	0.003	0.036	0.006	0.0005
14	0.450	0.550	0.490	0.420	0.500
	0.010	0.002	<0.008	0.002	0.005
21	0.570	0.570	0.730	0.690	0.737
	<0.001	<0.001	<0.0001	<0.0001	<0.0001

**Table 3.7.** Correlation between VN and ELISA titers (trial 2). <sup>A</sup> = Spearman's coefficient of correlation value. <sup>B</sup> = p- value.

<b>T</b> 7 <b>•</b>			E	ELISA kits		
Virus and dose	Days PI	A 1	A 2	B 1	B 2	С
STC <sup>A</sup>	7	10/10 <sup>B</sup>	10/10	10/10	10/10	10/10
10 <sup>5</sup> EID <sub>50</sub>	14	10/10	10/10	10/10	10/10	10/10
	21	10/10	10/10	10/10	10/10	10/10
STC	7	1/10	1/10	2/10	2/10	5/10
10 <sup>2</sup> EID <sub>50</sub>	14	10/10	10/10	10/10	10/10	10/10
	21	10/10	10/10	10/10	10/10	10/10
OH	7	0/4	0/4	0/4	0/4	0/4
10 <sup>5</sup> EID <sub>50</sub>	14	2/4	3/4	2/4	3/4	3/4
	21	2/4	2/4	2/4	2/4	2/4
	7	0/4	0/4	0/4	0/4	0/4
OH	14	0/4	0/4	0/4	1/4	0/4
10 <sup>2</sup> EID <sub>50</sub>	21	0/4	0/4	0/4	0/4	0/4
Neg cont	7	0/10	0/10	0/10	0/10	0/10
-	14	0/10	0/10	0/10	0/10	0/10
	21	0/10	0/10	0/10	0/10	0/10

**Table 3.8.** Serum samples tested for antibodies against IBDV by commercial ELISA kits (trial.3). <sup>A</sup> Two-week old SPF chickens were orally inoculated with 0.2 ml of the STC or the OH strains of IBDV and sera tested 7, 14 and 21 days PI.<sup>B</sup> No. Positive/ No. Tested.

			ELISA kits				VN	
Virus and dose	Days PI	A 1	A 2	B 1	B 2	С	STC strain	OH strain
STC	7	1066 <sup>A</sup>	4949	1571	7520	5084	651 <sup>в</sup>	< 10
10 <sup>5</sup> EID <sub>50</sub>	14	2911	11518	3275	13607	16195	808	< 10
	21	4764	13686	5463	17585	26794	3405	< 10
STC	7	307	597	260	320	445	10	< 10
$10^{2}$ EID 50	14	2415	10902	2732	12952	9041	736	< 10
	21	5339	13625	5598	16283	24925	2077	< 10
ОН	7	275	275	198	198	200	20	< 10
10 <sup>5</sup> EID <sub>50</sub>	14	524	1012	428	2016	1173	35	< 10
	21	412	697	340	1014	850	40	< 10
ОН	7	275	499	198	198	200	< 10	< 10
	14	275	499	198	241	200	< 10	< 10
10 <sup>2</sup> EID 50	21	275	499	198	198	200	< 10	< 10
Neg cont		275 <sup>C</sup>	499	198	198	200	<10	< 10

**Table 3.9.** Geometric mean ELISA and VN titers from serum samples tested for antibodies against IBDV (trial. 3). <sup>A</sup> Two weeks old SPF chickens were orally inoculated with 0.2 ml of STC or OH strains of IBDV. <sup>A</sup> GMT from ELISA. <sup>B</sup> GMT from VN. <sup>C</sup> The values assigned to the negative controls are half of the cutoff titer values provided by the manufacturer.

