PATHOLOGICAL INVESTIGATION AND MOLECULAR IDENTIFICATION OF INFECTIOUS BURSAL DISEASE IN BROILER CHICKENS

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Roll No.: 0121/06

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Khulshi, Chattogram 4225, Bangladesh

June, 2023

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Umme Aysha Habiba
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ABSTRACT

Infectious Bursal Disease (IBD) represents a highly contagious viral illness affecting young chickens, leading to immunosuppression, increased mortality rates, and stunted growth. The widespread impact of this disease has significantly undermined the poultry industry. This cross-sectional study aimed to explore the gross and histopathological alterations within various organs in suspected cases of IBD, subsequently confirmed through reverse transcription (RT-PCR) for molecular validation. This study was conducted at the Pathology Laboratory of Chattogram Veterinary and Animal Sciences University from December 2021 to February 2023. Out of 200 suspected cases of IBD identified based on gross lesions, 30 cases (comprising bursa, kidney, and spleen samples from each case) were randomly selected for histopathological investigation, while 8 samples among them were chosen specifically for molecular diagnosis. The clinical signs observed in IBD infected chickens were whitish diarrhea mixed with blood, ruffled feather, and massive death within short period. Necropsy findings revealed petechial hemorrhage in the bursa of fabricius, kidneys, thymus, spleen, and thigh muscle. Over the disease course, the bursa of fabricius displayed edematous changes in its serosal and mucosal regions, transitioning to a whitish creamy appearance and subsequent atrophy. The kidneys exhibited paleness, edema, and hemorrhagic manifestations. Depletion of follicular lymphocytes as well as edema, formation of cortical rim, interfollicular hemorrhage were the major histopathological changes found in bursa of fabricius and spleen. Kidney sections revealed hemorrhage, congestion, infiltration of inflammatory cells between renal tubules, edema in renal tubules, partially detached lining epithelium from basement membrane of many tubules. Out of thirty clinically diagnosed samples, eight samples were selected and all detected positive by RT-PCR for the hyper variable region of *VP2* gene. The consistent correlation among these diagnostic modalities strengthens the confidence in utilizing gross lesion assessments, supported by histopathological and molecular analyses, as effective and reliable means for identifying Infectious Bursal Disease Virus (IBDV) infected chicken.

Keywords: Infectious Bursal Disease (IBD), Necropsy findings, Histopathological examinations, RT-PCR.

CHAPTER 1: INTRODUCTION

Poultry farming stands as a critical cornerstone in addressing the protein deficit derived from animal sources. It is estimated that today 1 million entrepreneurs and 8 million people commercially produce 10.22 billion eggs and 1.46 million tons of poultry meat annually. Latest figures show 16 grandparent farms and 206 breeder farms, with up to 70,000 commercial farms. These are mainly small-scale, typically having 500- 2,500 birds. Commercial poultry farms are growing at a rate of 15% a year, with investment in the sector expected to double in the next decade. The poultry sector is gearing up for exporting by 2024. Backyard poultry still accounts for 65-70%, of the country's poultry population (DLS, 2022). This growth underscores the pivotal role of poultry farming, both in the agricultural economy and in catering to the dietary protein needs of the population. The major constraints in poultry farming are the outbreak of several devastating diseases causing economic loss and discouraging poultry rearing (Das et al., 2015). Among the various diseases, it is a major poultry pathogen in the poultry industry (Hein et al., 2002). The first outbreak of IBD occurred at the end of 1992 and caused up to 80% mortality in the field outbreaks (Chowdhury et al., 1997). Currently, this disease is reported throughout the globe and is an economical important disease causing 100% morbidity and mortality reaching up to 90% in susceptible flock. IBD is a highly contagious acute viral disease of young chickens of 3-6 weeks old that causes a fatality or immunosuppression by damaging bursa of fabricius and impaired growth of young chickens which results significant economic losses in the poultry industry (Islam et al., 2005).

The causal agent of IBD is infectious bursal disease virus (IBDV), a non-enveloped double stranded RNA (dsRNA) virus belonging to the family Birnaviridae (Jackwood et al., 1984). The disease is manifested by dehydration, development of depression with watery diarrhea, swollen and blood-stained vent (Islam and Samad, 2004). Severity of the signs depends on the virus strain and the age and breed of the chickens (Van den Berg et al., 1991). Infection with less virulent strains may not show obvious clinical signs but the birds may have fibrotic or cystic bursa of fabricius that become atrophied prematurely and may die of infections by agents that would not usually cause disease in immune competent birds. The frequent postmortem findings are

hemorrhage in the thigh/pectoral muscles, enlarged, edematous and hyperemic bursa or atrophic in chronic cases and hemorrhage in the junction between gizzard and proventriculus (Chettele et al., 1989). Though the gross lesions of IBD affected poultry are considered sufficient for diagnosis but is sometimes confused with other diseases (Banda, 2002). Molecular techniques like conventional reverse transcriptase polymerase chain reaction (RT-PCR) have been frequently used all over the world to detect viruses from field samples (Gohm et al., 2000 and Mathivanan et al., 2004). IBD is economically important for the poultry industry in function of the immune suppression that it affects dividing B-lymphocytes bearing cell surface IgM developing the severe morphological alteration of bursa of fabricius (Lukert and Saif, 1997; Ivan et al., 2001).

Despite the highly significant detrimental impact of IBD on the poultry sector, there remains a notable scarcity in achieving diagnostic efficacy through both pathological investigation and molecular approaches. Extensive epidemiological studies have been conducted on this disease within our country (Rashid et al., 2013; Shovon, 2015; Islam et al., 2015; Islam et al., 2016 and Akter et al., 2018), along with Sero epidemiological investigations. Furthermore, the molecular characterization of crucial virus isolates has been undertaken to advance our comprehension of its virulence by researchers (Islam et al., 2012 and 2021). However, despite these efforts, accurate diagnosis through the comprehensive assessment of postmortem findings and histopathological analysis remains pivotal in understanding the disease pattern. Recognizing the critical need to focus on postmortem findings and histopathology for diagnosing this disease accurately, our present study was conducted with a specific emphasis on elucidating these aspects to better comprehend the pathological manifestations. Considering the current important situation, we conducted the present study with the following objectives.

a) To investigate the gross and histopathological changes in different organs developed due to Infectious Bursal Disease (IBD)

b) To identify the IBDV from suspected cases by RT-PCR

CHAPTER 2: LITERATURE REVIEW

Infectious Bursal Disease (IBD), caused by the highly contagious IBD virus (IBDV), stands as a significant threat to the global poultry industry due to its severe impact on young chickens and other susceptible avian species. Extensively documented as a viral infection affecting the bursa of fabricius, this affliction presents a complex pathogenesis characterized by immunosuppression and the consequent heightened susceptibility to secondary infections. The extensive literature surrounding IBD provides a comprehensive understanding of its epidemiology, clinical manifestations, histopathological changes, and the intricate interplay between viral virulence factors and the avian immune system. Previous studies have elucidated the gross and histopathological alterations within affected organs, particularly the bursa of fabricius, kidney, and spleen, highlighting the systemic nature of the disease. Additionally, research efforts have focused on elucidating vaccination strategies, disease transmission dynamics, and control measures aimed at minimizing the economic losses incurred by the poultry industry due to IBD outbreaks. This literature review aims to synthesize and critically analyze the existing body of knowledge, thereby establishing a comprehensive foundation upon which the present study contributes novel insights into the pathological manifestations and systemic impact of IBD in avian populations.

