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CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE LESION AND MOLECULAR DETECTION OF THE VIRUS FROM OUTBREAK CASES IN POULTRY FARMS IN SELECTED TOWNS IN CENTRAL ETHIOPIA

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SIGNED DECLARATION SHEET

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LIST OF ABBREVATIONS

BF	Bursa of Fabricius
bp	Base pair
cDNA	Complementary Deoxyribonucleic acid
CSA	Central Statistical Agency
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
NVI	National Veterinary Institute
OIE	World Organization for Animal Health
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain reaction
VP2	Viral Protein
VTM	Virus transport media
vvIBDV	Very virulent infectious bursal disease virus

ABSTRACT

In Ethiopia, chicken production is challenged by infectious diseases among which infectious bursal disease frequently occurs as outbreaks and causes significant number of deaths. It causes mortality to chickens aged between 3 to 6 weeks old. This cross sectional study was conducted in Bishoftu, Gelan, Mojo and Dukem poultry farms that had infectious bursal disease outbreak between December 2022 and April 2023. Purposive sampling method was employed in which samples were collected from 100 chickens showing clinical signs and indicative gross pathological lesions during postmortem. Based on questionnaire, 74% of the respondents said they regularly vaccinate their chickens for infectious bursal disease properly whereas 26% didn't have proper follow up and gave vaccines repeatedly. Based on occurrence of diseases, 52% of respondents said, they had gumboro like disease in their poultry farm previously. And 74% of owners burn or buried dead birds but 26% used to throw the dead chickens out. Vascular disorder mainly hemorrhage and organ swelling were the most frequent gross lesions observed in many organs. The depletion of the lymphoid follicles and formation of cystic structure both in the cortical and medullary regions of bursa with accumulation of necrotic debris in the cystic structures was frequently examined microscopic lesion in bursa. Hyperplasias of the bursal inter-follicular epithelial and formation false glandular structure were also observed. In spleen, lymphocytic depletion in both white pulp and red pulp and hemorrhages in the trabecular sinuses were the common microscopic lesions. Severe hemorrhages in the interstitial space and necrosis of renal tubular epithelium were the repeatedly observed lesion of kidneys. The common microscopic lesions of the proventriculus were hemorrhage into the inter-glandular connective tissue, necrosis and slough off of the proventricular epithelium. Liver showed multifocal hepatocellular necrosis. Based on lesions and molecular detection result, it could be concluded that infectious bursal disease is still the real challenge to poultry producers by causing mortality and reduced growth in young and reduced production in adult chickens. Scheduled vaccination and strict biosecurity should be practiced to reduce the occurrence of the infectious bursal disease in poultry farms.

Key words: *Central Ethiopia, farms, Infectious bursal disease, lesion, outbreak, poultry*

1. INTRODUCTION

Poultry production is an important agricultural activity in almost all communities in Ethiopia. Since chicken production can be started by relatively small capital in small areas, chicken production is a good source of income to smallholder producers. Similarly medium and large-scale commercial chicken production is increasing. Total number of poultry is estimated to be 57 million in Ethiopia of which 78.85%, 12.02% and 9.11% are respectively indigenous, hybrid and exotic breeds (CSA, 2021).

Infectious bursal disease (IBD) has been identified as a major constraint to the development of poultry farming in Ethiopia as it causes frequent outbreaks and serious threats and challenges to poultry industry (Zeleke *et al.,* 2005; Woldemariam and Wossene, 2007; Chaka *et al.,* 2012; Mazengia, 2012). Infectious bursal disease, also called Gumboro disease, is classified as an economically important poultry disease (Touzani *et al.,* 2018). It is an acute, highly contagious, immunosuppressive viral disease that occurs in growing chickens especially those between three to six weeks of age. In chickens below three weeks of age, it results in immunosuppression, increased susceptibility to other diseases, and lack of a humoral response to vaccination in addition to mortalities (Khan *et al.,* 2009).

The causative agent of infectious bursal disease is the infectious bursal disease virus (IBDV). The excessive mutation rate of the IBDV genome causes the virus to alter its properties, including antigenic variation with accelerated virulence. The bursa of Fabricius (BF) is the main target organ of the virus (Michael and Jackwood, 2017). The virus primarily infects and destroys actively dividing pre-B lymphocytes within the BF. This leads to immunosuppression that occurs in clinical and asymptomatic forms, affecting both humoral and cell-mediated immune responses, resulting in birds being immunosuppressed to respond to a variety of secondary infections and susceptibility to bird diseases such as infectious bronchitis and Marek's disease (Apilak, 2006; Musa *et al.,* 2010).

Macroscopic lesions are observed principally in the BF and it becomes turgid, edematous and hemorrhagic in chickens that died by acute stage of very virulent IBD through postmortem examination. Diseased chickens are severely dehydrated, and have hypertrophic and pale 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. Severe depletion of a lymphoid cell is recognized both in the BF and in non- bursal lymphoid tissues microscopically (Tulu, 2019).

Clinical IBD can be diagnosed by a combination of characteristic signs and postmortem lesions, molecular detection, serological test, virology and histopathology. Asymptomatic IBD can be confirmed in the laboratory by exhibiting a humoral immune response in unvaccinated chickens or by exhibiting viral antigens or viral genomes in tissues (OIE, 2018).

The disease causes direct or indirect economic losses to the poultry sector. Direct losses are associated with mortality and reduced egg production in hens (Zachar *et al.,* 2016) while the indirect economic loss arises from secondary infections, growth retardation, carcass condemnation, and vaccination failures (Rauw, 2011; Qin and Zheng, 2017). Infectious bursal disease was reported for the first time in Ethiopia by Zeleke *et al.* (2003) with a mortality rate of 45-50%. The mortality rate was 56% in broiler and 25.08% in layer type chicken (Zeleke *et al*., 2005).

The overall prevalence of the IBD antibodies in different districts reached up to 83.1% in Ethiopia (Jenberie *et al.,* 2014). Since then, regular outbreaks of IBDV pose serious threats and challenges to the growing Ethiopian poultry sector. Wider areas of the country have experienced outbreaks of the disease, resulting in massive deaths in private, commercial and government poultry farms (Hailu *et al.,* 2009). A national survey showed that the rate of IBDV seropositivity in domestic chickens is almost 92% (Chaka *et al.,* 2012; Jenbreie *et al.,* 2012), and IBDV isolates happen to be clonal and highly virulent.

The objectives of this study are

*To characterize and describe gross and histopathological lesions from IBD suspected chicken

*Detection of IBDV genome from tissue with lesions suggestive of IBD from outbreak cases using a one-step RT-PCR kit.

*To assess poultry producers activities and biosecurity measures to prevent IBD in the study areas

2. LITERATURE REVIEW

2.1. Etiology

Infectious bursal disease (IBD) is caused by an acute, highly contagious infectious bursal disease virus (IBDV), which causes mortality and immunosuppression in chickens. IBDV is classified in the family *Birnaviridae* (Fauquet *et al.,* 2005). This family includes three genera: *Aquavirnavirus,* which is the infectious pancreatic necrosis virus that infects fish, mollusks, and crustaceans; *Avibirnavirus*, a typical species of which is the infectious bursal disease virus (IBDV) that infects birds and *Entomobirnavirus* which infects insects (Delmas, 2011). Infectious bursal disease virus (IBDV) and other birnaviruses are single-shelled, icosahedral, non-enveloped capsid viruses containing a bi-segmented double-stranded RNA genome that is 65 nm in diameter (Jenberie *et al.,* 2014). The viral capsid is composed of a single protein called VP2 (ICTV, 2012).

The virus is divided into two distinct serotypes (serotype 1 and serotype 2) that show negligible cross-protection. Serotype 1 viruses are pathogenic in young chickens, whereas serotype 2 viruses are neither pathogenic nor immunosuppressive in chickens (Eterradossi, 2000). IBD serotype 1 viruses are classified based on their phenotypic characteristics (such as antigenicity and virulence) and genetic characteristics (VP2 amino acid sequence); as attenuated (vaccine strains), classical (standard), antigenic variant, and extremely virulent (hypervirulent) strains (Van Den Berg *et al.,* 2004). Of the four serotypes phenotypic trait found in the field, the highly virulent or very virulent IBD viruses can infect chickens in the presence of maternal or high levels of vaccine antibodies, and the result is bursa damage with very high mortality and severe economic loss (Fragher, 2001; Maclach and Dubovi, 2001).

Serotype 2 antibodies are widely distributed in turkeys and can also be found in chickens and ducks. There are no reports of clinical disease caused by serotype 2 v irus infection (Naqi *et al.,* 2001). Serotype 2 viruses are immunologically distinct from serotype viruses. This is because vaccination with serotype 2 viruses does not confer protection against

serotype 1 nor does it induce immunity against pathogenic serotype 1 strains (Van den Berg *et al.,* 2004; Vera *et al.,* 2015).

2.2. Epidemiology

2.2.1. Host range and Transmission

The natural hosts of the disease are chickens and turkeys. The virus is rarely isolated from ducks and other poultry (Aregitu, 2015). However, only chickens develop the disease after infection with serotype 1 viruses. The disease primarily affects chickens between 3 and 6 weeks of age. Turkeys can be asymptomatic carriers of serotype 2 viruses and in some cases can be asymptomatic carriers of serotype 1 viruses, but their pathogenicity in turkeys is unknown. Anti-IBDV antibodies have been detected in guinea fowl, pheasants, and ostriches, which have also been revealed to harbor serotype 2 viruses. Neutralizing or precipitating antibodies have been detected in various species of wild ducks, geese, puffins and penguins, which may suggest that wild birds act as reservoirs or vectors (Van Den Berg *et al.,* 2004).

Infected individuals shed virus in their dropping within 48 hours of infection and for at least 14 days (Baxendale, 2002). It then contaminates the water, feed and bedding in which it is present, from where it often spreads. The most common route of infection is the oral route but the conjunctiva and respiratory tract may also be involved (Sharma *et al.,* 2000). The virus is so contagious that direct contact with excreting individuals; indirect contact and contaminated vectors between infected and susceptible flocks transmit it (OIE, 2016). Although there is no confirmation that IBDV spreads by trans ovarian infection (Eterradossi and Saif, 2008), it can survive on eggshell surfaces (Maclach and Dubovi, 2001).

No specific vector or carrier for IBDV has been identified, but the virus has been isolated from mosquitoes (*Aedesvexans*), rats, and the mealworm (*Alphitobiusdiaperinus*) (Eterradossi and Saif, 2008). Viable vvIBDV was recovered after 2 days from faeces of dogs fed tissue from experimentally infected chickens, suggesting that dogs may function as mechanical vectors of the virus (Pages-Mante *et al.,* 2004). Also households with outbreaks remain contagious for 54 to 122 days (Van Den Berg *et al.,* 2004).

2.2.2. Morbidity and Mortality

Flock morbidity is very high and most birds have severe depression lasting 5-7 days. Acute or subclinical forms of the disease are less common in chickens less than 3 weeks old. All breeds are susceptible to serotype 1 virus infections, but the highest clinical signs, lesions and high mortality are observed in white leghorns (Aregitu, 2015). Mortality begins on the third day of infection, peaks on the fourth day, and then declines rapidly, and surviving chickens recover to apparent health after 5-7 days. The severity of the disease depends on the age of the infected chickens, the susceptibility of the breed, the virulence of the strain and the degree of passive immunity (Teshome *et al.,* 2015).

Flock morbidity is usually 100% and mortality can vary from 5% to over 60% depending on the virus strain and chicken breed. Mortality is usually higher in layers than in broilers (Dey *et al.,* 2019). Mortality vary among serotype 1 IBDV strains, ranging from no mortality for variant strains to approximately 20% mortality for classical strains and more than 50% mortality for vvIBDV (Jiang *et al.,* 2021).