			ELISA kits			
Virus and dose	Days PI	A 1	A 2	R 1	R 2	C
	7	A 0 411 b	0.764 <sup>b</sup>	0 111 <sup>b</sup>	0.829 <sup>b</sup>	0.859 <sup>b</sup>
	,	<sup>B</sup> (0.058)	(0.111)	(0.026)	(0.340)	(0.145)
STC		(0.02.0)	(0111)	(0:020)	(010 10)	(01110)
10 <sup>5</sup> EID <sub>50</sub>	14	$0.757^{b}$	1.463 <sup>b</sup>	0.193 <sup>b</sup>	1.091 <sup>b</sup>	1.423 <sup>b</sup>
		(0.151)	(0.161)	(0.041)	(0.329)	(0.164)
	21	1.048 <sup>b</sup>	2.143 <sup>b</sup>	0.530 b	1.495 <sup>b</sup>	1.270 <sup>b</sup>
		(0.212)	(0.135)	(0.119)	(0.366)	(0.213)
	7	0.144 <sup>b</sup>	0.153 <sup>b</sup>	0.055 b	0.106 °	0.290 °
		(0.086)	(0.133)	(0.020)	(0.129)	(0.184)
STC	1.4	o ccoh	1.40.4 h	o 1 coh	1 101 h	1.000 h
10 <sup>-</sup> EID <sub>50</sub>	14	0.668	1.434	$0.168^{\circ}$	$1.101^{\circ}$	1.329
		(0.121)	(0.332)	(0.033)	(0.391)	(0.235)
	21	1 117 <sup>b</sup>	2 133 <sup>b</sup>	0 549 <sup>b</sup>	1 /192 <sup>b</sup>	1 201 <sup>b</sup>
	21	(0.110)	(0.077)	(0.150)	(0.561)	(0.195)
	7	$0.082^{a}$	$0.098^{a}$	$0.050^{a}$	$0.055^{a}$	$0.296^{a}$
	,	(0.012)	(0.015)	(0.010)	(0.0061)	(0.005)
		(0.012)	(0.010)	(01010)	(0.0001)	(01002)
ОН	14	0.266 <sup>a</sup>	0.238 <sup>b</sup>	0.053 <sup>b</sup>	$0.049^{a}$	0.424 <sup>b</sup>
10 <sup>5</sup> EID <sub>50</sub>		(0.188)	(0.107)	(0.005)	(0.003)	(0.205)
		,				
	21	0.230 <sup>b</sup>	0.216 °	0.106 °	0.036 °	0.428 <sup>b</sup>
		(0.140)	(0.102)	(0.047)	(0.001)	(0.090)
	-	0.107	0 111 h	0.0503	0.00058	0.005
	/	$0.127^{2}$	$0.111^{\circ}$	0.058	0.0685	0.296
<u>о</u> ц		(0.039)	(0.016)	(0.008)	(0.028)	(0.008)
Un	14	0 080 <sup>a</sup>	0.136 <sup>b</sup>	0 102 <sup>b</sup>	0 309 <sup>b</sup>	0 314 <sup>b</sup>
$10^{2}$ EID 70	14	(0.012)	(0.016)	(0.044)	(0.286)	(0.005)
10 110 30		(0.012)	(0.010)	(0.011)	(0.200)	(0.005)
	21	$0.0877^{a}$	0.149 <sup>b</sup>	$0.071^{b}$	0.045 <sup>a</sup>	0.326 <sup>b</sup>
		(0.015)	(0.034)	(0.013)	(0.010)	(0.005)
	7	0.065 <sup>a</sup>	0.090 <sup>a</sup>	0.041 <sup>a</sup>	0.051 <sup>a</sup>	$0.175^{a}$
		(0.008)	(0.006)	(0.002)	(0.006)	(0.002)
<b>N</b> T :		0.455 %	0.001	0.0.1.3	0.0703	0.4== 9
Neg cont	14	0.122 ª	0.091 "	0.041 "	0.050 *	0.177 <sup>a</sup>
		(0.165)	(0.010)	(0.001)	(0.001)	(0.010)
	21	0 071 <sup>a</sup>	0.000.8	0.042 <sup>a</sup>	0.051 <sup>a</sup>	0 312 <sup>a</sup>
	21	(0.071)	(0.090)	(0.042)	(0.001)	(0.013)
		(0.015)	(0.051)	(0.002)	(0.000)	(0.001)

**Table 3.10.** Mean OD and standard deviations of the ELISA values (trial.3). Two weeks old SPF chickens were orally inoculated with 0.2 ml of STC or OH strains of IBDV (trial. 3). <sup>A</sup> Mean OD values from the individual samples. <sup>B</sup> Standard deviations. OD values from a group statistically different from negative controls (p < 0.05) by Wilcoxon rank sum test are denoted by different superscripted uppercase letters.