2.1 Etiology

Infectious Bursal disease virus (IBDV) is classified as a member of the *Birnaviridae* family. The family includes 3 genera: *Aquabirnavirus* which type species is infectious pancreatic necrosis virus (IPNV), which infects fish, mollusks and crustaceans; *Avibirnavirus* which type species is infectious bursal disease virus (IBDV), which infects birds; and *Entomobirnavirus* whose type species is *Drosophila* X virus (DXV), which infects insects (Viruses in this family possess bi-segmented, double-stranded RNA (dsRNA) genomes, which are packaged into single shelled, non-enveloped virions. The capsid shell exhibits icosahedral symmetry composed of 32 capsomeres and a diameter ranging from 55 to 65 nm (Brown et al., 1994). IBDV strains have been classified into two distinct serotypes 1, pathogenic and 2, non-pathogenic (Van

den Berg, 2000). Two serotypes of the virus have been described; these are Serotype 1 IBDV strains, pathogenic to chickens (Muller et al., 2003; Van Den Berg et al., 2004), whereas serotype 2 strains are non-pathogenic. Serotype 1 IBDV isolates comprise the variant, classical virulent and vvIBDV strains, which widely differ in their pathogenicity to chickens. Variant IBDVs do not cause mortality, whereas the classical strains cause up to 20% mortality (Muller et al., 2003). VvIBDV causes mortality exceeding 50% in susceptible chickens (Chettle et al., 1989; Muller et al., 2003). Infectious bursal disease virus is highly resistant to adverse environmental conditions. It is more resistant to heat and ultraviolet light than reovirus and is resistant to ether and chloroform. Once infected with IBDV, chickens can shed the virus in feces for as long as 16 days. It is reported that poultry houses which previously harbored infected flocks remained infective for at least 122 days and that fomites (water, feed, droppings) contaminated with IBDV contribute to viral dissemination. Therefore, the control of this disease depends mainly on vaccination, Al Natour et al., (2004), but in some cases vaccinations have been ineffective in protecting birds (Islam et al., 2003).

2.2 Epidemiology

Cosgrove, (1962) reported a specific disease, (IBD) that affects the bursa of fabricius in chickens. The first cases were seen in area of Gumboro, United States of America (USA), which is the name derived, even if the terms 'IBD' or 'infectious bursitis' are more accurate descriptions. In the year of 1960 and 1964, the disease was observed in most parts of the USA (Lasher and Davis, 1997), and become a devastating disease in Europe in the years 1962 to 1971. With its pandemic movement from the year 1966 to 1974, the disease was reported in the southern and western Africa, Far East, Middle East, India and Australia (Lasher and Shane, 1994; Vander Sluis, 1999; Van den Berg, 2000;). Infectious Bursal Disease currently become an international issue, 95% of the 65 countries that responded to a survey conducted by the (OIE, 1995) announced presence of infection (Eterradossi, 1999), including New Zealand which had been free of disease until 1993. Only chickens develop IBD after infection by serotype 1 viruses. Turkeys may be asymptomatic carriers of serotype 2, and at times, of serotype 1 viruses whose pathogenicity for turkeys is ill-defined (Reddy and Silim, 1991; Owoade and Durojaiye, 1995). Anti-IBDV antibodies have been detected in

guinea fowl, common pheasants and ostriches, which have also been found to carry serotype 2 viruses (Guittet et al., 1992). Neutralizing or precipitating antibodies have been detected, *inter alia,* in various species of wild duck, goose, tern, puffin, crow and penguin, which may mean that wild birds act as reservoirs or vectors (Ogawa et al., 1998). The age of maximum susceptibility to IBDV is between 3 and 6 weeks, which is the period of maximum bursa development, during which the acute clinical signs are observed. Infections occurring before the age of three weeks are generally subclinical and immunosuppressive. Clinical cases may be observed up to the age of fifteen to twenty weeks (Okoye and Uzoukwu, 1981). Light strains of laying stock are more susceptible to disease than the heavy broiler strains (VandenBerg and Meulemans, 1991; Hassan and Saif, 1996). IBD transmits in a horizontal way only, with healthy subjects being infected by the oral or respiratory pathway. Infected subjects excrete the virus in faces as early as 48 hours after infection and may transmit the disease by contact over a sixteen-day period. The possibility of persistent infection in recovered animals has not been researched. The disease is transmitted by direct contact with excreting subjects, or by indirect contact with any inanimate or animate contaminated vectors. Some researchers have suggested that insects may also act as vectors (Howie and Thorsen, 1981). The extreme resistance of the virus to the outside environment enhances the potential for indirect transmission. The virus can survive for four months in contaminated bedding and premises, and up to fifty-six days in lesser mealworms taken from a contaminated building (Lucio and Hitchner, 1980). In the absence of effective cleaning, disinfection and insect control, the resistance of the virus leads to perennial contamination of infected farm.

2.2.1 Morbidity and mortality

Infectious bursal disease is extremely contagious and in infected flocks, morbidity is high, with up to 100 % serological conversion, after infection, whilst mortality is variable. Until 1987, the field strains isolated was of low virulence and caused only 1 % to 2 % of specific mortality. However, since 1987 an increase in specific mortality has been reported in different parts of the world. In the USA, new strains responsible for up to 5%of specific mortality were described (Rosenberger and Cloud, 1986). At the same time, in Europe, Africa and subsequently in Japan, high mortality rates of 50 % to 60 % in laying hens and 25 % to 30 % in broilers were observed. These hyper

virulent field strains caused up to 100 % mortality in specific pathogen free (SPF) chickens (VandenBerg et al., 1991; Nunoya et al., 1992).

2.3 Clinical signs

The incubation period is very short which range from 2 to 3 days. In acute cases, the chickens are tired, prostrated, dehydrated, suffer from watery diarrhea, and their feathers are ruffled. Mortality commences on the third day of infection, reaches a peak by day four, then drops rapidly, and the surviving chickens recover a state of apparent health after five to seven days. Disease severity depends on the age and breed sensitivity of the infected birds, the virulence of the strain, and the degree of passive immunity. Initial infection on a given farm is generally very acute, with very high mortality rates if a very virulent strain is involved. If the virus persists on the farm and is transmitted to successive flocks, the clinical forms of the disease appear earlier and are gradually replaced by subclinical forms. Nonetheless, acute episodes may still occur. Moreover, a primary infection may also be apparent when the viral strain is of low pathogenicity or if maternal antibodies are present. The clinical signs of IBD vary considerably from one farm, region, country or even continent to another. Schematically, the global situation can be divided into three principal clinical forms, these are; the classical form, caused by the classical virulent strains of IBDV. Specific mortality is relatively low, and the disease is most often subclinical, occurring after a decline in the level of passive antibodies. The second is immunosuppressive form, principally described in the USA, is caused by low pathogenicity strains of IBDV, as well as by variant strains, such as the Delaware variant E or GLS strains, which partially resist neutralization by antibodies against the ‘classical’ viruses (Snyder, 1990). The acute form, first described in Europe, Africa and then in Asia, is caused by 'hyper virulent' strains of IBDV, and is characterized by an acute progressive clinical disease, leading to high mortality rates on affected farms (Chettle et al., 1989; Stuart, 1989; VandenBerg et al.,1991).