2.2.3. Physico-chemical nature of the virus

Infectious bursal disease virus is highly resistant to adverse environmental conditions and many types of chemicals and disinfectants. More resistant to heat, UV light, ethers, chloroform, phenol derivatives, and quaternary ammonium compounds (Rashid *et al.,* 2013). The virus is highly stable and tends to persist in the environment despite thorough cleaning and disinfection (Sharma *et al.,* 2000). The virus is also impermeable to 1-hour exposure to 0.5% to 30% phenol and 0.125% trimersal. Exposure to 0.5% formalin for 6 hours markedly reduced viral infectivity (Teshome *et al.,* 2015). IBDV is also heat stable and at 56°C and remains viable after 5 hours of treatment but iodine complexes adversely affect the virus (Dwight *et al.,* 2004).

2.3. Clinical sign

Infectious bursal disease virus has a short incubation period of 2-3 days and infection usually lasts 5-7 days. Clinical manifestations include acute onset of depression, trembling, watery diarrhea, anorexia, weakness, ruffled feathers, and vent pecking. Affected chickens develop transient immunosuppression. In severe cases, the birds became dehydrated and in terminal stages subnormal temperature and death (Deresse, 2017).

2.4. Pathogenesis

Pathogenesis is the mechanism by which viruses cause host injury, disease, resulting in death, or immunosuppression in infected hosts (Van Den Berg *et al.,* 2000). IBDV results vary by strain type, dose of infectious virus, age, chicken breed, route of infection, and presence or absence of neutralizing antibodies (Kebede, 2018). During fetal development and around 10 weeks of age, immune system cells (lymphocytes) migrate to the BF and are programmed to become antibody-producing cells (Khan *et al.,* 2018).

Four to five hours after the fecal-oral route and inhalation, IBDV initiates infection and the virus replicates primarily in gut-associated macrophages and lymphoid cells in the cecum, duodenum, jejunum, and kuffer cells (Tulu, 2019). Infected macrophages transport virus to the BF, that is a major focus organ, for widespread IBDV replication in the cytoplasm of intrabursal B cells (OIE, 2016). Primary viremia develops and virus reaches BF within 11 hours after infection. A second round of viremia occurs 16 hours after infection, leading to illness and death, or the virus destroys the lymphoid follicles in the BF and secondary lymphoid tissues such as gut associated lymphoid tissue, conjunctiva associated lymphoid tissue, bronchial associated lymphoid tissue and cecal tonsil (Van den Berg, 2000). It then multiplies within BF and B cells, allowing the virus to enter the bloodstream that causes secondary viremia. Spread of the virus to other lymphoid organs such as the spleen, Peyer's patches, thymus, bone marrow, cecal tonsils and Harderian glands can occur during vvIBDV infection of predominantly susceptible chickens (Eterradossi and Saif, 2008). The virus

spreads to other organs, such as kidneys and muscle tissue, causing characteristic clinical symptoms and death (Dey *et al.,* 2019).

The acute phase of IBD lasts 7 to 10 days, during which time the bursa cortex and medulla, peripheral blood, thymic medulla B cells are depleted, and the BF atrophies. When not lethal, IBD causes immunosuppression with decreased antibody responses due to destruction of immature B-lymphocytes within the bursa, becoming more severe in young birds (Dey *et al.,* 2019). Three days post infection, the bursa starts to increase in size and weight due to body fluid accumulation (edema and increased blood in the organ). By the fourth day, the bursa is usually twice its normal weight and size, after which it begins to decrease in size. From the 8th day onwards, they are about one-third of their normal weight (Musa *et al.,* 2012).

2.5. Immune response

The virus infection in chickens activates all branches of the immune system. However, the degree of activation depends on the virulence of the infecting strain, age, immune status and genetic background of the affected chickens. Maternal antibodies may alter immune responses, and highly virulent vaccine strains may negate higher antibody levels. Offspring of parental herds vaccinated with classical IBDV strains may exhibit weak maternal immunity to the virus strain. High levels of maternal antibodies protect young chickens from vvIBDV infection for up to 3 weeks after hatch. When the IBD virus damages the BF in a young chicken, the BF can no longer program a sufficient number of lymphocytes. This causes a reduced immune system performance and immunosuppression in chickens (Orakpoghenor, 2019).

Apart from immunosuppressive effects, both humoral and cellular functions of the immunity are impaired during IBDV infection due to lysis of B cells and alterations of antigen-presenting cells. Viruses prefer actively dividing B cells compared to mature B cells (OIE, 2016). Thus, IBDV causes severe immunosuppression through lympholytic effects on surface IgM-bearing B cells (Sharma *et al.,* 2000).

Infection with the virus at young age impairs the chicken's immune response. All of these led to early observations of the immunologically destructive potential of IBDV. The degree of immunosuppressive effect depends on the age at the time of infection. Maximum damage happens when infection occurs during the first two to three weeks after hatching. Immunosuppression resulted in poor flock performance, many secondary infections, poor feed conversion, poor protective responses to vaccination, and increased condemnation rates in process-level carcasses (Van Den Berg *et al.,* 2004).

2.6. Gross lesions

The extent of disease distribution in tissues and lesion severity caused by IBD depends on the subtype and pathogenicity of the virus (Regenmortel, 2003). Gross lesions are mainly observed in the bursa and signify all phases of inflammation after acute infection (Muller *et al.,* 2003). Based on the virulence and pathogenicity, IBD can cause more or less severe lesions in the BF and other organs such as the spleen, thymus, and kidney, leading to immunosuppression and death in birds (Sharma *et al.,* 2000; Eteradossi*,* 2000). Infected birds become dehydrated and have dark discoloration of the pectoral muscles and hemorrhages occur in the thigh and pectoral muscles (Saif, 2008).

The cloaca bursa is most affected, since it is the major organ for IBDV replication (Regenmortel, 2003). BF undergoes profound changes starting at 3 days post-infection. Pathologic lesions noted on BF included enlargement and swelling with yellowish discoloration, edematous, swollen, sporadic hemorrhagic lesions, and finally atrophy are included. An increase in size is accompanied by a red color (Mossie, 2021). Office of international des epizootics states that the bursa of chickens infected with highly virulent serotype 1 IBDV has a black cherry appearance and has a prominent striation. In addition, necrotic foci of the mucosal surface and petechial or ecchymosis may be spotted in infected bursa (Sali, 2019).

The most severe cases in BF are described by severe mucosal infection and serous exudation, often escorted by yellowing of the bursal surface and petechial hemorrhage. By day 5, the bursa returns to normal size, and by day 8 it atrophies to less than one-third of its normal size

(Tanimura, 2022). Infection with classic IBDV strains causes inflammation and enlargement of the bursa within 3 days after infection. In contrast, variant strains of IBDV usually cause rapid atrophy, mucosal edema, and bursa firmness despite the absence of inflammation (Qin and Zheng, 2017).

The gross appearance of kidneys of birds necropsied during infection may appear normal. In chickens that have died or are in later stages of the disease, urate builds up in the renal tubules and ureters, often resulting in swollen, pale kidneys (Deresse, 2017). The spleen is enlarged with evenly distributed small gray foci (Orakpoghenor *et al.,* 2020). Pathologic changes in the spleen and thymus are less pronounced than those in the bursa. The liver is usually swollen, and patchy congestion has patchy effects. After death, the liver may be swollen and greenish with areas of necrosis. Hemorrhage in breast and leg muscles, dark discoloration of the pectoral muscles, and occasional hemorrhage in the leg, pectoral, and thigh muscles are also common and frequently observed, probably due to coagulation disorders (Sindu *et al.,* 2015). Increased mucus in the intestine of diseased birds is possibly due to dehydration (Baxendale, 2002).

Very virulent infections are characterized by severe clinical symptoms, high mortality, and sharp mortality curve followed by quick recovery. The vvIBDV strain has similar clinical presentation and 4-day incubation period as classical viruses, but the acute phase is worse (Tang and Saif, 2004). The vvIBDV strain causes more severe changes in the cecal tonsil, thymus, spleen and bone marrow and a greater decrease in the thymus weight index in comparison to the moderately pathogenic strain, but similar bursal lesions. The virulence of field strains of IBDV has been shown to relate with the manifestation of lesions in non-bursal lymphoid organs (Snyder *et al.,* 1988).

2.7.Histopathological lesions

The main lymphoid structures affected by IBDV are BF, spleen, thymus, Harderian gland, cecal tonsil, gut and head-associated lymphoid tissue (Oluwayelu *et al.,* 2002). B-lymphocyte degeneration and necrosis in the medullary region of the bursal follicles occurs within 1 day

after exposure. Depleted lymphocytes are rapidly replaced by hetrophil and hyperplastic reticuloendothelial cells. At 3 or 4 days post infection, all bursal follicles show IBDV-associated lesions (Saif and Barnes, 2003). Heterophil and plasma cell necrosis and infiltration occur within the follicular and interfollicular connective tissue. In addition, fibroplasia and the interfollicular connective tissue can occur, causing the surface epithelium of the bursa to become involuted and abnormal (Eterradossi, 2000).

Proliferation of the bursal epithelial layer gives rise to glandular structures of columnar epithelial cells containing mucin globules. At this stage of infection, scattered foci of repopulating lymphocytes are observed. However, these did not develop into healthy follicles. Inflammatory edema and cellular infiltrates extend from the bursal follicle to the muscular layer and subserosal tissue. In capillaries and venues; monocytosis and hyperemia are observed, sometimes accompanied by hyaline thrombosis and hemorrhage. In the spleen, hyperplasia of reticulo-endothelial cells surrounding the arteries of the adenoid sheath is seen early in infection. Lymphatic necrosis appears in the periarteriolar lymphatic sheath 3 days after infection. The spleen recovers rapidly without lasting damage to the germinal follicle (Ingrao *et al.,* 2013).

Alterations in the thymus and cecal tonsils occur shortly after infection and include areas of lymphoid necrosis and hyperplasia of reticular and epithelial components in the medullary regions of thymic follicles. Histological lesions in the kidney are non-specific and possibly due to severe dehydration of the affected chickens. Observed lesions consisted of large casts and glomerular hypercellularity of homogeneous material infiltrated with heterotrophils (Tanimura, 2022).

2.8. Diagnosis

Diagnosis includes consideration of flock history, clinical signs, and postmortem lesions. Because IBDV is difficult to isolate, immunological methods are best used to confirm clinical disease or detect subclinical disease. In chickens infected with IBD, pathological lesions in the

BF are characteristic, and histopathological studies combined with detection of viral antigens by immunohistochemistry confirm IBDV infection (OIE, 2018).

2.8.1. Histological diagnosis

Histological diagnosis is centered on detection of alterations in the bursa. The ability to induce histological lesions in non-bursal lymphoid organs such as the thymus (Inoue *et al.,* 1999), spleen or bone marrow has been described as a potential feature of highly virulent IBDV strains. The advantage of histological diagnosis lies in the probability of diagnosing both acute and chronic or subclinical forms of the disease. A typical feature of vvIBDV strains is their ability to cause lesions in the thymus, spleen, or bone marrow (Van Den Berg *et al.,* 2000).

Detection of viral antigens: thin sections of the BF prepared to detect viral antigens specific to IBDV done by direct and indirect immunofluorescence or by immunoperoxidase staining in the bursal follicles of infected chickens. The use of monoclonal antibodies in Immunohistochemistry techniques for detection of the virus enhances the specificity of the test (Teshome *et al.,* 2015).

2.8.2. Virological diagnosis

The virus can be detected in the BF in chicks during the acute phase of infection, ideally within the first 3 days after the onset of clinical symptoms. For isolation, inoculate 9- to 11-day-old embryonated eggs from chickens without anti-IBDV antibodies with a filtered homogenate of the BF. The most profound route of inoculation is the chorioallantoic membrane. The yolk sac route is also viable, but the intra-allantoic route is the least sensitive (Tulu, 2019). Neutralizing the viral effects with a monospecific anti-IBDV serum must prove the specificity of the observed lesions. Isolation in embryonated eggs does not require viral adaptation by serial passaging and is suitable for vvIBDV. In the absence of lesions, the first passage embryos should be homogenized and clarified under sterile conditions and two additional serial passages performed (Mekuriaw *et al.,* 2017).

2.8.3. 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, in order to measure the titre of passive antibodies and determine the appropriate date for vaccination or in laying hens to verify success of vaccination (Sharma *et al.,* 2000).