Days PI	A 1	A 2	B 1	B 2	С
7	0.848 <sup>A</sup>	0.862	0.870	0.840	0.800
	<0.001 <sup>B</sup>	<0.001	<0.001	<0.001	<0.001
14	0.749	0.803	0.705	0.455	0.560
	<0.001	<0.001	<0.001	0.0003	0.001
21	0.600	0.590	0.658	0.644	0.550
	0.007	0.0009	0.0001	0.0001	0.002

**Table 3.11.** Correlation between VN and ELISA titers (trial 3). <sup>A</sup>= Spearman's coefficient of correlation value. <sup>B</sup>= p- value.

		ELISA Kits used						
							VN t	iters
Chickens		A 1	A 2	<b>B1</b>	B2	С	STC	OH
Broilers n=20	Pos/total	20/20	20/20	20/20	20/20	20/20	20/20	13/20
	s GMT <sup>A</sup>	7688	17487	4527	7797	6148	1235	276
	Pooled <sup>B</sup> sera	5919	16032	6700	7543	3701	5120	160
Layers n=30	Pos/total	30/30	30/30	30/30	30/30	30/30	30/30	30/30
	GMT <sup>A</sup>	5548	13650	6471	10323	9043	5574	445
	Pooled <sup>®</sup> sera	5993	15543	3800	11431	8929	20480	1280

**Table 3.12.** ELISA and VN titers of naturally infected chickens. <sup>A</sup> Individual serum samples were assayed by ELISA and VN. The GMT were calculated using the individual titer values. <sup>B</sup> Individual serum samples were pooled together in equal volume and tested by ELISA and VN.

Chickens	Virus	A 1	A 2	B 1	B 2	С
-	STC	0.210 <sup>A</sup>	0.300	0.680	0.570	0.440
		0.350 <sup>B</sup>	0.190	0.0009	0.0085	0.046
Broilers						
	OH	0.640	0.060	0.390	0.490	0.388
		0.002	0.771	0.080	0.026	0.090
	STC	0.390	0.460	0.350	-0.011	0.316
		0.0291	0.0099	0.052	0.950	0.087
Layers						
	ОН	0.125	0.327	0.216	0.056	-0.069
		0.508	0.0777	0.250	0.767	0.715

**Table 3.13.** Correlation between ELISA and VN antibody titers for the naturally infected broilers and layers sera. <sup>A</sup> = Spearman's coefficient of correlation. <sup>B</sup> = p - value.

# **CHAPTER 4**

# DEVELOPMENT OF A DIFFERENTIAL RT-PCR ASSAY FOR INFECTIOUS BURSAL DISEASE VIRUS STRAINS.

## 4.1 SUMMARY

Several antigenic and pathogenic subtypes of Infectious bursal disease virus (IBDV) exist worldwide including classic, variant, very virulent (vv), serotype 1 and serotype 2 viruses. Simple and quick diagnostic assays are vital for implementing control and prevention strategies for Infectious bursal disease. Currently available assays like chain reverse-transcriptase polymerase reaction/restriction fragment length polymorphism analysis (RT-PCR/RFLP) or real time detection are expensive and not feasible for a large number of samples. The reliable indicator for vvIBDV remains the in vivo pathogenicity testing due to the lack of a virulence marker for vvIBDV. Simple RT-PCR assays were developed for differentiating various strains of IBDV. Primer sets were designed for this study. Primer set one targeted a portion of segment A and was specifically designed to amplify serotype 2 strains of the IBDV while primer set 2 targeted the segment B and amplified only the vvIBDVs. A total of 26 previously characterized virus strains including 11 classics, 5 variants, 5 vv and 5 serotype 2 strains were used to validate differential RT-PCR assays. The results indicated that primer set 1 amplified a 415 bp RT-PCR product for the serotype 2 viruses and primer set 2 162

specifically amplified a 715 RT-PCR product for vvIBDV except for two vv Taiwan strains. Subsequent sequencing of the vv Taiwan strains revealed their high nucleotide similarity with the classic viruses. To further confirm the specificity of primer set 2 for the vvIBDVs, twenty field samples suspected to be vvIBDV from different geographic locations were tested. All but one of the Korean strain (91108) of the suspected vvIBDV tested positive by this primer set.