2.4 Pathology

Even although IBD affected different lymphoid organs (Sharma, 1993; Tanimura et al., 1995; Tanimura and Sharma, 1997), the principal target of the virus is the bursa of fabricius, which is the reservoir of B lymphocytes in birds. Indeed, the target cell is the B lymphocyte in active division, for which the infection is cytolytic (Burkhardt and Müller, 1987). Cell sorting studies have demonstrated that the B lymphocyte is susceptible in the immature stage, during which immunoglobulin M is carried on the surface of the lymphocyte (Nakai and Hirai, 1981). This accounts for the paradoxical immune response to IBDV, in which immunosuppression co-exists with high anti-IBDV antibody titre. The mature and competent lymphocytes will expand because of stimulation by the virus whereas the immature lymphocytes will be destroyed. The virus subsequently enters the general circulation via the hepatic portal vein. A phase of primary viraemia ensues, during which the virus reaches the bursa, 11 hours after infection, and a major secondary replication cycle occurs. A phase of secondary viraemia then occurs, and the other lymphoid organs become massively infected. Macroscopic lesions are observed principally in the bursa which presents all stages of inflammation following acute infection (McFerran , 1993). Necropsy performed on birds that died during the acute phase (three to four days following infection) reveal hypertrophic, hyperemic and edematous bursas. The most severe cases are characterized by a major infection of the mucous membrane and a serous transudate, giving the bursal surface a yellowish color. This appearance is often accompanied by petechiae and hemorrhages. By the fifth day, the bursa reverts to normal size and by the eighth day becomes atrophied to less than a third of the normal size. The affected animals are severely dehydrated, and many birds have hypertrophic and whitish kidneys containing deposits of urate crystals and cell debris. Hemorrhages in the pectoral muscles and thighs are frequently observed, probably due to a coagulation disorder. Certain variants from the USA are reported that causes rapid atrophy of the bursa without a previous inflammatory phase (Lukert and Saif, 1997). Moreover, in the acute form of the disease caused by hyper virulent strains, macroscopic lesions may also be observed in other lymphoid organs like thymus, spleen, cecal tonsils, Harderian glands, Peyer's patches and bone marrow. (Hiraga et al., 1994; Inoue et al., 1994; Tsukamoto et al., 1995; Inoue et al., 1999). Pathological study in Bangladesh on naturally infected chickens by Islam and Samad, (2004) observed gross lesions in bursa which was swollen, edematous and streaks of hemorrhagic on outer and inner

surface of bursa. The cut surface of the bursa revealed slimy and gelatinous material. Thigh muscle also revealed petechial hemorrhage and in addition spleen was hemorrhagic and swollen. This investigation also indicated that hemorrhages at the junction of proventriculus and gizzard. The B lymphocytes are destroyed in the follicles of the bursa as well as in the germinal centres and the perivascular cuff of the spleen. The bursa is infiltrated by heterophils and undergoes hyperplasia of the reticuloendothelial cells and of the intermolecular tissue. As the disease evolves, the surface epithelium disappears, and cystic cavities develop in the follicles. Severe panleukopenia is also observed and these microscopic lesions are exacerbated in the acute forms of the disease.

2.5 Immunosuppression

The destruction of immature B lymphocytes IBDV in the bursa creates an immunosuppression, which will be more severe in younger birds (Giambrone et al., 1976). In addition to the impact on production and role in the development of secondary infections, this will affect the immune response of the chicken to subsequent vaccinations which are essential in all types of intensive chicken production (Giambrone et al., 1976) The most severe and longest duration immunosuppression occurs when day-old chicks are infected by IBDV (Sharma et al., 1989 and 1994). In field conditions, this rarely occurs since chickens tend to become infected at approximately two to three weeks, when maternal antibodies decline. Evidence suggests that the virus has an immunosuppressive effect at least up to the age of six weeks (Lucio and Hitchner, 1980).

2.6 Diagnosis

The clinical diagnosis of the acute forms of IBD is based on disease evolution of a mortality peak followed by recovery in five to seven days and relies on the observation of the symptoms and post-mortem examination of the pathognomonic lesions, of the bursa of Fabricius. Diseases like avian coccidiosis, Newcastle disease in some visceral forms, stunting syndrome, mycotoxicosis, chicken infectious anemia and nephropathogenic forms of infectious bronchitis are the differential diagnosis for IBD. In all acute cases, the presence of bursal lesions allows for a diagnosis of IBD. In subclinical cases, an atrophy of the bursa may be confused with other diseases such as Marek's disease or infectious anemia. A histological examination of the bursa will allow differentiation between these diseases (Lukert and Saif, 1997).

2.6.1 Histological diagnosis

Histological diagnosis is based on the detection of modifications occurring in the bursa. The ability to cause histological lesions in the non-bursal lymphoid organs, such as the thymus (Inoue et al., 1994), spleen or bone marrow (Inoue et al., 1999) has been reported as a potential characteristic of hyper virulent IBDV strains. The histological diagnostic method has the advantage of allowing for diagnosis of both the acute and chronic or subclinical forms of the disease. Detection of viral antigens: thin sections of the bursa of fabricius prepared to detect viral antigens specific to IBDV done by direct and indirect immunofluorescence or by immune peroxidase staining in the bursal follicles of infected chickens between the fourth and sixth day after inoculation. No viral antigen is detectable from the tenth day. However, the virus can be isolated from bursa sampled from the second to the tenth day, with a maximum infectious titer after four days. The use of monoclonal antibodies in IHC techniques for detection of the virus enhances the specificity of the test.

2.6.2 Embryo inoculation

Infectious bursal disease virus may be detected in the bursa of Fabricius of chicks in the acute phase of infection, ideally within the first three days following the appearance of clinical signs. Isolation: A filtered homogenate of the bursa of fabricius is inoculated in nine to eleven days old embryonated eggs originating from hens free of anti- BDV antibodies. The most sensitive route of inoculation is the chorioallantoic

membrane (CAM); the yolk sac route is also practicable, and the intra-allantoic route is the least sensitive. The specificity of the lesions observed must be demonstrated by neutralizing the effect of the virus with a mono specific anti-IBDV serum. Isolation in embryonated eggs does not require adaptation of the virus by serial passages and is suitable for vvIBDV. In the absence of lesions, the embryos from the first passage should be homogenized in sterile conditions and clarified, and two additional serial passages should be performed (Rosenberger, 1989; Lukert and Saif, 1997).

2.6.3 Serological diagnosis

In areas contaminated by IBDV, most broiler flocks have anti-IBDV antibodies when leaving the farm. Current serological tests cannot distinguish between the antibodies induced by pathogenic IBDV and those induced by attenuated vaccine viruses, so serological diagnosis is of little interest in endemic zones. Nonetheless, the quantification of IBDV-induced antibodies is important for the medical prophylaxis of the disease in young animals, to measure the titer of passive antibodies and determine the appropriate date for vaccination (Kouwenhoven and vandenBos, 1994 ; Dewit, 1999) or in laying hens to verify success of vaccination. (Lucio,1987; Meulemans et al., 1987). Serology is likewise essential to confirm the disease-free status of flocks. Each serological analysis must include enough (at least twenty) of individual serum samples representative of the flock under study. A kinetic study requires at least two serological analyses separated by an interval of three weeks (paired sera).