Serology is likewise essential to confirm the disease-free status of flocks. Each serological analysis must include a sufficient number (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). Little attention has been paid to serodiagnosis in endemic areas because test cannot distinguish between antibodies elicited by virulent IBDV and those elicited by attenuated vaccine viruses (Flensburg, 2002).

2.8.4. Molecular detection

Most molecular identification efforts have determined on characterizing the larger segment of IBDV (segment A), particularly the coding region. Several protocols have been published for characterizing RT-PCR products using restriction endonucleases (Zierenberg *et al.,* 2001). RT-PCR is one of the most usual molecular diagnostic tests to detect the IBDV genome within the BF. It helps to detect viral RNA in homogenates of infected organs/embryos and cell cultures without considering the viability of the virus present. RT-PCR can recognize the viral genome without replication in cell culture, eliminating the need to grow the virus prior to amplification (Aliy *et al.,* 2020).

RT-PCR combined with restriction fragment length polymorphism (RFLP) is a highly functional and rapid method for characterizing and detecting existing and emerging virus strains. RT-PCR products are sequenced for advanced characterization of IBDV strains. The

outer capsid protein of the VP2 gene contains a hypervariable region, suggesting a possible location for differentiation of the IBDV strain (Zierenberg *et al.,* 2000).

It is also used to detect non-replicating viral genomes in cell culture, as it does not demand propagation of the virus prior to amplification. RT-PCR is performed in three steps. These are; extraction of nucleic acids from test samples, conversion of IBDV RNA to cDNA by reverse transcription (RT), and amplification of cDNA by PCR. In dissimilarity to single-stranded RNA, IBDV double-stranded RNA (dsRNA) cannot be degraded by RNAase (Msomi *et al.,* 2017).

2.9. Differential diagnosis

Avian coccidiosis, Newcastle disease, Infectious bronchitis, Hemorrhagic syndrome and Marek's disease are differential diagnoses of IBD. The virus can be detected in all acute forms of the disease by the appearance of bursal lesions. When asymptomatic, bursal atrophy is difficult to distinguish from Marek's disease and other Infectious anemia. In this case, histopathological observation of the bursa can help distinguish between these diseases (Aregitu, 2015; OIE, 2018).

The lesions and symptoms of coccidiosis are very related to those of IBD. Like IBD, coccidiosis is correlated with abrupt onset, ruffled wings, and bloody droppings, but no bursal lesions. Also, muscle hemorrhage and bursal edema can distinguish IBD from coccidiosis. Non-infectious diseases such as mycotoxins are also responsible for bursal atrophy. With aflatoxins, there is degeneration of both the thymus and bursa. High doses of zearalenone also lead to decreased bursa weight. As in other animal species, steroidal anti-inflammatory drugs such as corticosteroids induce apoptosis and decrease lymphocyte production. Poorly nourished or stressed animals may develop smaller bursa (Sali, 2019).

2.10. Prevention and control

A strong conflict between the virus and physico-chemical factors causes the virus to persist in the external environment. It seems unrealistic to eradicate the disease in affected countries. There is no exact treatment for IBD. Antibiotics are used to treat or prevent secondary infections of the disease. Vitamins, electrolytes and minerals are administered to support acid-base balance (Deresse, 2017). Vaccines alone will not solve the problem unless a combination of strategies is used (Van Den Berg *et al.,* 2000). Therefore, a successful prevention and control program must include an effective vaccination programme, hygiene measures, medical prophylaxis, good biosecurity, cleaning and disinfection, and adequate chick sources are critical for disease prevention and control (Muller *et al.,* 2012). With strict hygiene measures, localized prevention of IBD outbreaks has been achieved worldwide through vaccination (Bulcha *et al.,* 2021).

Vaccines and vaccination programs vary widely and depend on several local factors (modes of production, level of biosecurity, local disease patterns, maternal antibody status, vaccine availability, costs and potential losses). After an outbreak of IBD, the farm should be evacuated and thoroughly cleaned and disinfected. Houses should be left empty for at least 6 months after disinfection with iodophor and formalin. Very strict restrictions should be imposed on vehicle, equipment and visitor access to farms (Teshome *et al.,* 2015).

2.10.1. Vaccination

The precise approach to prevent and control IBD depends on hygiene practices, vaccination schedules, maternal antibody levels and variability, and vaccine strain choice. And, as reported in various studies, standard vaccination programs and stringent biosecurity measures are essential tools to prevent and control this disease (Sze *et al.,* 2016; Shegu, 2019). Immunization programs depend on several factors. These factors include the type of production system, level of biosecurity, maternal antibody levels, vaccine availability, cost, and potential losses from IBD (Mossie, 2021).

Live-attenuated vaccines can be prepared from fully or partially attenuated classical and mutant strains of IBDV by passaging the virus in tissue culture or embryonated chicken eggs (Jackwood and Sommer-Wagner, 2011). Classical live attenuated vaccines are suitable for mass vaccination and can induce strong humoral and cellular immunity in 3–6 week old chickens. Mild or intermediate vaccines are used in hens to induce a primary response prior to immunization. The vaccine is administered by intramuscular injection, spray, or use of drinking water at 8 weeks of age (OIE, 2018). Mild vaccines specifically for pathogen-free chickens are suitable for vaccination of breeding hens. It is not particularly effective in the presence of high levels of maternal antibodies or against vvIBDV strains. Therefore, a mild vaccine should be administered after maternal antibodies have disappeared, i.e. between 4th week and 8th week (Van Den Berg *et al.,* 2000).

2.10.2. Biosecurity

Developing and implementing routine biosecurity procedures as best management practices on poultry farms reduces the likelihood of introducing infectious diseases. The risk of disease transmission between farms can be mitigated through proper management of farms. Disease outbreaks in poultry can spread between farms and severely impact poultry farms. The risk of developing disease on farms is influenced by many factors such as bedding, feed and water management, disinfection of sheds, pest control, disposal of used litter and bird carcasses; and the effectiveness of biosecurity measures for people and equipment entering the farm (Stephen, 2012). Biosecurity in small-scale farms is not practiced well as there is no adequate practice to isolate sick birds from livestock herds, and dead birds are left to livestock and wild predators. Chickens and eggs are sold on the open market along with other food items. Current systems for selling live birds pose significant and potential hazards to both buyers and sellers, but it is generally difficult to introduce biosecurity and sanitation practices into such systems (Solomon 2007).

2.11. Status of IBD in Ethiopia

Frequent disease outbreaks and the emergence of new strains of infectious diseases became a challenge for Ethiopia's young poultry sector. In Ethiopia, IBD was first reported in 2002 in 20–45-year-old chickens and chickens from commercial farms (Jenberie *et al.,* 2014). The disease later became a major problem in poultry systems in Ethiopia (Mossie, 2021). The mortality rate of IBD in different poultry farms varies between 45-50% and the average mortality rate is 49.89%. In recent years, 25-75% of deaths/losses of exotic and crossbred chickens have been attributed to IBD (Woldemariam and Wossene, 2007). Several cases have been reported from different parts of the country. Consequently, Amajo *et al.* (2022) reported 48.96% from the Wolaita zone of southern Ethiopia. A national survey showed that the rate of IBDV seropositivity in domestic chickens is almost 92% (Chaka *et al.,* 2012; Jenberie *et al.,* 2012), and IBDV isolates happen to be clonal and highly virulent. As research has shown, the mortality of chickens and due to the disease is 58.09% and 25.08% respectively (Zeleke *et al.,* 2005).

The seroprevalence of IBD is significantly higher in crossbred and exotic breeds compared to indigenous chickens (Tadesse *et al.,* 2014). The disease occurs more in intensive production systems (85.9%) than in extensive production systems (81.6%). Broilers had a mortality rate of 56.09% while layers had a mortality rate of 25.08% (Zeleke *et al.,* 2005). The seroprevalence of IBD in the country is 83.1% (Jenberie *et al.,* 2012). Reports Woldemariam and Wossene (2007) showed a high seroprevalence of IBD of 100%. Mazengia (2008) reported an overall prevalence of 51.1% in Bahir Dar and Farta districts. There are also studies by various authors who have found the disease to be common in different parts of our country. A seroprevalence of 45.05% for IBD in chickens reared in a backyard poultry production system in Mek'ele city was reported (Zegeye *et al.,* 2015).

In backyard chickens taken from a market in central Ethiopia, the prevalence was 91.9% in the dry season and 96.3% in the rainy season (Chaka *et al.,* 2012). Another study in Central Oromia reported a seroprevalence of 82.2% and Kebeles of Bishoftu reported 27.8% mortality in the absence of vaccination (Tesfaheywot and Getnet, 2012) and Nigussie (2007) reported a

high seroprevalence of 61% among backyard chickens in Addis Ababa and Adami Tulu districts. A study conducted in both the farmers' associations and the Bishoftu Kebele showed the presence of IBDV-specific antibodies without vaccination, indicating exposure of domestic chickens to the virus. According to this study, the total seroprevalence of IBD in Bishoftu was 82.20% (Zeryehun and Fekadu, 2012).

2.12. Economic importance

Infectious bursal disease has a tremendous economic impact on production, from direct to indirect losses. Direct losses in IBD are due to deaths from the disease and secondary infections and the indirect loss is economic losses due to decreased production parameters and due to subclinical infections. The economic impact of the disease is also significant, causing most of the economic destruction associated with IBD due to secondary infections, growth retardation and immunosuppressive effects that lead to carcass destruction (Tulu, 2019). In addition, the increased use of antibiotics to combat co-infections has increased public health concerns, with variable mortality rates affecting both layers and broilers. Mortality can reach 100 percent in highly virulent disease strains. Even if the bird survives, the resulting immunosuppression and impact on laying hens' egg production is significant (Muller *et al.,* 2003).

In Ethiopia, an outbreak investigation was conducted in 2002 on a suspected case of IBD reported from a commercial poultry farm in the city of Bishoftu (Zeleke *et al.,* 2005). At the time of the survey, 22,437 broilers and 2,508 broilers had died, and within 4 weeks, 40,000 Hubbard broilers and 10,000 Roman Brown eggs were at risk of on-farm outbreaks. It has been previously reported that IBD is the leading cause of economic losses in the poultry trade. Poultry producers are usually concerned that if birds survive, these economic mortality costs will be incurred and not exceeded for lost flocks. The sum of these losses, when clearly quantified as above, tends to be unimaginably large and alarming. IBD remains a serious disease problem in domestic and farm chickens, posing a significant danger to poultry production (Tulu, 2019).

3. MATERIALS AND METHODS

3.1. Study areas and study period

The study was conducted in Bishoftu, Mojo, Dukem and Gelan poultry farms based on IBD outbreak report from December 2022 to April 2023.

Bishoftu is located in Oromia National Regional State about 45 km south east of Addis Ababa and is at an altitude of 1850 meter above sea level. The area has an average annual rainfall of 800 mm and average maximum and minimum temperature of 28^{o}C and 12.3^{o}C, respectively. It is found at 9^{o}N latitude and 39^{o}E longitude (https://en.wikipedia.org/wiki/Bishoftu accessed in May 18, 2023).

Mojo is located at about 65 km away from Addis Ababa in the East Shewa Zone of the Oromia. It has latitude and longitude of 8°N and 39°E with an elevation between 1788 and 1825 meters above sea level. The average minimum and maximum temperature is 21^{o}C and 30^{o}C respectively with average annual rainfall of 243mm (30^{o}C respectively with average annual rainfall of 243mm (https://en.wikipedia.org/wiki/Mojo,Ethiopia accessed in May 18, 2023).

Dukem is located in the Oromia Special Zone surrounding Finfinne at 37 km southeast of Addis Ababa and 10 km northwest of Bishoftu. This town has a latitude and longitude of 9^{o}N 39^{o}E respectively and an elevation of 1950 meters above sea level. The average minimum and maximum temperature is 9^{o}C and 26^{o}C respectively with an average annual rainfall of 800mm (https://en.wikipedia.org/wiki/Dukem accessed in May 18, 2023).