#### **4.2 INTRODUCTION**

Infectious bursal disease (IBD) is an acute and contagious disease affecting young chickens from 3-6 weeks of age. The etiologic agent is Infectious bursal disease virus (IBDV) a bi-segmented, double stranded RNA virus belonging to the Birnavirideae family. The disease affects young chickens and manifests itself in terms of morbidity and mortality. Two serotypes of the virus are recognized. Serotype 1 viruses are pathogenic to chickens while serotype 2 viruses are non-pathogenic to chickens and are common in the field. Antigenic and pathogenic forms of serotype 1 viruses include classic, variant and the very virulent (vv) are recognized. The vvIBDV strains were first reported from Europe in 1980 and are marked by increased virulence and an ability to break through the protective barrier provided by vaccination with the classic strains (16). The vv strains spread to different continents very rapidly and have been described from South America (1). The US, Canada and New Zealand are still free from these strains but there is a concern that these strains could spread in these countries too.

The reverse-transcriptase polymerase chain reaction/restriction fragment length polymorphism analysis (RT-PCR/RFLP) analysis has been used to categorize IBDVs
including the vv strains to different molecular groups and for the differentiation of classics and vvIBDV(10,15,18). A real time PCR assay for differentiation of majority of the vvIBDVs from the classic viruses has been described (7). These methods utilize expensive enzymes and equipment, are time consuming and not feasible for assaying large numbers of samples.

A simple RT-PCR assay to differentiate the classic viruses from the vv viruses would be highly desirable for monitoring the spread of vvIBDV. Therefore, the objective of the present study was to develop differential RT-PCR assays to differentiate serotype 1, serotype 2, and the vv strains of IBDV.

### **4.3 MATERIALS AND METHODS**

**Viruses**. All the viruses used to standardize the RT-PCR assay in this study have been maintained in our laboratory. These viruses were either propagated in baby grivet monkey cells (BGM-70), chicken embryos or harvested from bursas. A list of these viruses and their sources is included in Table 4.1. These viruses represent known classic, variant, very virulent (vv) and serotype 2 IBDV strains and were previously characterized in our laboratory (Table 4.1). The viral RNA from the field strains suspected to be vvIBDV were kindly provided by Dr. D. Jackwood. These strains were molecularly characterized recently (7).

**Primer design.** All the primers used in this study were designed manually. The sequences used for designing these primers were downloaded from GenBank. The sequence from segment A of the OH strain was used to design the primers specific for the serotype 2 viruses. The sequences from segment B were used for designing the primer

specific for the vv viruses Table 4.2. These sequences were aligned and visualized by using Molecular Evolutionary Genetics Analysis Software (MEGA 3) program (Fig 4.1) (13). The primer sequences, polarity, nucleotide positions they encompass and the expected product sizes are presented in Table 4.3. The primer sites were chosen so that the 3' position consisted of the nucleotide(s) mismatched to other subtypes of IBDV. The primers were designed by keeping the G C content as close to 50% as possible and without any complementarity with each other to avoid primer dimers. Two conserved primers were also designed from the segment B sequences to amplify the vv strains not amplified by the specific primer (set 2). The primers were synthesized by IDT (Integrated DNA Technologies, Inc. Coralville, IA, USA).

**RNA extraction.** RNA from all the samples was extracted by the Trizol reagent (TRIZOL LS reagent, Life Technologies, Grand Island, NY) according to the manufacturer's instructions.

**RT-PCR.** The RT-PCR amplification was carried out with Gene Amp PCR System 9700 (Perkin Elmer, Foster City, CA) in a 50µl volume. Reagents were used in the final concentration of 1X PCR buffer, 2.5 mM of MgCl<sub>2</sub>, 20 units of Rnasin ribonuclease inhibitor, 5 units of AMV and 2.5 units of Taq polymerase (Promega, Madison, WI); and 0.2mM of dNTPs (Invitrogen, Carslbad, CA).

The amplification cycle for serotype 2 strains included the RT step at 45 C for 60 min, followed by 5 min at 94 C and PCR steps consisting of 35 cycles of 94 C for 30 sec, 57 C for 30 sec, 72 C for 30 sec. A final elongation step of 72 C for 7 min was included.

A similar amplification cycle was used for the vvIBDV except for the lower annealing temperature of 48C. The RT-PCR products were separated on 1% agarose gel and visualized using ethidium bromide stain.