2.6.4 Molecular identification

Most efforts at molecular identification have focused on the characterization of the larger segment of IBDV (segment A) and especially of the vVP2 encoding region. Several protocols have been published on characterization using restriction endonucleases of RT-PCR products. These approaches are known as RT-PCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism) Jackwood, (1990); Lin et al., (1993). The usefulness of the information they provide depends on the identification of enzymes that cut in restriction sites that are phenotypically relevant. Some sites involved in antigenicity have already been identified, however, restriction sites reliably related to virulence still need to be defined and validated. Nucleotide sequencing of RT-PCR products, although more expensive than restriction analysis,

provides an approach to asses more precisely the genetic relatedness among IBDV strains. Markers have been demonstrated experimentally, using a reverse genetics approach, for cell culture adapted strains, which exhibit amino acid pairs 279 N–284 T (Lim et al., 1999) or 253 H–284 T (Mundt , 1999). In most very virulent viruses, four typical amino acids are present (222 A, 256 I, 294 Iand 299 S) (Lin et al., 1993; Brown et al., 1994; Eterradossi et al., 1999). However, it is not yet known whether these amino acids play a role in virulence or if they are merely an indication of the clonal origin of most vvIBDV isolates. Several recent studies indicate that although VP2 is an important virulence determinant, it may not be the only one. It has been reported that segment A and B of IBDV mostly co-evolve (i.e. most significant IBDV clusters, such as vvIBDV-related strains, may be identified by analysis of both genome segments), however some potentially reassortant viruses have been identified (Lenouen et al., 2006)

CHAPTER 3: MATERIALS AND METHODS

3.1 Study location and Study period

Samples were collected from the birds brought from different parts of Chattogram district to Department of Pathology and Parasitology, CVASU for postmortem examination. This study was conducted over the period of December 2021 to February 2023.

3.2 Study population

During the study period, 200 broiler chickens displaying symptoms such as tiredness, prostration, dehydration, watery diarrhea, ruffled feathers, and sudden death underwent postmortem examination. Samples were collected from all 200 chickens, with 30 samples randomly selected for histopathological investigation. Concurrently, eight samples (bursa, spleen and kidney from each bird) were chosen for molecular assessment according to having maximum pathological lesion.

3.3 Sample collection and Preservation

After postmortem examination of dead broiler birds suspected to be infected with IBDV, bursa, kidney and spleen samples were collected for molecular and histopathological examination. Bursa and spleen samples were kept frozen at -80°C for further molecular diagnosis by reverse transcription PCR (RT-PCR). For histological examination samples were fixed in Bouin's solution (10 Folds of the tissue size) and kept in labeled plastic containers.

3.4 Virus isolation method

3.4.1 Extraction of viral RNA

As IBD is an RNA virus, the Viral RNA was extracted from frozen bursa and spleen samples, using easy-RED^{TM} RNA extraction kit, (iNtRON Biotechnology, Inc. is a Korea-based manufacturer company) according to the manufacturer's procedure. Finally, the RNA was extracted in 60 µl of elution buffer and used as template for conventional RT-PCR assay and also stored at -80°C until further use.

3.4.2 Selection of oligonucleotide primers

Infectious bursal disease virus specific primers were used from a previously reported work by Mohammed et al., (2013).

Table 1. Primers used for RT-PCR of IBDV

Primer	Primers Sequence (5´-3´)	Amplicon Size
Vvfp 775 (Sense)	-AATTCTCATCACAGTACCAAG -	253bp
Vvrp 1028 (Antisense)	-GCTGGTTGGAATCACAAT -	

3.4.3 Complementary DNA (cDNA) synthesis

The cDNAs were synthesized from the extracted RNAs using a cDNA synthesis kit (Addbio^{TM}). According to the kit instruction, master mixes (10 µL) containing reverse transcriptase, dNTPs, and random hexamer primers were mixed with extracted RNAs (10 µL) and the reaction was performed as follows: 45 °C for 60 min, 80 °C for 5 min, and 12 °C for 10 min. The cDNAs were kept in −20° C freezers for later use.

3.4.4 Amplification of DNA from cDNA of IBDV using *VP2* gene primer

The cDNA samples (4 µL) were mixed with 10 µL of Thermo Scientific PCR Master mix (2x) with the addition of 0.5 µL of each forward (Vvrf) and reverse (Vvrf) primers, and 10 µL nuclease free water for making 25 µL volume for each reaction. The PCR reaction was then maintained in following temperatures in the Biometra PCR thermocycler: an initial denaturation at 95°C for 5 min, then 40 cycles as follows: 20 s at 95°C, 30 s at 60°C, and 35 s at 72°C and a final extension at 72°C for 7 min. The PCR products were run on 1.5 % agarose gel and the presence of a 253bp band was considered a successful amplification.

3.4.5 Agar gel electrophoresis

1.5% agarose gel was made by using 0.5 g agarose powder and 50 ml TAE buffer with ethidium bromide. The DNA amplicons were visualized using 4 µl of the final PCR product and 2 µl standard 100 bp plus DNA markers at 120 V/100 mA for 30 min. Gels were photographed using a gel documentation system. Positive or negative

amplifications were evaluated as presence or absence of visible orange color bands on agarose gels under UV light.

3.5 Histopathological slide preparation

Grossly affected tissues were collected, identified, and preserved in Bouin's solution (10 Folds of the tissue size) in labeled plastic containers. Thickness of the tissue sample was 4-5 mm. Tissues were preserved for at least 7 days before processing.

3.5.1 Processing of tissue

Preserved tissues were processed following removal of fixative, dehydration, clearing, impregnation, and embedding.

Sample identification marks were made by a soft lead pencil and a garland (tissue string) of tissues was made considering the cut surface for sectioning. Then the tissue garlands were placed for an overnight wash in running tap water to remove the fixative. Dehydration was done by moving the tissues through ascending concentration of ethanol series (80% alcohol- two hours, 95% alcohol- two changes one hour each, 100% alcohol- three changes one hour each) for appropriate time to prevent shrinkage of cells. Clearing reagents should be miscible with the dehydrant and the paraffin. Xylene was used as a clearing reagent to replace alcohol (xylene- two changes one hour each, xylene- two hours. Impregnation of tissue by paraffin for complete removal of the clearing agent was done by three changes in paraffin bath (56-58oC), two hours each. The cooked tissues were kept overnight to rest. Embedding was done by placing the tissue in melted paraffin to make a block, which after solidification provided a firm medium for keeping all parts of the tissue intact when sections were cut.

3.5.2 Preparation of sections

Tissue block embedded in paraffin was set in the rotary microtome machine and sections were cut at 3-5μm thickness until suitable ribbon was formed. The ribbon of tissue sections was placed in a warm water bath (55-58oC) and allowed to spread. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. Sections were picked up on grease-free clear slides. Sections were the air-dried and placed on a rack.

3.5.3 Staining of tissue slides

A regressive staining procedure was followed to stain the tissue slides. In the regressive staining technique, the sections were first overstained with a relatively neutral solution of hematoxylin. Then the excess stain was removed by using an acid alcohol solution. After that sections were neutralized with an alkaline solution (weak ammonia water) for better differentiation. Then the sections were counterstained with eosin followed by the removal of excess eosin by alcohol.

After staining and mounting cover slip the slides were air dried and then examined under microscope.

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CHAPTER 4: RESULTS

4.1 Clinical history and signs

In the affected birds a spectrum of clinical signs became evident, collectively indicating the severity of the illness. These signs included depression and dullness. Additionally, watery diarrhea, ruffled feathers, anorexia, where birds exhibited reduced appetite or refused to eat, exacerbated their weakened state. Trembling, gasping for breath and prostration further signified the extent of illness, with some birds being unable to stand and preferring to lie down. Furthermore, affected birds displayed reluctance or inability to move normally, alongside signs of discomfort soiled vents and vents picking were also found.

4.2 Gross pathological changes

Postmortem examination of affected birds revealed several gross changes. The birds were found dehydrated and there were petechial hemorrhages in the leg and thigh muscle during postmortem examination. The bursa of fabricius was swollen, hemorrhagic and sometimes covered in gelatinous substances. The hemorrhage was observed in bursal folds. In some cases, bursal folds were edematous and bursal lumen was filled with pus. The kidney became swollen, petechial hemorrhagic and pale. Spleen was found to be swollen, hemorrhagic and dark in color.