Galan is a town located in the Akaki district of the Oromia special zone surrounding Finfine and 25 km South East from Addis Ababa-Adama highway crossing galan town. This town has a latitude of 8^{o} 52’0” N and longitude of 37^{o} 46’59” (https://en.wikipedia.org/wiki/Galan_(town) accessed in May 18, 2023).

3.2. Study animals

Chickens showing clinical signs of IBD in the susceptible age group and different breeds located in poultry farms in the study areas were study animals. Saso, Bovans brown, and Cob 500 breed type chickens between 22 and 45 days old, and from both sexes were included into this study due to outbreak reports. Privately owned chickens from small and medium-scale production systems were included. Chickens, which were diseased or dead recently were assessed.

3.3. Study Design and Sampling method

An outbreak based cross sectional study design was carried out from December 2022 to April 2023 on suspected IBD outbreaks. It was purposive sampling in which farms were visited based on information on the occurrence of outbreaks of IBD during the study period. Outbreaks were reported from 20 farms, and 100 chickens were examined. Five chickens were assessed per outbreak per farm according to OIE (2018) and the total numbers of samples in the study were determined based on the frequency of outbreaks reported. A questionnaire survey was prepared and administered to 20 farm owners who faced this suspected IBD outbreak and to 30 immediate neighbor farms without outbreak to assess factors associated with the occurrence of IBD and to evaluate the level of biosecurity measures taken by farm owners.

3.4. Sample collection and processing

3.4.1. Questionnaire survey

Questionnaire survey (Annex I) was administered to 50 poultry farm owners from the 20 outbreak suspected farms and from 30 of their respective nearby farms; information gathered included production type, location, contact with other farms, previous exposure to the disease, biosecurity practices, and practice of vaccination. Personal observation was used to assess the general status of the poultry farms.

3.4.2. Clinical and Postmortem examination

All moribund chickens were thoroughly observed for clinical signs after taking histories in 20 farms suspected of IBD outbreak. Representative chickens within the expected age range (3-6 weeks), which showed indicative clinical signs of IBD like rapid onset of the disease, whitish diarrhea, depression and marked drop in feed; were used for further investigation. Accordingly 100 chickens were opened following a standard post mortem examination procedure (Butcher and Miles, 2015). The observed gross pathological lesions in the bursa of fabricius, spleen, kidney, pectoral and thigh muscle, liver, thymus, proventriculus and gizzard muscle were recorded (Annex II).

The gross lesions of bursa and other lymphoid organs were categorized according to Prabhu *et al*. (2020) where (-) stands for no gross lesions and appearing normal; (+) for bursa slightly enlarged and edematous; (++) for edematous, enlarged or hemorrhagic bursa with changes in any of the lymphoid organs (hemorrhages in thymus/ necrotic foci in spleen/hemorrhage in caecal tonsils) and (+++) for gross changes in the lymphoid organs including other organs (liver, kidney, muscle and proventriculus).

3.4.3. Molecular detection

The bursal tissue samples for molecular detection were collected from chickens aged 3-6 weeks, which had clinical signs and gross pathological features suggestive of IBD in the farms suspected of having the disease outbreaks. The bursal tissues were collected aseptically in pre-sterilized cryovials with virus transport media (VTM) and transported on icebox to national veterinary institute (NVI), and kept at -80^{O}c until to be processed (OIE, 2016). From purposively selected 20 poultry farms, a total of 20 pooled bursa samples (bursal tissues from five different chicken per pool), were collected from 100 suspected chickens. Accordingly 8 pools from Bishoftu, 5 from Dukem, 4 pool from Mojo and, 3 pool from Gelan were collected. But due to resource limitations, only 5 pool (selected based on lesion score) were processed for molecular detection.

Sample preparation

The viral antigen was prepared from the collected suspected BF samples and samples were thawed and taken out from the labeled sample bottle in the class II Biosafety cabinet and took 1 gram of tissue aseptically. The tissue sample was chopped into small pieces using a sterile scalpel blade, and scissors, and also minced using a mortar and pestle. A 10% (W/V) suspension of each bursa sample was prepared in sterile phosphate buffer saline solution supplemented with penicillin and streptomycin (1000 µg/ml each). The suspension was transferred into sterile centrifuge tube and centrifuged at 3000 rpm for 10 minutes. The upper aqueous phase (supernatant) fluid was harvested aseptically to sterile test tubes and stored at -80^{o}C for the next process.

RNA extraction

The field sample was subjected to processing prior to a molecular biology analysis, whereby the tissue suspension was subsequently dispatched to the National Veterinary Institute's molecular biology laboratory. RNA extraction was performed in the molecular biology laboratory. The ribonucleic acid of the IBDV virus was isolated from the suspension sample using the RNeasy® Mini Kit manufactured by QIAGEN, Germany, in accordance with the manufacturer's instructions for RNA extraction. The extraction step was as follows: A volume 350 µl of the homogenized sample suspension were transferred to Eppendorf tube and 350 µl of RLT buffer was added and homogenized by vortex then centrifuged at 13,000 rpm/min for 3 minutes. Again, 1 volume of 70% ethanol was added to the lysate, and mix well by pipetting with no centrifugation.

The next step was to transfer 700 µl of the sample to a column placed in a 2ml collection tube, close the lid, and centrifuge for 15 s at 13,000 rpm/min then, discard the flow through. After centrifugation 700 µl Buffer RW1 was added to the RNeasy spin column, close the lid, and centrifuge for 15s at 13,000 rpm/min. discard the flow through. Then add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15s at 13,000 rpm/min. Discard the flow through. 500 µl Buffer RPE was added to the RNeasy spin column, Close the lid, and

centrifuge at 13,000 rpm/min. the RNeasy spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1min to dry the membrane. As optional step places the RNeasy spin column in a new 1.5ml collection tube. Then 30-50 µl of RNase-free water was added directly to the spin column membrane, close the lid, and centrifuge for 1 min at 13,000 rpm/min to elute the RNA. Finally, the extracted RNA underwent RT-PCR analysis to assess the existence of IBDV using custom-designed Forward primer IBDF3 design (5'TCT TGG GTA TGT GAG GCT TG -3') and Reverse primer design IBDR3 (5'CCC GGA TTA TGT CTT TGA -3') primers specific to the V2 gene, in accordance with the guidelines of the World Organization for Animal Health (OIE, 2018).

Conventional Polymerase chain reaction (PCR)

The PCR technique was carried out in conventional one step RT-PCR. The master mix preparation and reaction volume was described as following. The viral suspensions were subjected to VP2 gene amplification analysis after addition of 5µl template RNA to the master mix in amplification room. Touchdown PCR was conducted in a final reaction volume of 25 µl using a 250 µl capacity thin wall PCR tube containing 5 µl 5X PCR Buffer with MgSO4 (Fermentas), 1 µl 10 mM dNTPs (Thermo Scientific), 4 µl RNase-free water, 1 µl RT-PCR enzyme (Taq DNA polymerase), 2 µl of each primer, and 5 µl of cDNA template. The PCR reaction was done one cycle at 50°C for 30 minutes for cDNA template followed by initial denaturation of the PCR reactions for one cycle at 95^0c for 15 minute; subsequent done at 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds for 35 cycles; and the final extension for one cycle was done at 72 °C for 7 minutes. In accordance with the Kit protocol, an RT-PCR single-step process was performed for the synthesis of complementary DNA (cDNA). Subsequently, the amplified fragment was subjected to visualization and subsequent comparison with bands derived from molecular markers.

Agarose gel electrophoresis of PCR product

PCR products were analyzed by 1.5 % (w/v) Agarose electrophoresis gel stained with gel red and 100mL TAE buffer. Briefly, 5 µl each PCR products was mixed with 1 µl 6X loading

buffer and loaded in to separate well of the pre-prepared gel and 100bp DNA molecular marker was also added onto the first lane and run at 120 volt for about 1:20 hours in electrophoresis apparatus. The PCR products band was visualized by gel documentation under UV-lamp camera and the size of the PCR products was estimated by comparing with the band size of the molecular marker 100bp ladder (marker) that was loaded on a separate lane (OIE, 2012).

3.4.4.Histopathological procedure

For histopathological examination, tissues with gross lesions (bursa, proventriculus, spleen, liver and kidney) were trimmed (5 mm up to 1cm in size) and fixed in 10% buffered formalin. Tissue samples were processed by automated tissue processor starting with formalin in the 1st utensil followed by dehydration in a series of increasing alcohol concentrations (70%, 80%, 100%, 100%, 100%), and clearing by two passages in xylene I and II. Then, the tissue was impregnated by molten paraffin wax one and two at melting point temperature of paraffin wax, which is 54-60^{o}C. Finally a tissue block was made (embedded) using molten paraffin mixed with bee wax. The embedded tissues were sectioned by rotary microtome (5µm) according to Bancroft and Gamble, (2008).

The sectioned ribbon were collected and placed on water bath at 40-50^{0}C .The relaxed ribbons on water bath were picked up on clean microscopic slide and labeled. The labeled slides were deparaffinized (hot oven & xylene) and rehydrated in descending gradient of ethyl alcohol (100%, 100%, 100% 80%, and 70%). The rehydrated tissues were stained with Meyer's hematoxylin and eosin dye and, mounted with cover slide using Dipex. The stained tissue scanned at 10x and microscopic changes were examined at 40x & 100x objective. Major histopathological changes were recorded. All the histpathological procedures were done in Animal Health Institute, Veterinary Pathology Laboratory.

3.6. Data analysis

The collected data was coded and recorded in Microsoft office Excel spreadsheet 2010. Descriptive statistics was used to summarize data of lesion and questionnaire using Stata Version 14.

3.7. Ethical statement

Ethical clearance with certificate reference number VM/ERC/28/02/14/2023 for this research was obtained from Addis Ababa University College of Veterinary Medicine and Agriculture Animal Research Ethical Review Committee, and all procedures were conducted according to animal research ethics (Annex III).

4. RESULTS

4.1.Questionnaire survey findings

From the visited farms in the study areas, their production types were 62% broiler and 38% layer. According to these survey 70% poultry producers rented the poultry house and often change their location, and only 30% of respondents owned their own poultry house. The educational statuses of the owners were 20% in primary school, 30% in secondary school and 50% of them were above secondary school.

From the respondents, 52% said, they had IBD like disease in their poultry farm previously while 18% never had IBD like disease. Thirty percent of the respondents have rented the poultry house recently and hence do not know whether IBD like disease outbreak occurred in the farm. Fifty four percent of poultry producers had different supplier for their source of day old chicks while 46% always use the same supplier. Seventy four percent of the respondents said they regularly vaccinate their chickens for IBD properly whereas 26% didn't have proper follow up on their chicken vaccination schedule.

Seventy percent of the respondents reported the practice of isolation of sick chickens into separate house; while 30% of respondents did not practice isolation of sick chickens. When they were asked if they sell diseased or recovered chickens, 40% of the respondents said they sold but 60% responded they don't. Seventy-four percents of owners burn or buried dead chickens but 26% of them threw dead chickens out on the road. Personal observation indicated that, the poultry premises were poorly ventilated with dusty litter; the chickens were over-crowded and managed under poor hygienic conditions on a floor system. Poultry producer's response on biosecurity practice is given in the following table.

Table: Biosecurity practice of the respondents

Biosecurity practices	Response	%
Practice of all-in all-out	Applied	72
	Not applied	28
Cloth & shoe change room	Have	80
	No	20
Hand wash & disinfect	Practiced	54
	Not practiced	46
Foot bath	Yes	68
	No	32
Access to the farm	Access never granted	56
	Conditional Permission	44
Contact with other farm	Yes	42
	No	58
House disinfection	Yes	52
	No	48

4.2. Clinical findings

In farms with occurrence of out break and visited, the chickens were in age range of 22-45 day olds. Of sick chicken that manifested clinical sign, 11% were within age range of 22-28days, 61% were within age range of 29-35 days, 25% were within age range of 36-42 days and only 3% were within age range of 43-45days.

The most frequent clinical symptoms observed were; depression (Fig. 1A), marked drop in feed and water intake, rapid onset of the disease followed by mass death (Fig. 1B), white creamy diarrhea, soiling of the vent and loss of body weight.