The RNA extracted from all the classic, variant, vv and serotype 2 IBDVs were tested with both primer sets. Twenty field samples suspected to be vvIBDV were tested with primer set 2 to confirm their identity as the vvIBDV.

Cloning of the RT-PCR product into the pCR-XL-TOPO vector. The Taiwan strains (PT and IL) were amplified using the conserved primer set and amplicons of the appropriate size were excised from the gel and extracted with the QIAquick <sup>TM</sup> gel extraction kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. The purified PCR product was cloned into pCR-XL-TOPO<sup>®</sup> vector by using the TOPO<sup>®</sup> XL PCR cloning kit (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. Ten transformed colonies were subcultured in Luria-Bertani (LB) broth medium containing 50 µg/ml of Kanamycin (Invitrogen, Co., Carlsbad, CA). The plasmid DNA was extracted by using the QIA prep<sup>®</sup> Spin Miniprep kit (QIAGEN Inc., Valencia, CA). The plasmid DNA was digested with EcoR1 enzyme (Promega, Madison, WI) to confirm the presence of the insert according to the manufacturer's instructions. The DNA concentration was measured by spectrophotometer at 260 nm. The DNA was sequenced using M13 forward and reverse primers by an ABI 3710 automated DNA sequencer (Applied Biosystems, Foster City, CA) using the MCIC facilities (Molecular and Cellular Imaging Center, OARDC, The Ohio State University, Wooster, Ohio).

**Data analysis**. The sequence data were downloaded by Chromas 2.4 (Technelysium Pty Ltd., Queensland, Australia). The sequences obtained were edited and the contigs was

assembled using the Cap assembler software available at the web (<u>http://pbil.univ-lyon1.fr/cap3.php</u>). The contigs were added to the MEGA 3 for analysis.

## **4.4 RESULTS**

**Primer specific to the serotype 2 strains.** The primer designed for the serotype 2 strains amplified specifically the serotype 2 strains only yielding an RT-PCR product of 415 base pairs (bp) in size (Fig 4.2). None of the serotype 1 viruses were amplified with the primer set 1 (Table 4.4). One of the serotype 2 strains, SW, had a weak band with this primer set.

**Primer specific to the vvIBDV.** The primer specific to the vvIBDV amplified all the vvIBDV except for the Taiwan strains (PT and IL) yielding an RT-PCR product of 715 bp in size (Fig 4.3. and 4.4). None of the classics, variants or the serotype 2 strains tested positive with this primer pair (Table 4.4).

A conserved primer set 3 designed from segment B sequences was used to amplify the Taiwan strains yielding an RT-PCR product of 749 bp. The RT-PCR products were cloned, sequenced and analyzed in the area encompassing the primer area. The nucleotide sequences of the Taiwan strains were similar to the classic viruses and therefore primer set 2 was unable to identify this strain as vvIBDV.

**Suspect Field samples of vvIBDV.** All field samples from the suspected vvIBDV tested positive with the vvIBDV specific primer set except one of the Korean strains (91108) of vvIBDV (Table 4.5).

#### 4.5 DISCUSSION

The primer set designed for the serotype 2 strains identified all 5 strains including OH, MO, GK-15, KM and SW of serotype 2 IBDV. None of the serotype 1 viruses including classics, variants or vvIBDV were amplified by this primer set. The SW strain yielded a faint band which could be due to a point mutation in the area encompassing this primer set. The primer set designed in this study is an improvement over a previously designed multiplex RT-PCR for differentiating the serotype 1 and serotype 2 strains that targeted the VP4 sequences of IBDV(11).

The genome segment A of the vvIBDV has been extensively sequenced in an attempt to identify the genetic basis for virulence of these strains. To date, no genetic basis for virulence of the vvIBDV has been described. The vv strains are antigenically similar to the classic strains. Our attempts to design a specific primer for the vvIBDV from the segment A did not succeed since the nucleotide sequences of segment A did not fall in the distinct pattern unique for all the vvIBDV.

Recently, the VP1 sequences of the IBDV have become available for many classic, variant and the vvIBDVs. Analysis of the published VP1 sequences revealed that the nucleotide sequences of the vvIBDVs form a distinct pattern and group separately from all the classics, variants and the serotype 2 strains of the IBDV(3,17). The nucleotides unique for the vvIBDV are dispersed over the entire segment B of the virus. The primers were designed to make use of the nucleotide consistency of the vvIBDV at these positions so that they could identify only the vvIBDVs.