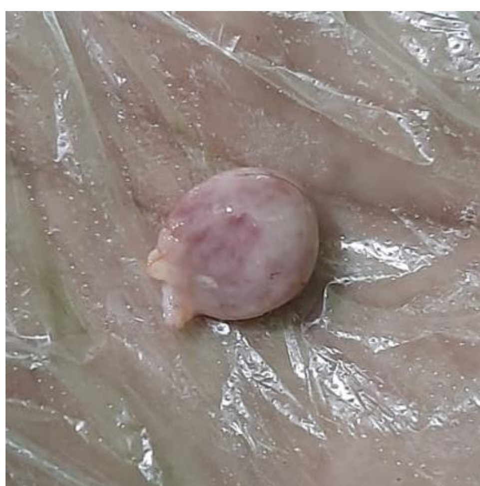

Figure 1: Hemorrhagic bursa.

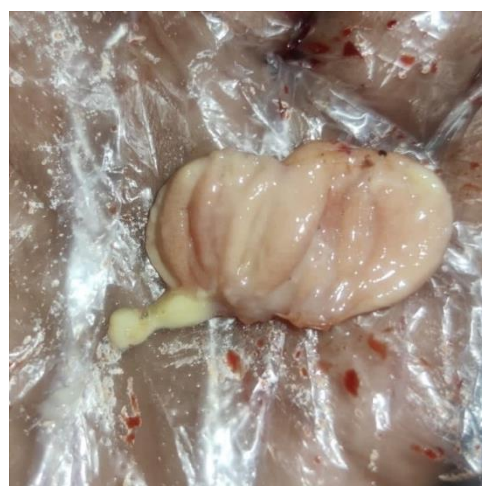

Figure 2: Edematous bursal folds with pus.

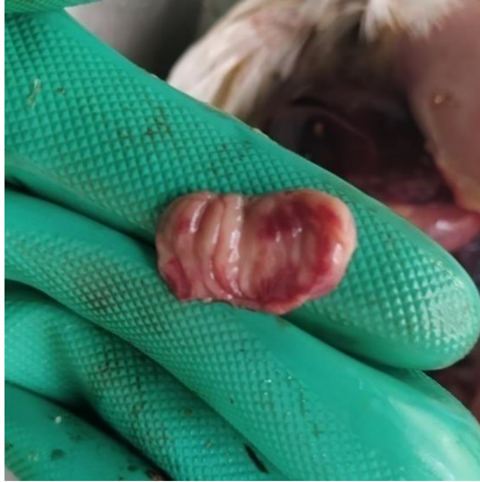

Figure 3: Hemorrhage in bursal folds.

Figure 4: Hemorrhage in bursal folds with pus.

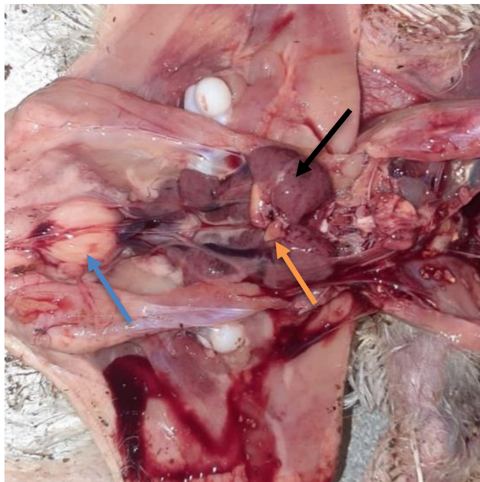

Figure 5: Swollen spleen (black arrow), petechial hemorrhagic (yellow arrow) kidney; swollen bursa covered in gelatinous substances (blue arrow).

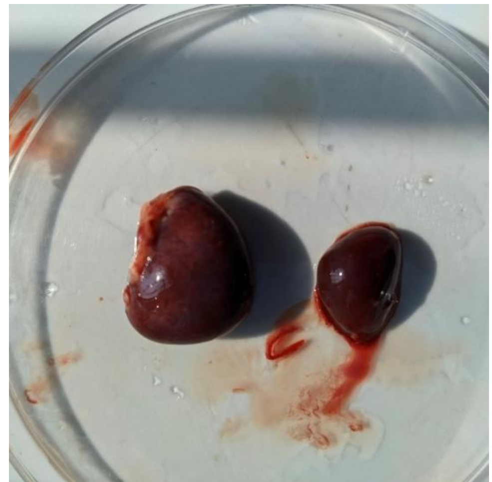

Figure 6: Swollen, hemorrhagic and dark spleen (left side).

4.3 Histopathological changes

Major microscopic lesions were found in bursa. Microscopic changes found in bursa include depletion of follicular lymphocytes leading to atrophied lymphatic follicle as well as edema, formation of cortical rim, inter follicular hemorrhage, congestion, infiltration of heterophilic inflammatory cells in inter follicular space, fibrosis and thickening of inter follicular space.

Histopathological slides of kidney sections revealed hemorrhage, congestion, infiltration of inflammatory cells between renal tubules, edema in renal tubules, partially detached lining epithelium from basement membrane of many tubules and collecting ducts. There was fragmentation and sloughing of the epithelium in few tubules and collecting ducts as well as epithelial destruction. The histological sections of spleen tissue revealed hemorrhage, congestion, lymphoid cell depletion and inflammatory cell infiltration.

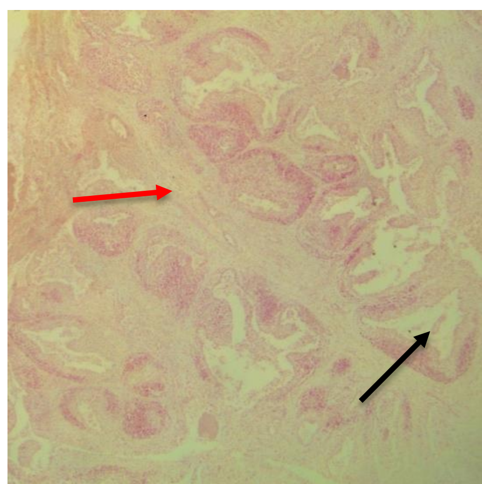

Figure 7: Edema (red arrow) and depletion of lymphoid cell in lymphoid follicle of bursa (black arrow).

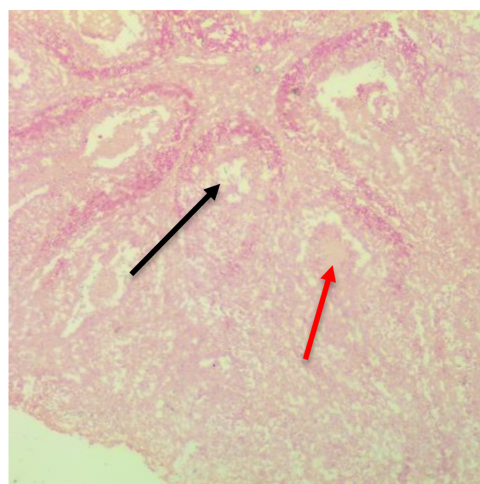

Figure 8: Depletion of lymphoid cells in lymphoid follicle (black arrow) fibrosis and thickening of interfollicular space (red arrow) in bursa.