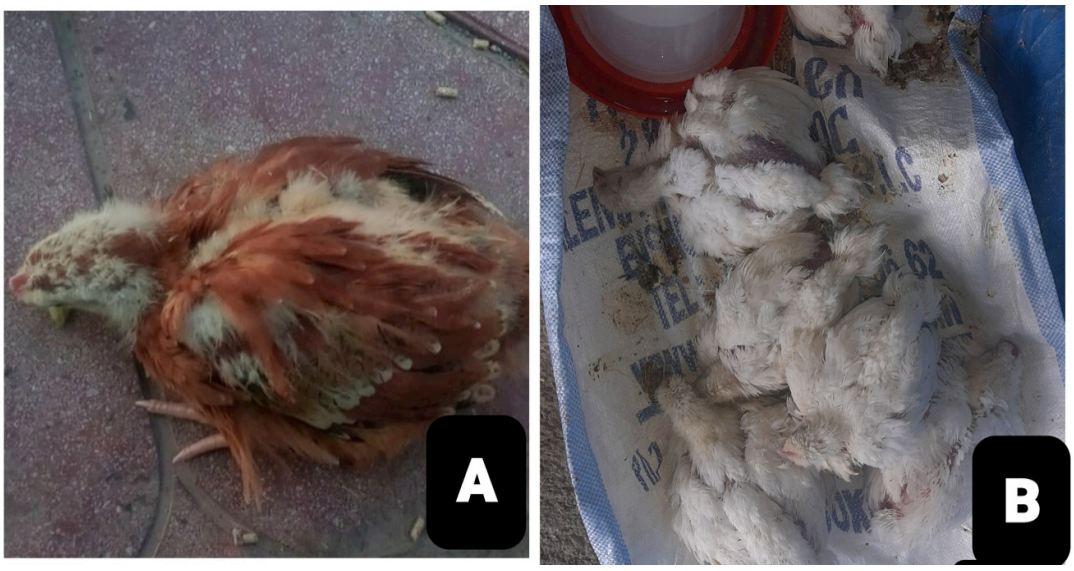

Figure 1: Clinical manifestations of IBD: Depression **(A)**, mass death **(B)**

4.3. Characterization of gross pathological lesions

The frequently found gross lesions in IBD suspected chickens were in BF, spleen, cecal tonsil, proventriculus, gizzard, kidney, liver, pectoral muscle and thigh muscle. The organs with gross lesions and their frequency of occurrence from the 100 chickens with necropsies are indicated in Fig. 2.

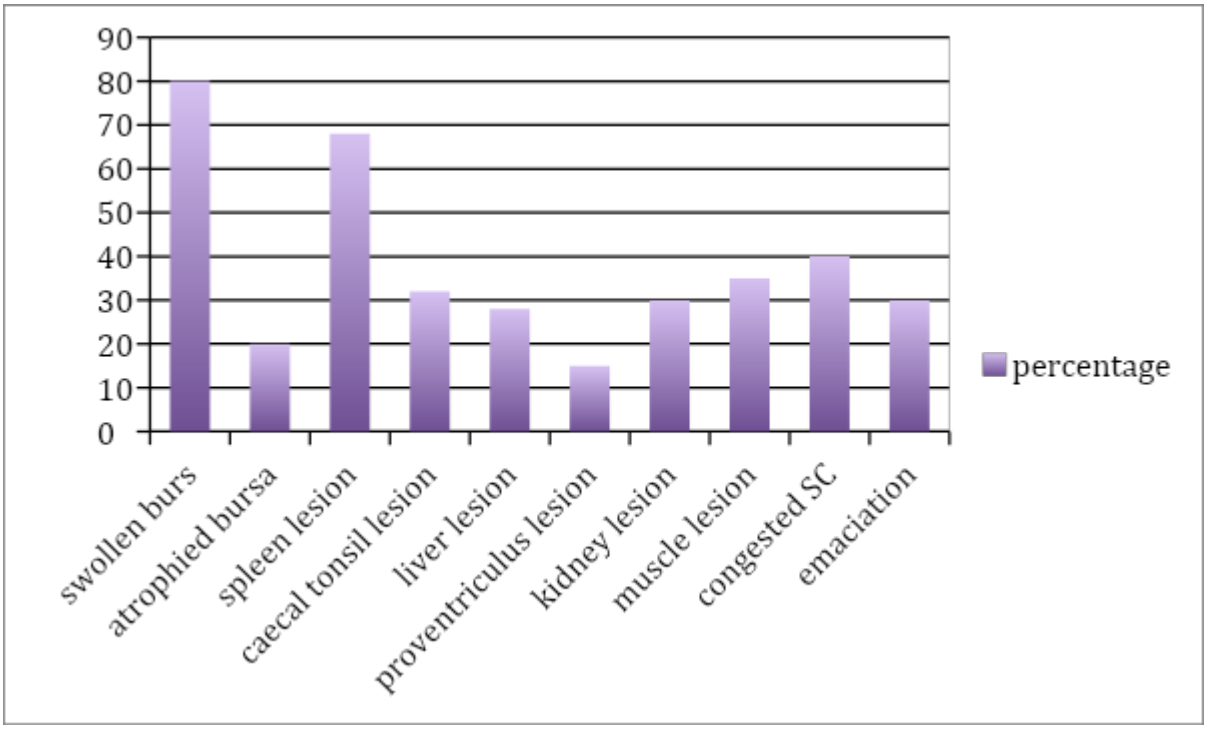

Figure 2: Gross pathological change frequency of IBD in different organs

In present study, based on Prabhu *et al*. (2020) lymphoid organ gross lesion score category, 11% of examined bursa showed only slight enlargement and were edematous (category +). In 43% of grossly examined cases, bursas were edematous, enlarged and hemorrhagic; caecal tonsils were hemorrhagic and swollen and the spleens were either congested or hemorrhagic (category ++). And in the remaining 46% of the examined cases; in addition the bursa, caecal tonsil and spleen; the gross lesions were observed in liver, kidney, muscle and proventriculus (category +++).

The most frequent (80%) gross bursal lesion was swelling, and hemorrhages. Sometimes the bursal hemorrhage was observed even from serosa of the bursa. In some cases, bursas were slightly hemorrhagic although they were swollen. Upon incision, severely enlarged and hemorrhagic BF and bursal folds (Fig. 3A) were appreciated. Severe petechial and ecchymotic haemorrhages resulting in complete discoloration from red to blackish red (Fig. 3B) was also observed. The BF usually had a yellowish gelatinous film around it. In some cases bursas were atrophied, and contained whitish-creamy exudates.

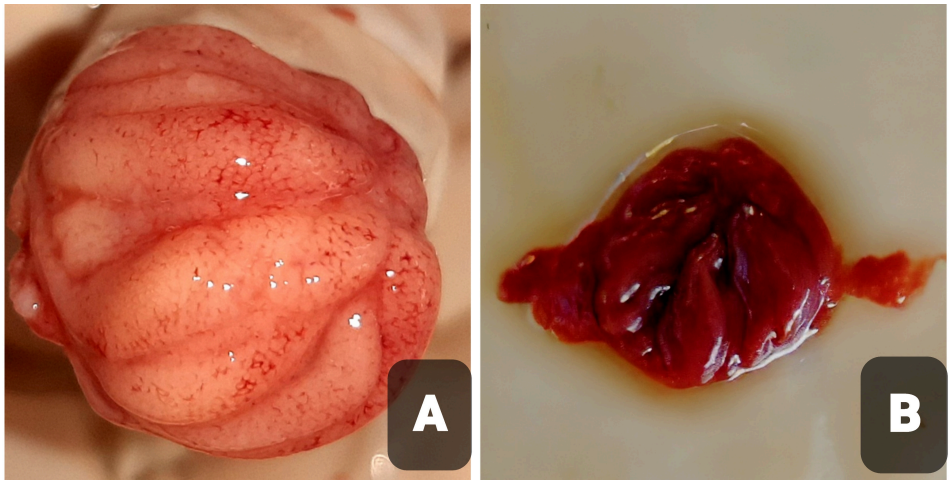

Figure 3: Lesions in the BF. Enlarged, edematous and haemorrhagic BF **(A, B)**

The spleen became swollen, congested and dark red in 68% of chickens (Fig. 4). Moderate to severe splenomegaly with small gray foci uniformly distributed on the surface has been observed.

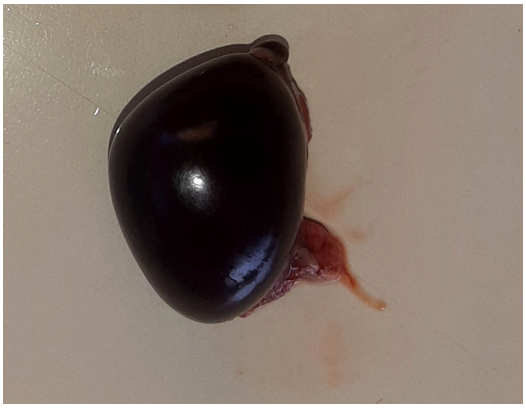

Figure 4: Gross lesion of spleen: congested and swollen spleen

Liver was pale and moderately swollen in 28% of the affected chickens (Fig. 5A) while the remaining livers were apparently normal or had no visible gross lesions. Some of the livers were bile stained. There were diffuse paleness in the lobes of affected livers and hepatomegaly was frequently observed. Grossly, kidneys were swollen, hyperemic or hemorrhagic in 30% of the cases (Fig. 5B). Kidneys were slightly hypertrophied in caudal part, and ureters were

distended with urates. The proventriculus and gizzard showed petechial hemorrhagic lesions in 15% of the cases (Fig. 5C). Cecal tonsils were also enlarged and petechial hemorrhage was commonly found in 32% of the cases (Fig. 5D). The thigh and pectoral muscles showed petichial and ecchymotic hemorrhage (35%). And 30% of the affected chickens were highly emaciated and dehydrated. Congestion of subcutaneous blood vessels were observed in 40% of the affected chickens.

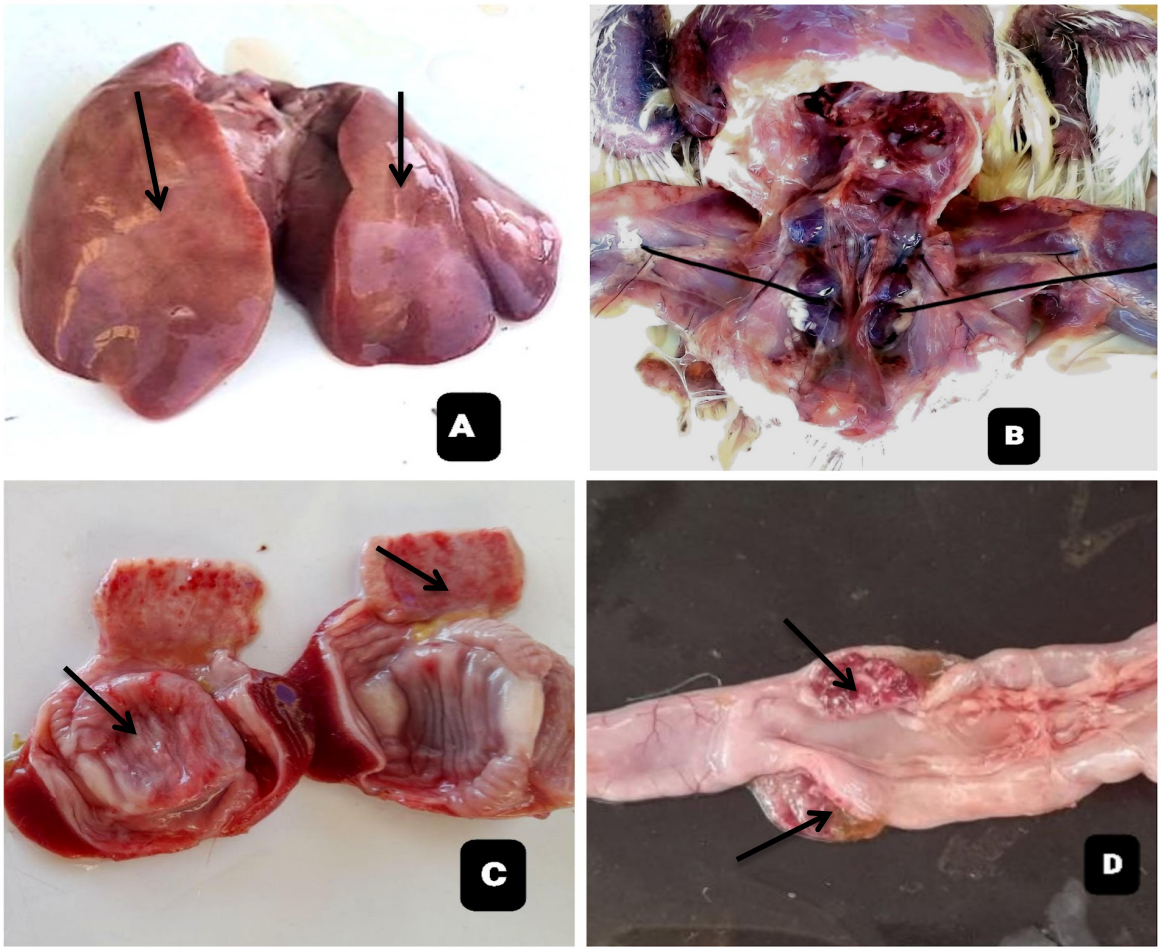

Figure 5: Gross lesions of different organs: diffuse pale and swollen liver **(A)** (black arrow), enlarged and hemorrhagic kidney **(B)** (black arrow), petechial hemorrhage in proventriculus and gizzard **(C)** (black arrow), swollen and haemorrhagic caecal tonsil **(D)** (black arrow)