Primer set 2 did not detect the Taiwan strains (PT and IL) and one of the suspected vvIBDV (Korean strain (91108)). Sequence data from the Taiwan strains (PT,IL)

revealed the nucleotide sequences similarity of this strain with the classic strains. The two strains not amplified by primer set 2 belonged to a geographically close area. The molecular studies with the vvIBDVs have indicated that the European and Asian vvIBDV have evolved from a common ancestor and evolved independently of each other (16). The comparison of the VP1 sequences also points to the same conclusion. All but one of the field isolates suspected to be vvIBDV tested positive with primer set 2. These isolates were identified recently as vvIBDV by probes designed from the segment A sequences by the real time assay (7). As more sequence data becomes available for the VP1 of the Asian strains it might be possible to develop a multiplex RT-PCR assay specific for the vvIBDVs.

Attempts were also made to design primers from other sites of segment B genome that were unique for the vvIBDV. However, none of the primer sets from these sites were specific for the vvIBDV when tested with the 26 isolates maintained in our laboratory and amplified different classic and variant strains as well. The Taiwan strains also showed a different RFLP profile than the Turkish (OA,OE) and Holland (Hol)strains of vvIBDV when cut with the *Mbo*1 enzyme (15). The nucleotide sequencing or the molecular methods to identify IBDV show the similarities or differences among the classics and the vvIBDV but actual pathogenicity studies *in vivo* are required for confirming the vv phenotype.

The annealing temperatures described in this assay are crucial since the primers mismatches the other strains at the 3' position and lowering the annealing temperatures too much would increase the chances of nonspecific binding with the other strains. The rapid and quick diagnostic methods described in this study could be used to identify European, Middle Eastern and Latin American vvIBDV from the classics, variants and the serotype 2 IBDVs. The RT-PCR method described here is fast (1 day) and could differentiate the field isolates with high sensitivity and specificity. Rapid screening for the vvIBDV is especially important for control or prevention of IBDV.

## **4.6 ACKNOWLEDGMENTS**

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**Fig 4.1.** Schematic representation of partial ClustalW alignment from segment B of 12 IBDVs including 6 classics, 1 variant and 5 vvIBDVs. The highlighted region is the primer sites for the upstream and downstream primers (primer set 2). The dotted region represents the flank area. The primers were designed to have unique nucleotide for the vvIBDV at the 3' end.



**Fig. 4.2.** RT-PCR results of the representative samples with primer set 1. M=Marker; 1= STC; 2= IN; 3= Del-E; 4= Hol; 5= OA; 6= PT; 7= OH; 8= MO; 9= GK 15; 10= KM; 11= SW.

## $M \ 1 \ 2 \ 3 \ 4 \ 5 \ 6 \ 7 \ 8 \ 9 \ 10 \ 11 \ 12 \ 13 \ 14 \ 15$



**Fig 4.3.** RT-PCR products from primer set 2. M=Marker; 1=STC; 2= SAL; 3= UV; 4=D-78; 5=BVM; 6=NC; 7=LVN; 8=BLEN,9= BATTS;10= MNA; 11=BB; 12= IN; 13=MD;14= Del-E; 15=GLS.

## M 16 17 18 19 20 21



**Fig.4.4.** RT-PCR products from primer set 2. 16= Ev; 17= HOL; 18=OA; 19 = OE; 20= PT; 21= IL.

	Strain name	Subtype	Origin	Reference
	STC	Classic	US	(8)
	SAL	Classic	US	(6)
Serotype 1	UV (Univax-IBD)	Classic	US	(6)
	D-78	Classic	US	(6)
	BVM (Bursa-Vac-M)	Classic	US	(6)
	NC	Classic	US	(9)
	LVN	Classic	US	(9)
	BLN	Classic	US	(2)
	BATTS	Classic	US	*
	MNA	Unknown	US	*
	BB (Bio-Burs)	Classic	US	(6)
	IN	Variant	US	(5)
	MD	Variant	US	(8,14)
	DEL-E	Variant	US	(12)
	GLS	Variant	US	(2)
	Ev	Variant	US	(12)
	HOL	Very virulent	Holland	(15)
	OA/G1	Very virulent	Turkey	(15)
	OE/G2	Very virulent	Turkey	(15)
	РТ	Very virulent	Taiwan	(15)
	IL	Very virulent	Taiwan	(15)
	OH	Serotype 2	US	(4)
Serotype 2	MO	Serotype 2	US	(4)
	GK15	Serotype 2	US	(4)
	KM	Serotype 2	US	(4)
	SW	Serotype 2	US	(4)