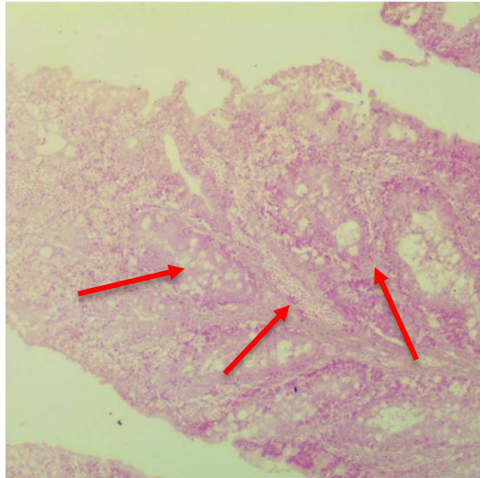

Figure 9: Hemorrhage, congestion, and inflammatory cellular infiltration (red arrow).

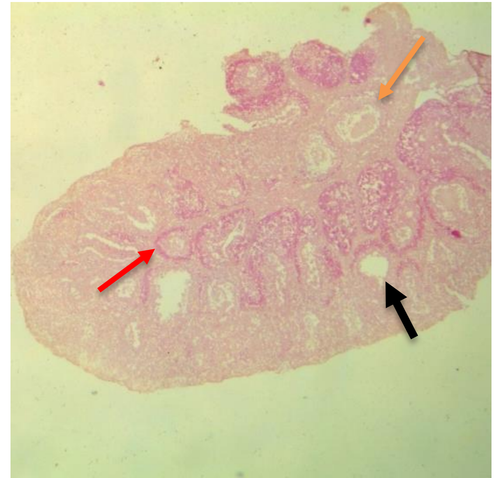

Figure 10: Depletion of lymphocytes in atrophied follicle (black arrow), formation of cortical rim (red arrow), edema (orange arrow) in bursa.

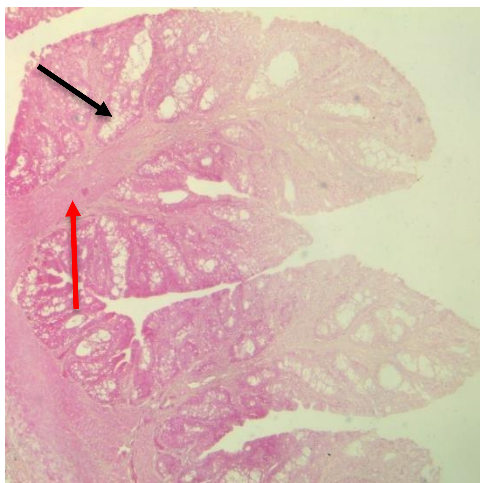

Figure 11: Follicular atrophy (black arrow), fibrosis and thickening of interfollicular space (red arrow).

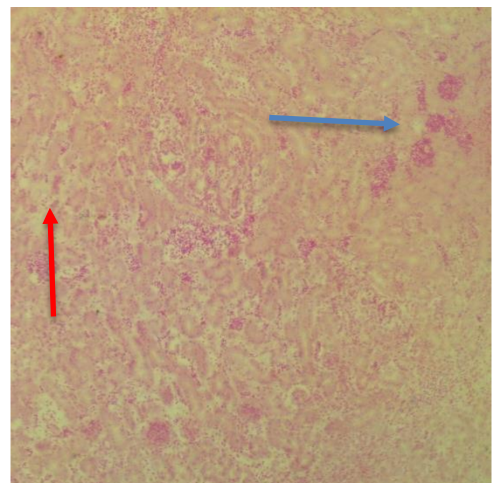

Figure 12: Congestion in peritubular capillaries (red arrow), hemorrhage and infiltration of inflammatory cells in peritubular spaces (blue arrow).

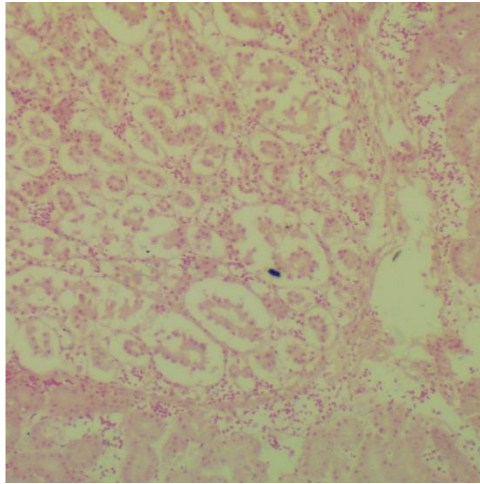

Figure 13: Detachment, fragmentation and sloughing of lining epithelia of renal tubules and collecting ducts.

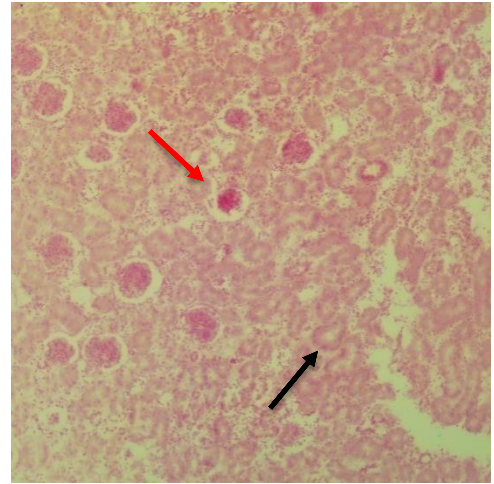

Figure 14: Hyperemic renal glomerulus (red arrow), swelling of tubules (black arrow).

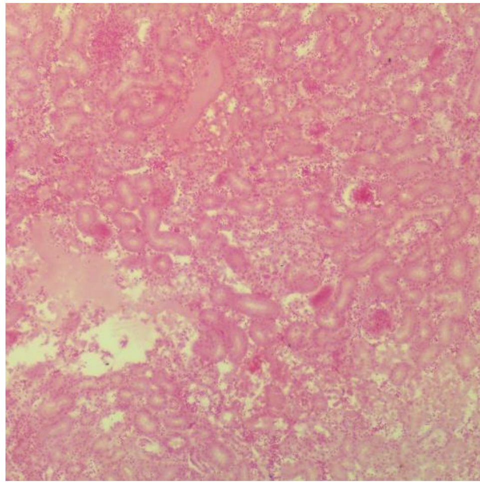

Figure 15: Edema in renal tubules and peritubular spaces.

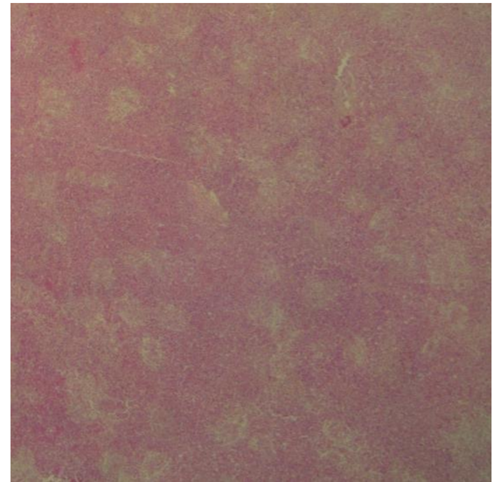

Figure 16: Lymphocytic depletion in spleen.

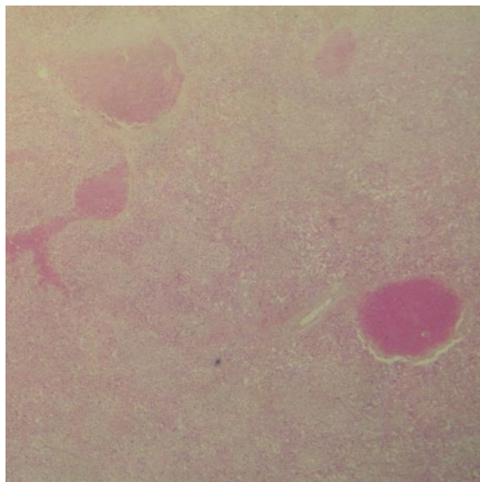

Figure 17: Congestion in spleen.