4.5. Histopathological findings

Microscopic lesions of the bursa include vascular changes in which the veins and capillaries were congestion (dilated and filled with excessive RBCs) (Fig. 6), massive multi focal lymphoid necrosis in the lymphoid follicles of bursal plicae. Hyperplasia of the bursal interfollicular epithelium gives rise to typical false glandular structure (Fig. 7A). The depletion of the lymphoid follicles gives cystic structure both in the cortical and medulary regions with accumulation of necrotic debris in the cystic structures (Fig. 7B). In grossly atrophied bursa there were interfollicular proliferation of fibrous connective tissue.

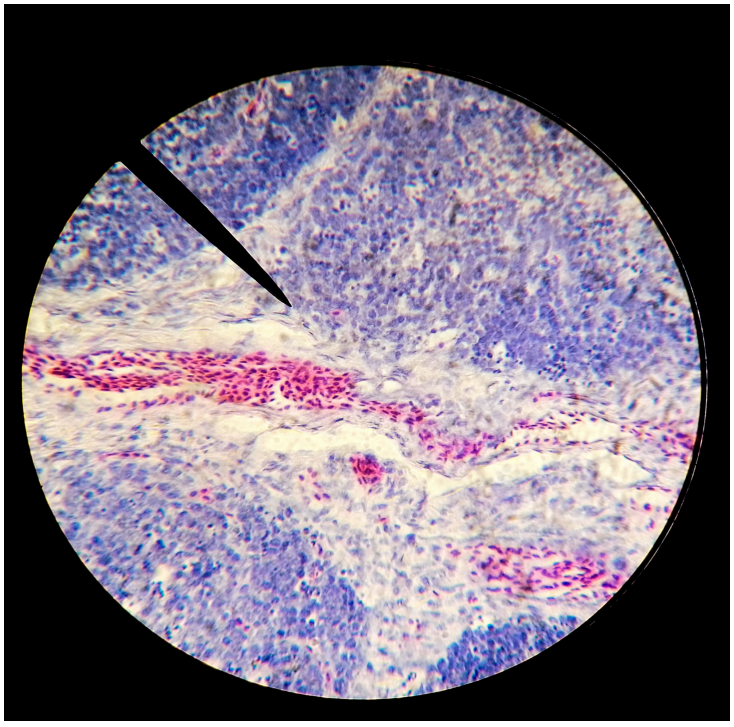

Figure 6: Haemorrhagic bursa**.** Severe distributed hemorrhage in interfollicular spaces of BF (microscope arrow)

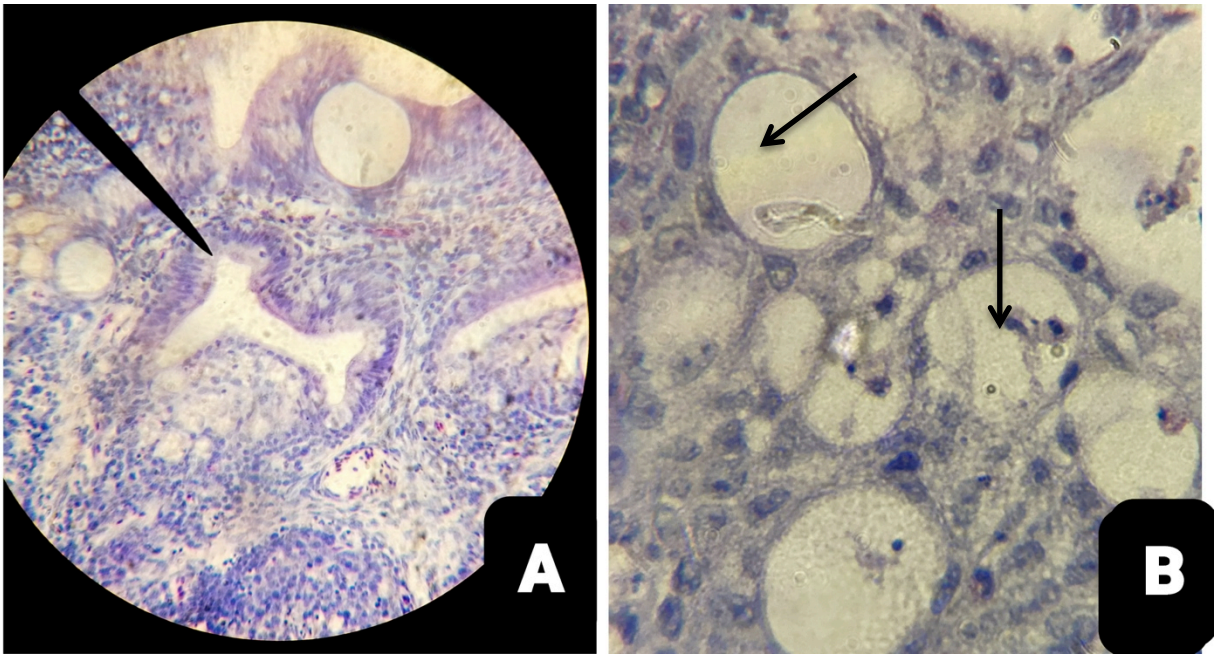

Figure 7: Microscopic lesion of Bursa: Typical interfollicular epithelium hyperplasia that give false glandular structure **(A)** (microscope arrow), cystic structure and severe necrosis of lymphocyte **(B)** (black arrow)

In spleen, among the microscopic lesion observed there were lymphocitic depletion in both white pulp and red pulp but severe necrosis of the lymphocytes was in the lymphoid follicle of the white pulp. There were hemorrhages in the trabecular sinuses (Fig. 8A). There were formation of multiple or multifocal lymphoproliferative nodules (Fig. 8B) There were multiple areas of lymphocytic necrosis that gives empty spaces in dense cortical region of spleen with few lymphocytes showing nuclear pyknosis and the rest without nucleus (Fig. 8C & 8D).

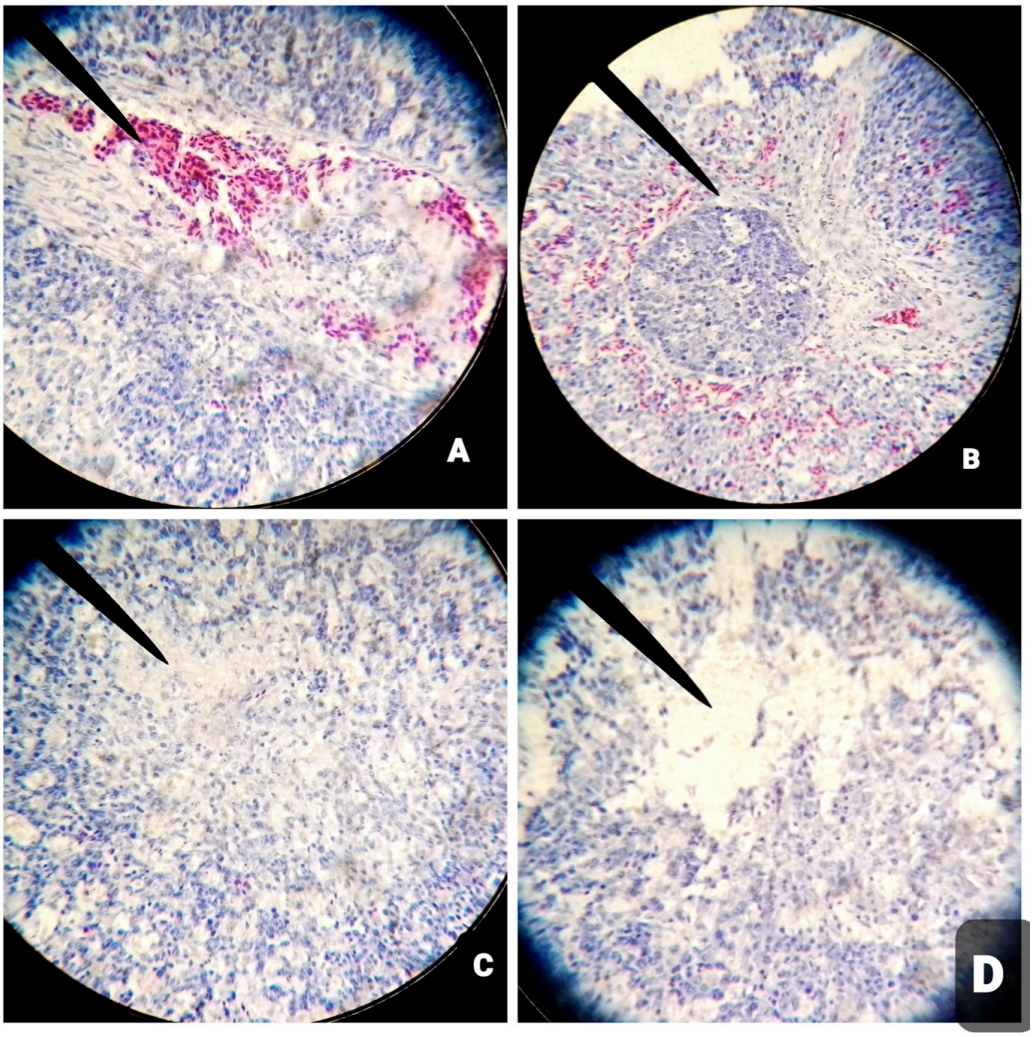

Figure 8: Microscopic lesion of spleen: Hemorrhage in the trabecular sinuses **(A)** (microscope arrow), Multiple or multifocal lymphoproliferative nodules **(B)** (microscope arrow), Multiple areas of lymphocytic necrosis in the dense cortical region of spleen, nuclear pyknosis and rhexis **(C)** (microscope arrow), some areas of total loss of lymphocytes give an empty space **(D)** (microscope arrow)

The microscopic lesion in the kidney included; severe hemorrhage with large amounts of red blood cells present in the interstitial space, degenerated tubules and hemorrhage between and within tubules, necrosis of renal tubular epithelium, and severe diffused glomerular nephritis (Fig. 9). The renal tubular epitheliums were totally necrotized and only tubular basement membranes were seen containing cell debris in the tubules. In some of the necrotized renal tubules even the basement membranes were lost.