**Table 4.1.** IBDV strains used in the study.\* Isolated in our laboratory. Unpublished data.

Strain designation	Subtype	Segment A Accession numbers	Segment B Accession numbers	Geographic location
Winterfield 2512	Classic		AF 083092	US
D-78	Classic		AF499930	US
Edgar	Classic		AY459320	US
IM	Classic		AY029165	US
P2	Classic		X 84035	Germany
Autralian	Classic		M19336	Australia
GLS	Variant		AY368654	US
OKYM	vvIBDV		D49707	Japan
UK661	vvIBDV		X92761	UK
IL3	vvIBDV		AF083093	Israel
HK46	vvIBDV		AF092944	Hong Kong
D6948	vvIBDV		AF240687	The Notherlands
STC	Classic	D00499		LIGA
ОН	Serotype 2	U30818		USA USA

**Table 4.2.** Sequence data of VP 1 protein from GenBank used for designing primers specific to IBDV.

PRIMER	TARGET AREA	POLARITY	SEQUENCE $(5' \rightarrow 3')$	Expected Product size	LOCATION
Primer set 1	Segment A (serotype 2	Upstream	GCCTGAGTGAGTTGACTGACTAC	415	526-548
	specific)	Downstream	AGAGGTCACAGTCAAAGCTGTTG		941-963
Primer set 2	Segment B (Very virulent	Upstream	CCGAGGCCACAGATAACCTTAAA	715	538-560
	specific)	Downstream	CCTCTAAACGGGTTGAAC		1235-1252
Primer set 3	Segment B (conserved)	Upstream	CTACGGGAGTGGGACCTACA	749	602-621
		Downstream	ACCACGTGTTGGAGTGAACA		1331-1350

**Table 4.3.** Primer sets designed to amplify serotype 2 and serotype 1 strains of IBDV.

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		Strain	Subtype	Primer set 1	Primer set 2
	1	STC	Classic	-	-
	2	SAL	Classic	-	-
	3	UV	Classic	-	-
	4	D-78	Classic	-	-
	5	BVM	Classic	-	-
Serotype 1	6	NC	Classic	-	-
	7	LVN	Classic	-	-
	8	BLEN	Classic	-	-
	9	BATTS	Classic	-	-
	10	MNA	Classic	-	-
	11	BB	Classic	-	-
	12	IN	Variant	-	-
	13	MD	Variant	-	-
	14	DEL-E	Variant	-	-
	15	GLS	Variant	-	-
	16	Ev	Variant	-	-
	17	HOL	Very virulent	-	+++
	18	OA/G1	Very virulent	-	+++
	19	OE/G2	Very virulent	-	+++
	20	РТ	Very virulent	-	-
	21	IL	Very virulent	-	-
Serotype 2	22	OH	Serotype 2	+++	-
	23	MO	Serotype 2	+++	-
	24	GK15	Serotype 2	+++	-
	25	KM	Serotype 2	+++	-
	26	SW	Serotype 2	+	-

**Table. 4.4.** RT-PCR results with the primer sets specific for the serotype 2 and the very virulent strains of IBDV. <sup>A</sup> - = Negative; +++ = Positive sample; + = Weak positive sample.

Field samples	Origin	Sample	RT-PCR
		designation	results
	Dominican	DR4	A +++
	Republic		
		AK 2	+++
	France	AL 10	+++
		FD 7	+++
		Isr 4	+++
		Isr 6	+++
Suspect		Isr 7	+++
vvIBDV	Israel	Isr 10	+++
		Isr 23	+++
		Isr 24	+++
		Isr 28	+++
		Isr 29	+++
	Jordan	YMSE	+++
		9596	+++
	Korea	9664	+++
		91108	-
	Singapore	179	+++
		182	+++
	South Africa	SA 2	+++
	Spain	Spain 1	+++

**Table 4.5.** Field samples suspected to be vvIBDV tested with primer set 2 specific for the vvIBDV.  $^{A}$  - = Negative; +++ = Positive sample.

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