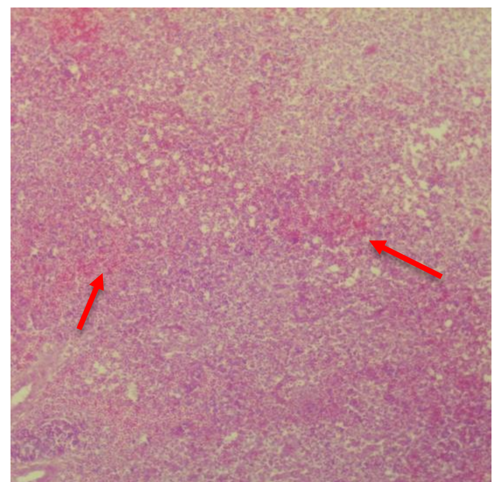

Figure 18: Hemorrhage in spleen.

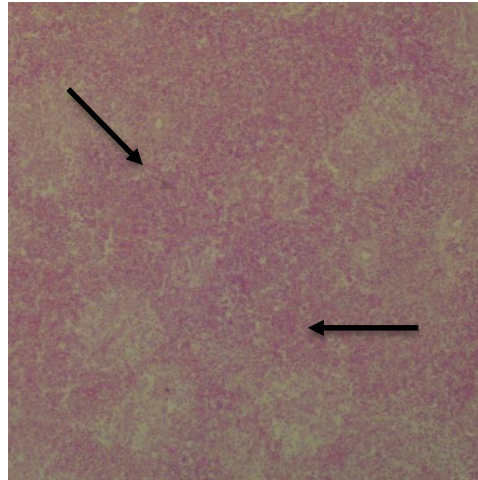

Figure 19: Infiltration of inflammatory cells in spleen (black arrow).

4.4 Molecular findings

For molecular confirmation of IBDV in bursal tissues , the *VP2* gene was selected as it plays important role in antigenicity, cell tropism, virulence and apoptosis and induces serotype-neutralizing antibodies (Jackwood et al., 2008 and Wu et al., 2020). cDNA of pooled tissue samples was positive with *VP2* gene specific primers by producing an amplicon size of 253 bp (Figure 20) approximately which confirms the IBD virus. Out of eight samples, all were found positive for IBDV

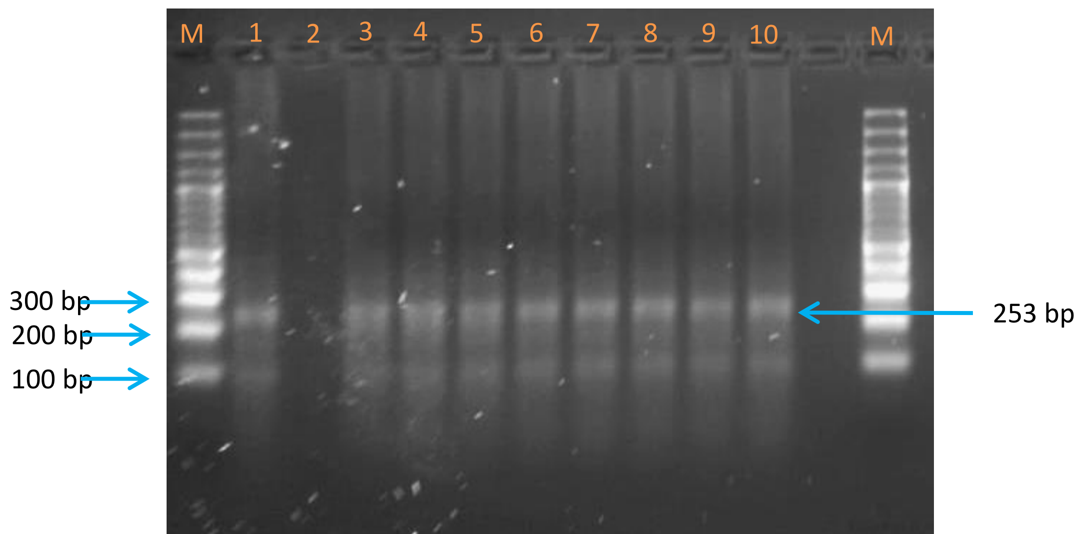

Figure 20: RT-PCR products (253 bp) of IBDV analyzed using 1.5% agarose gel electrophoresis. Lane M = 100 bp DNA ladder, Lane 1= Positive control, Lane 2= Negative control, Lane 3-10 = test samples.

CHAPTER 5: DISCUSSION

This study aimed to examine the pathological alterations occurring in various organs of broiler birds infected with Infectious Bursal Disease Virus (IBDV), validated through confirmation via RT-PCR testing.

In affected birds, the observed clinical signs encompassed depression and lethargy, marked by a notable lack of vigor and vitality in afflicted birds. Concurrently, profuse watery diarrhea resulted in dehydration, exacerbating their debilitated condition. The appearance of ruffled feathers indicated an uncharacteristically disheveled state, underscoring the compromised health of the birds. Anorexia, leading to diminished appetite or a complete refusal to eat, further weakened their condition, reflecting the extensive physiological compromise and organ pathology inflicted by IBDV. Additionally, manifestations such as trembling, labored breathing, and prostration highlighted the severity of illness, with some birds' incapable of standing opting to lie down instead. Moreover, affected birds exhibited reluctance or inability to move normally, coupled with signs of distress, evidenced by soiled vents and vents picking, indicative of the systemic inflammatory response triggered by the viral infection induces discomfort. These signs were like those described in the early outbreaks of IBD by (Dey et al., 2019; Pandey, 2021; Omer and Khalafalla, 2022 and Thippani et al., 2023). These clinical manifestations highlight the complexity of the disease's impact on avian health and welfare, emphasizing the need for comprehensive management and control strategies to mitigate its effects within poultry populations.

The gross pathological findings were hemorrhagic, enlarged, edematous and hyperemic bursa with bloody or mucoid contents which support the findings of Chettele et al., (1989); Islam and Samad, (2004); Mazengia et al., (2009); Khan et al., (2011); Rozina et al., (2014) reported an occurrence of hemorrhage in thigh muscles, with observed hemorrhage in the bursa, bursal swelling, bursal edema in and bursal congestion. These lesions are regarded as characteristic and indicative findings of this disease. The observed phenomenon results from the virus infiltrating and multiplying within the B lymphocytes located in the bursa, leading to the destruction of these cells. This viral attack causes hemorrhage, enlargement, and hyperemia due to the damage inflicted on the blood vessels and surrounding tissues (Qin and Zheng, 2017).

However, none of the investigators (Mazengia et al., 2009 and Rozina et al., 2014) reveal the gross pathological changes in spleen. In our case the spleens were found to be swollen, hemorrhagic and dark in color which matches with the findings reported in the study done by Teshager, (2015). This might be due to the compromised immune system resulting from IBDV infection that makes the bird susceptible to secondary infections. As a result, the spleen, being a critical organ in immune responses, might undergo enlargement as it actively engages in combating these secondary infections and trying to bolster the compromised immune function. After postmortem examination the kidneys were found swollen, hemorrhagic and pale which also supports the findings of Teshager, (2015). This signifies multifaceted pathological changes within avian renal tissues. This presentation is indicative of the complex impact of the Infectious Bursal Disease Virus (IBDV) on the kidneys. Direct viral infiltration and replication within renal cells potentially result in cellular damage, contributing to kidney swelling and hemorrhage. Moreover, the immunosuppression triggered by IBDV-induced B lymphocyte destruction in the bursa of Fabricius renders birds susceptible to secondary infections, including those affecting the kidneys.