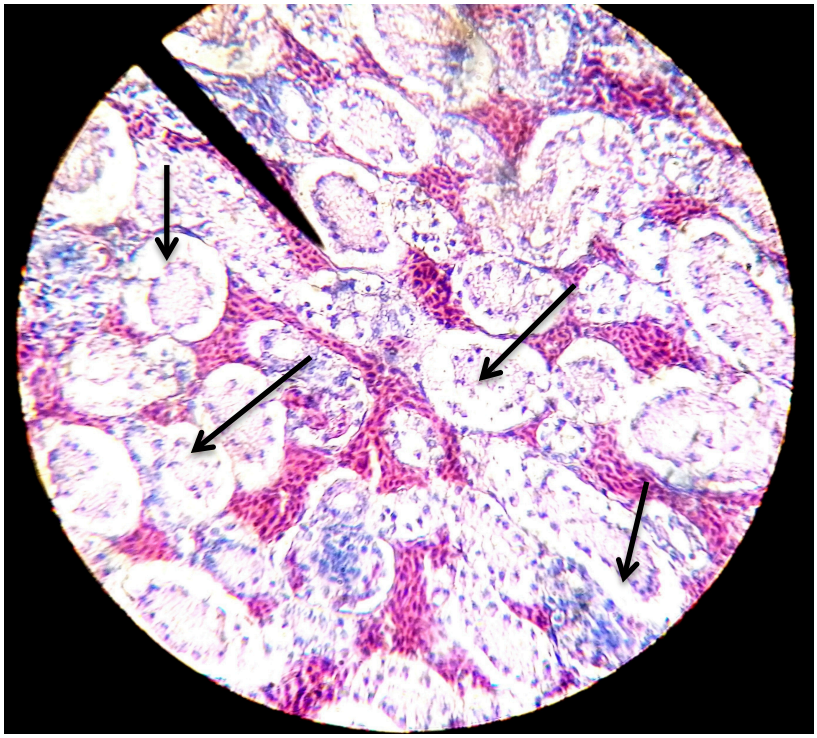

Figure 9: Microscopic lesion in kidney: Hemorrhage and severe diffused glomerular nephritis with both the tubules and glomerules lobe necrotized (black arrow)

The microscopic lesions of the proventriculus include hyperemia (capillaries dilated & filled by RBCs) and hemorrhage (free RBCs) in the interglandular connective tissue (Fig 10A & 10B). In some areas, the proventricular gland epithelium was severly necrotized and slough off (Fig. 10C). There were heavy proliferations of inflammatory cells especially of lymphocytes into the lamina properia. The proventricular glands were relatively normal that the glandular epitheliums were intact in most cases (Fig. 10D) but there were sparsely distributed inflammatory cells into sub epithelial region.

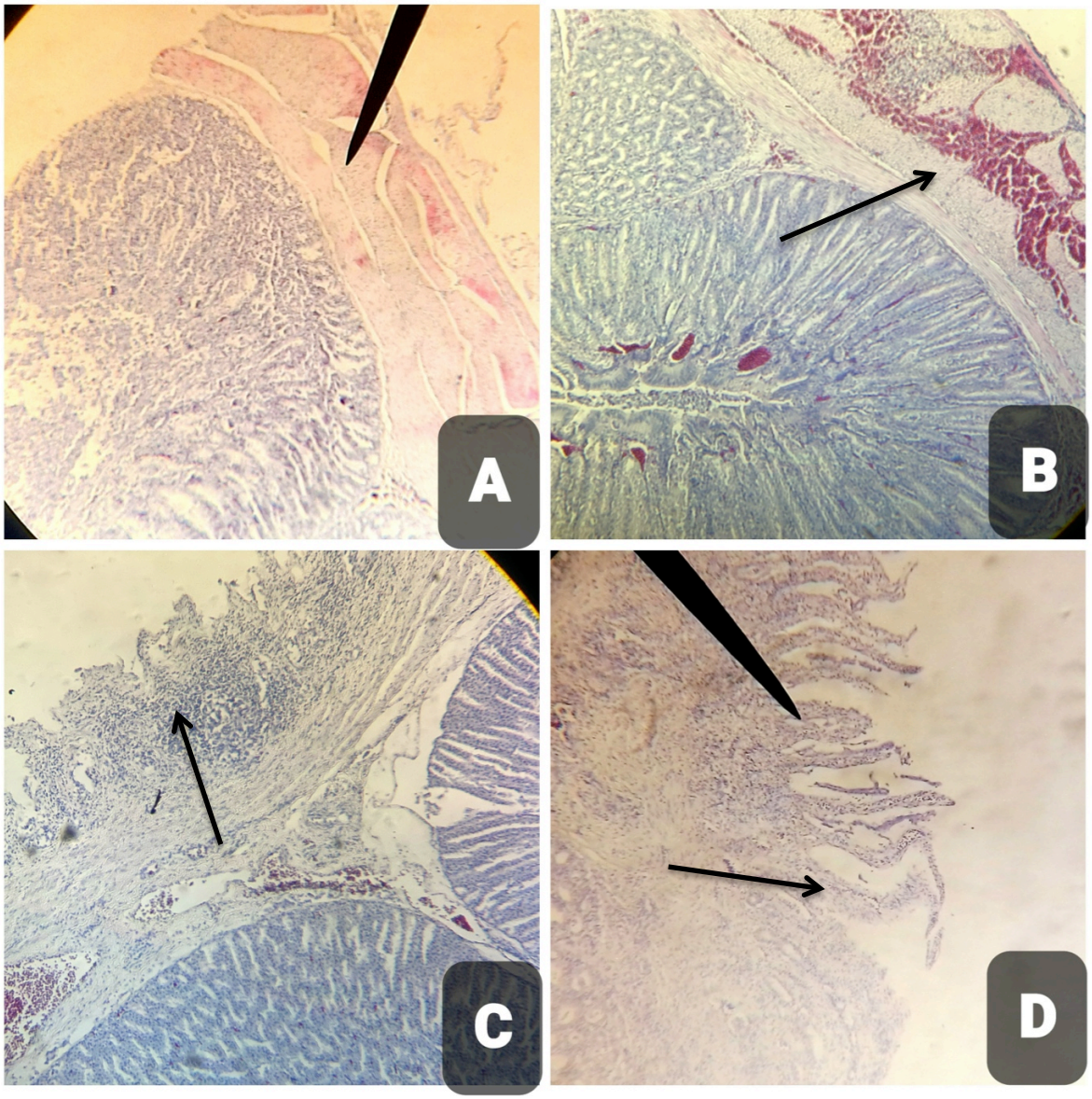

Figure 10: Microscopic lesion of Proventriculus: hyperemia and hemorrhage in the interglandular connective tissue (**A** (microscope arrow), **B** (black arrow)), in some areas, the proventricular gland epithelium was severly necrotized **(C)** (black arrow), slightly sloughed epithelium **(D)** (black arrow)

Among the microscopic lesion of liver, there were diffused hemorrhages in some parts and congestion with many capillaries, and veins were dilated and full of hemorrhage in another part of the same microscopic tissue of the liver. Multifocal hepatocellular necrosis with nuclear piknosis, rhexis and total loss of hepatocytes were observed. (Fig. 11)

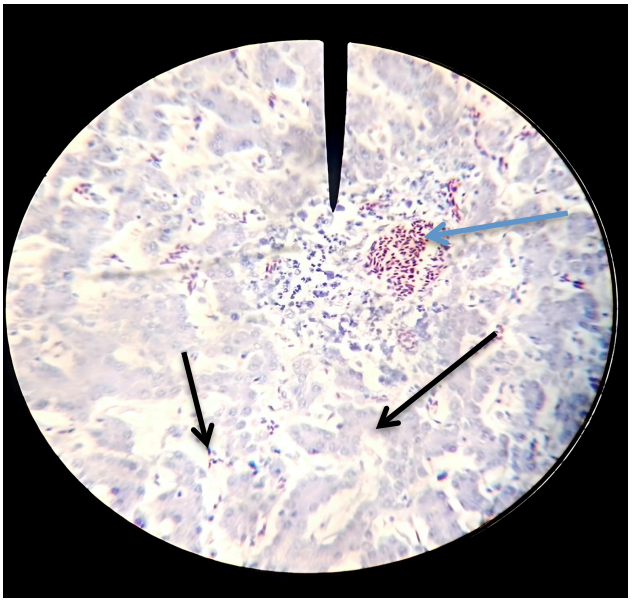

Figure 11: Microscopic lesion of liver: congestion in many capillaries and veins are dilated and are full of RBC (black arrow), multifocal hepato cellular necrosis (black arrow)

4.4. Molecular detection

Conventional polymerase chain reaction (PCR) results

Of 20 pooled samples taken from 100 chicken, only 5 of the pools were subjected to molecular detection of IBDV due to resource limitation. However, the 5 pools were selected purposively based on the lesion score (Prabhu *et al.,* 2020) in which 1 pool from (+) score, 2 pools from (++) score and 2 pools from (+++) score were subjected to PCR. Only pools from both (++) and (+++) score were found PCR positive (Fig. 12).

1	2	3	4	5	6	7	8

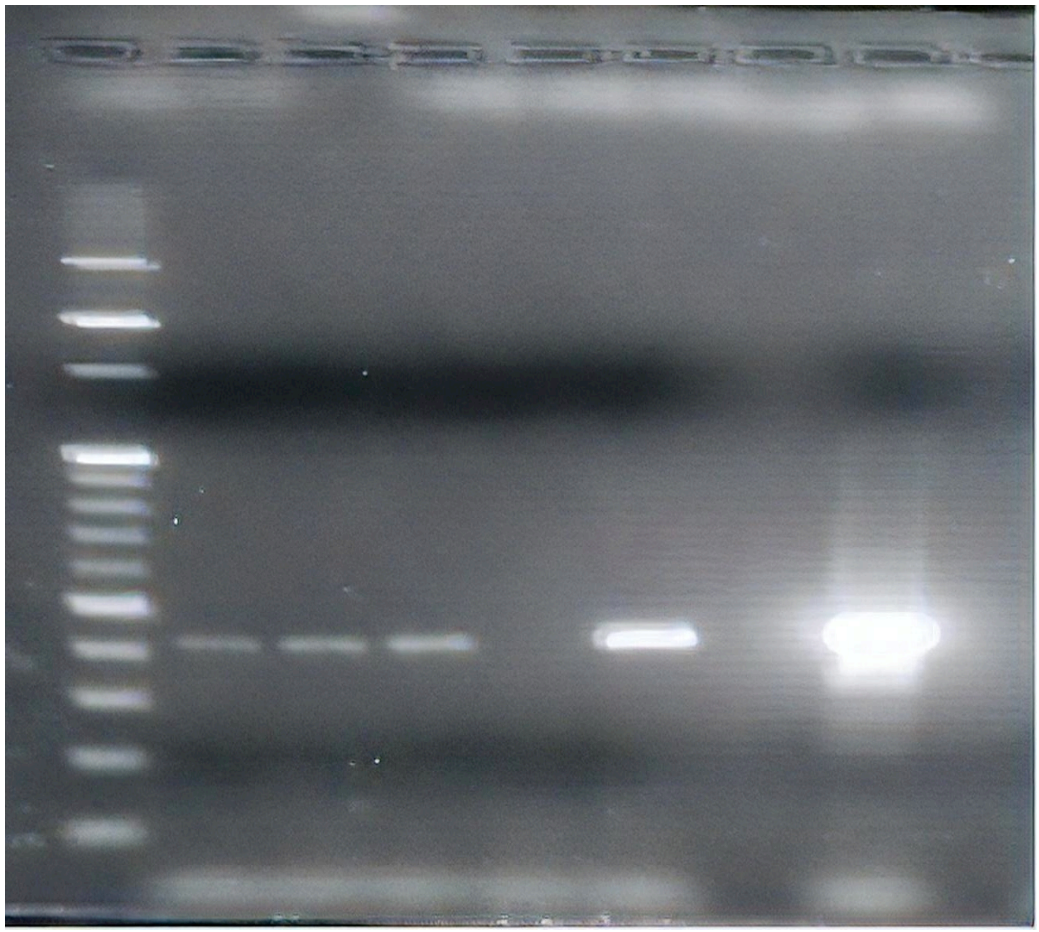

Figure 12: Conventional PCR agarose gel electrophoresis detection of IBDV. Lane 1: molecular marker started at 100bp, Lane 2 to Lane 6: suspected sample where Lane 2, 3, 4 and 6 were positive field sample (PCR band size) and Lane 5 was negative field sample, Lane 7: negative control without template and Lana 8: IBDV positive control

5. DISCUSSION

This study was conducted to characterize both gross and microscopic lesions of IBD as well as to detect the virus by molecular detection in chickens maintained under different productions systems in areas like Bishoftu, Mojo, Dukem and Gelan. Clinical signs, gross lesions, histopathological changes, virus detection of the disease; and biosecurity measures of farmers in the study area were assessed.