The main microscopic changes in the bursa of broiler chicken with IBD infection included the loss of lymphoid cells, development of the cortical rim, atrophied lymphoid follicles, intra follicular and inter follicular hemorrhage and congestion as IBDV primarily targets the B lymphocytes in the bursa of fabricius, resulting in the destruction of these immune cells. Also causes edema, inflammatory cellular infiltration and disruption of follicular architecture with fibrosis. The viral infection triggers a robust inflammatory response, leading to edema and infiltration of inflammatory cells within the lymphoid tissues. This inflammation contributes to tissue swelling and disrupts the normal architecture of the lymphoid follicles. The research work by Chowdhury et al., (2015) and Teshager, (2015) were related to these histological findings in bursa. Depletion of the lymphoid cells leads immunosuppression which prevents the birds from optimally responding to vaccine and ultimately leads to increase the incidence of numerous concurrent infections including Marek’s disease, Newcastle disease, coccidiosis, infectious bronchitis hemorrhagic-aplastic anemia and gangrenous dermatitis, infectious laryngotracheitis

inclusion body hepatitis, reovirus (Montgomery et al., 1991), chicken anemia agent, salmonellosis, colibacillosis and Mycoplasma synoviae (Giambrone et al., 1997).

In microscopic sections of spleen tissue revealed hemorrhage, congestion, lymphoid cell depletion and inflammatory cellular infiltration. These histological findings in IBD infected chicken's spleen were related with study of Hair-Bejo et al., (2004) and Gary and Butcher, (2013).

Microscopic examination of kidney tissue sections revealed hemorrhage, congestion, infiltration of inflammatory cells between renal tubules, edema in renal tubules. Partially detached lining epithelium from basement membrane of many tubules and collecting ducts were also observed. There was fragmentation and sloughing of the epithelium in few tubules and collecting ducts as well as epithelial destruction. These microscopic findings are like the findings mentioned in the studies by Teshager, (2015).

In this study, RT-PCR was conducted to confirm the diagnosis of IBDV, focusing on detecting the *VP2* gene and all samples tested positive for the specific gene, whereas Thippani et al. (2023) reported a detection rate of 85%, potentially attributed to variations in their sample selection. In our investigation, the tissue samples utilized for molecular detection were primarily identified as infected with bursal disease through postmortem examination. Islam et al., (2012), in their molecular investigation targeting the *VP2* gene, identified two isolates, GB1 and GB3, which clustered with other highly virulent IBDVs of European and American origin. However, our study did not achieve a similar analysis or classification and through this we couldn't identify the genotypes with different pathogenic profiles specially of this region.

Both the pathological and the molecular findings observed in this study were found effective in diagnosing the infection of IBDV in broiler chicken.

CHAPTER 6: CONCLUSION

In conclusion, the consistency observed between the diagnosis based on gross lesions, histopathological examination, and molecular diagnosis, notably validates the reliability of postmortem findings as a dependable method for diagnosing Infectious Bursal Disease (IBD). This comprehensive analysis emphasizes the complexity of IBD pathology and its detrimental effects on poultry, highlighting the urgency for stringent disease control measures, enhanced surveillance strategies, and continued research efforts toward improved diagnostic tools and vaccine development to mitigate the impact of Infectious Bursal Disease in poultry populations.

CHAPTER 7: LIMITATIONS

More extensive epidemiological study could be done using available data which could give us a new insight into this disease control, management, and vaccination profile. All the samples were not analyzed by molecular technique, by which we could have some wider variations in result.

Investigating the serological status of infected birds could aid in quicker and more accurate identification of the IBD virus. This could include the refinement of molecular diagnostic methods like RT-PCR or the exploration of advanced diagnostic technologies.

We could identify the emerging genotypes with different pathogenic profiles in Chattogram by sequencing and analyzing the available sample.

CHAPTER 8: FUTURE RECOMMENDATIONS

Studying the genetic variability and evolution of IBDV strains, especially in relation to their pathogenicity, could provide insights into the disease's changing dynamics. Analyzing viral strains and their virulence patterns may contribute to better disease management strategies.

Investing in the development of more sensitive and specific diagnostic tests like Rapid Antigen test, several serological test, gene analysis, gene sequencing for IBDV could aid in quicker and more accurate identification of the virus. This could include the refinement of molecular diagnostic methods like RT-PCR or the exploration of advanced diagnostic technologies.

Further research into the development and effectiveness of vaccines against diverse strains of IBDV could significantly contribute to disease prevention. Investigating novel vaccination approaches or improving existing vaccines might offer better protection and control against the disease.

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Appendix

Compositions of fixative used to preserve tissue sample

Bouin's solution (fixative)	
Composition	**Amount**
1. Picric acid, saturated aqueous solution	750 ml.
2. 37-40% formalin	250ml.
3. Glacial acetic acid	50ml.

Reagents and solutions used in staining of tissue sections

Harris Hematoxylin	**1% stock alcoholic eosin**
Hematoxylin crystals………………... 5 g. Alcohol 100 % ....,...... 50 ml. Ammonium or potassium alum…... 100 g. Distilled water……………...……. 1000 ml. Mercuric oxide…………………..... 2.5 g. After preparation of the stain 2-4 ml glacial acetic acid per 100 ml of solution was added. Stain was filtered before use.	Eosin Y …………………...…. 1 g. Distilled water …………...…. 20 ml. Dissolve and add; Alcohol 95% ……………..…. 80 ml. For working solution 1 part of Eosin stock solution was mixed with 3 parts of 80% alcohol. Just before use 0.5 ml of glacial acetic acid per 100ml of stain solution was added.
Acid alcohol	**Ammonia water**
Alcohol 70 % ……………………1000 ml. Hydrochloric acid, concentrated ……10 ml.	Distilled water………………1000 ml. Ammonium hydroxide, 28%.....2-3 ml.

Staining procedure:

i. Deparaffinization:
   a. Xylene ……………………2 changes, 5-10 minutes each.

ii. Rehydration through graded alcohol:
   a. Alcohol 100%...................2 changes, 5 minutes each.
   b. Alcohol 95%................2 minutes.
   c. Tap water ………………5 minutes.

iii. Harris hematoxylin……..10-15 minutes.

iv. Rinse in tap water………. 10 minutes.

v. Differentiate in acid alcohol……3-10 quick dips.

vi. Wash in tap water………… 5 minutes.

vii. Ammonia water (for bluing)……. 3-5 dips.

viii. Wash in tap water………. 10 minutes.

ix. Eosin…………………… 15 seconds to 2 minutes.

x. Alcohol 95% …………… 2 changes, 2 minutes each.

xi. Alcohol 100% …………… 2 changes, 3 minutes each.

xii. Xylene …………………… 2 changes, 2 minutes each.

xiii. Cover slip was placed on stained tissue after putting DPX.

The slides were then dried at room temperature and examined under microscope.

Biography

Umme Aysha Habiba, daughter of Md. Faridul Islam and Jaynab Begum was born in Chattogram, Bangladesh. She completed her Secondary School Certificate (SSC) examination from Rangunia Public School in 2012 and Higher Secondary Certificate (HSC) examination from Chattogram University of Engineering and Technology (CUET) School and College in 2014. She obtained her Doctor of Veterinary Medicine (DVM) Degree in 2020 from Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh. Currently, she is a candidate for the degree of Master of Science in Veterinary Pathology, under the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, CVASU.