The occurrence of IBD outbreak in this study indicated that it is higher in broilers than layers with regard to the production type, implying the age vulnerability of the broilers. These findings are consistent with Jenbreie *et al.* (2012). Almost half of respondents indicated that there was an IBD outbreak previously in their farm, which shows that IBD was concern in the study areas. Seventy percent of respondents had practice of isolating sick chickens. These results are consistent with the claim of previous study of Amigo *et al.* (2022), and this shows the farm owners were taking preventive measures but still it was not practiced fully by all producers. Similar to the finding of Ismael *et al.* (2021), the source of the chickens were different in many farms in this study, and this had contributed for the various vaccines type usage, unknown vaccine history and administration, which aided for the difficulty to give the chicken disease protection.

All in all out management system was relatively well done in the study area; however, Birhanu *et al.* (2015) reported that only some of the farms were practicing it in and around Mekelle Ethiopia; and only 63.3% of the producers dressed on protective cloth and shoe while most of farmers in this study area used proper clothe and shoe and also most of the farmers have used footbath in front of their farm entrance in the study area. This may be due to having better knowledge about disease prevention although it is not practiced by all of the respondents. Although Ismael *et al.* (2021) have found that most of farms allowed visitors entry; the present study has shown poultry farmers in the study areas were prohibiting visitor's entry to their farms. The practice of proper vaccination is similar to Ogbe *et al.* (2012) but when comparing our results to those of previous studies (Bereket *et al.,* 2014; Meskerem, 2017; Amijo *et al.,* 2022), whom reported that only some of the poultry farmers had full adherence to vaccination

schedules for their chickens, it must be pointed out that the farmers in the study area had awareness about importance of vaccination and had better access to vaccines.

Proper disposal of dead birds was practiced by majority of the farmers as a bio-security measure when their chickens died, by burning or burying them deep in the ground in line with (Ogbe *et al.,* 2020) finding. This finding contradicts a report by Ismael *et al.* (2021) and Augustine *et al.* (2014), which revealed that farms did not dispose carcasses of dead chicken properly. Dead birds are source of the virus and should be incinerated or composted to destroy infectious virus. The rest of respondents who didn't dispose dead chickens properly have contributed to disease transmission one way or another. Although biosecurity measures were practiced by majority of the respondents, it is still not fully and adequately done in the study areas, which contributed to the disease occurrence to their farms. Along with this, there was vaccination failure due to improper management and different chicken suppliers which leads to unknown vaccine history. Physico-chemical nature of the virus also made it difficult to destroy the virus from their poultry houses.

The clinical signs like depression, ruffled feather, marked drop in feed and water intake, white diarrhea sometimes tinged with blood, sudden death, emaciation, dropping of the wing and rapid onset of disease were among the major clinical manifestations. These clinical manifestations are consistent with what has been found in previous agreed findings (Zeleke *et al.,* 2005; Shekaro and Josiah, 2015; Ogbe *et al.,* 2020). Most of the active cases were observed in 29-35 days old chickens but the overall suspected cases were in age range between 22-45 days. Except 11 cases where only BF were affected, the rest of active cases were having lymphoid and non-lymphoid organs lesion along with affected BF.

The gross pathological findings observed in this study include hemorrhagic, edematous, enlarged and gelatinous lesions in BF, creamy and atrophied bursa in late stage of the disease, hemorrhage and congestion on the pectoral and thigh muscles (may be due to coagulation disorders), congestion of subcutaneous blood vessels in some parts of the affected chickens, enlarged and congested spleen, pale and enlarged liver. These changes tie well with previous studies (Mazengia *et al.,* 2009 and Rozina *et al.,* 2014, Kulsum *et al.,* 2018). However, in

contrast to this study finding, where swollen and pale livers were common findings; none of the investigators reported the gross pathological changes in liver. The expected lesions of swollen and hemorrhagic or atrophied bursas were also less frequent in Zeleke *et al.* (2005) findings. In line with the previous finding of (Babiker *et al.,* 2008; Ogbe *et al.,* 2020; Rahmaan *et al.,* 2019), kidney was found swollen, hemorrhagic, pale, and ureter were turgid with urate in a numbers of cases. Kidney damage is believed to be a consequence of diarrhea in IBD. Petechial and ecchymotic hemorrhage was also observed on the mucosa of caecal tonsils, proventriculus and gizzard; but contrary to their findings, this study did not find pin-point haemorrhages in the thymus. The haemorrhages in bursa and in other lymphoid organs in IBD has been suggested as an immune mediated damage to the blood vessels which is mainly due to the effect of macrophage derived tumor necrosis factor and interleukins or due to disseminated intravascular coagulation.

The major microscopic lesions were bursal lesions with moderate to severe lymphoid depletion, necrosis, intrafollicular and interfollicular hemorrhage. This report is similar to previous findings (Rahmaan *et al.,* 2019; Cheggag *et al.,* 2020). These histopathological findings of bursal inflammation and cellular infiltration indicate the impact of IBD, and for this reason the disease is named infectious bursal disease due to inflammation of the BF. The liver showed multifocal necrosis of hepatocytes and lymphocytic infiltrate into hepatic tissue. Similar to Cheggag *et al.* (2020) findings, microscopic changes in the proventriculus included congestion and hemorrhage as well as sloughed off epithelium. In spleen of IBD infected chicken, hemorrhage was observed in trabecule, and depletion of lymphocytes in germinal center was evident. This histological finding in IBD infected chickens spleen was related with study of (Kulsum *et al.,* 2018; Ogbe *et al.,* 2020). Since IBD mainly affected lymphoid organs, spleen is one of the affected organs.

Kidneys showed severe hemorrhage, with large amounts of red blood cells present in the interstitial space. The tubules of kidney were degenerated and hemorrhage was between and within tubules. Study conducted by Kaboudi *et al.* (2019) and Oluwayelu *et al.* (2002) encountered similar histological changes in kidney with the present findings. These results demonstrate that IBD exerts pathological effects on the kidney; hence the disease was

previously known as infectious avian nephrosis due to its renal effects. Renal damage can be explained by the accumulation of immune complexes in endothelial cells of renal glomeruli.

In the RT-PCR assays, the primer pairs specific to IBD serotypes were utilized in a single reaction using RNA extracted from bursa tissue sample. In accordance, the RNA was amplified and this suggests the circulation of the IBDV in the chicken flocks found in the study areas. This calls for strict review of the vaccination strategy found in the towns as well as the strict biosecurity measurement that should be taken.

6. CONCLUSION AND RECOMMANDATIONS

This study shows that the suspected outbreak was due to IBDV based on pathological lesions and molecular detection of IBDV. Prominent gross and microscopic lesions were observed in bursa of Fabricius, spleen, kidney, muscles (thigh and pectoral), liver, proventriculus and gizzard. The IBD clinical signs were seen in the age range of 22-45 days. Not all respondents regularly and repeatedly vaccinate chicken for IBD and similarly some producers used to throw out the dead chickens to the environment.

Based on aforementioned concluding remarks, the following recommendations are forwarded:

- Chickens should take the 1st IBDV vaccine at day old if not born from IBDV infected hens (maternal antibody) and should take booster doses. If chickens born from IBDV positive hens chickens should start the 1st IBDV vaccine at the end of 2nd week and should take booster vaccine repeatedly based on chicken types.

- Biosecurity practice like footbath, disinfection of materials used to transport chicken (especially day old) and regular sanitation of poultry houses should be strictly practiced.

- Chickens suspected of IBD should be properly & promptly diagnosed (Virus isolation/molecular) and those with diseases should be kept separate and those died should be properly disposed not to contaminate the environment.

- Producers should take continuous training on how to remove dead chickens, how and when to vaccinate chickens.

- Gross lesions particularly of bursa and muscular hemorrhages may help at least as tentative diagnosis, which can help proper disposal of dead chickens until IBDV isolation.

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8. APPENDICES

Annex I: Questionnaire survey collection format

Addis Ababa University College of veterinary medicine and agriculture

A questionnaire survey prepared to collect information about the practice of biosecurity and prevention methods related to infectious bursal disease in chicken flock at purposively selected areas in Bishoftu, Mojo, Gelan and Dukem

Date ...../...../..... Questionnaire No. .....

Answer the questionnaire by marking 'X' on correct answer or by circling the correct choice and by filling out the open questions corresponding to your chicken flock.

1. Farm location…….

2.Owner's education status:

a) Primary b) secondary c) above Secondary d) illiterate

3. Ownership status of the poultry premises:

a) Rented b) Owned premises

4. Farm capacity

a) Small scale b) Medium scale c) Large-scale

5. Farm types

a) Broiler b) Layer c) pullet

6. Chicken breed(s)

a) Local b) exotic

7. Where is the source of the day old chicks?

a) Always from the same supplier b) sometimes from different supplier c) always from different suppliers

8. Do you practice all in all out system in your farm?

a) Always b) sometimes c) never

9. Do you properly clean and disinfect your poultry house in down time between exit and entry of new batch of chickens?

a) Yes b) no

10. Is there cloth and shoe changing room in your farm?

a) Yes b) no

11. Is there practice of hand wash and disinfection before entering the farm?

a) Yes b) no

12. Is there foot bath at the entrance of your farm where you change regularly?

a) Yes b) no

13. Do visitors have access to your farm?

a) Access is never granted b) conditional permission

14. Does the farm have contact with other farms?

a) Yes b) No

15. Do you practice proper vaccination in your farm?

a) Yes b) no

16. Do you have separate room or area for isolation of sick chickens?

a) Yes b) no

17. What is the fate of dead chickens from your farm?

a) Burn or Buried b) Collected by rendering company c) Throw outside the farm
d) Other

18. Did you sell the diseased / recovered chicken at market?

a) Yes b) No

19. Did the farm have previous exposure to IBD?

a) Yes b) No c) I don't know

20. Is there any local name for gumboro in your area? Mention if you know____

Annex II: Pathological and molecular sample data collection sheet

Date _____/_____/_____

1. Case no. _____ Age _____ Breed_____ Location _____

2. History and clinical signs observed

A)_____
B)_____
C)_____
D)_____
E)_____

3.Postmortem findings in different organs and its gross interpretation

3.1) Bursa of fabricious A)_____ b)_____

3.2) Spleen A)_____ b)_____

3.3) Caecal tonsil A)_____ b)_____

3.4) Proventriculus and gizzard A)_____ b)_____

3.5) Thymus A)_____ b)_____

3.6) Kidney A)_____ b)_____

3.7) Liver A)_____ b)_____

3.8) Pectoral muscle A)_____ b)_____

3.9) Thigh muscle A)_____ b)_____

3.10) Body condition of carcass A)_____ b)_____

4. Tissue sample for molecular test ☐

5. Pathological sample taken for histopathology

Bursa of fabricious ☐ Proventriculus and gizzard ☐

Spleen ☐ Thymus ☐

Caecal tonsil ☐ Kidney ☐

Liver ☐ Muscle ☐

Annex III: Ethical clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/28/02/14/2023

Name and affiliation of applicant: **Dr Debebe Ashenafi (MSc, Assistant Professor)**
Department of Microbiology, Immunology and Veterinary Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Study on Vaccines against Major Viral and Bacterial Diseases of Poultry in Ethiopia*

Date of application:	**December, 2022**
Nature of the project:	**Field investigation and Experimental trial**
Target animal species:	**Chicken**
Number of animals involved:	**Depends on the number of outbreaks** **For experimental purposes: 200 chickens**
Study area:	**National Veterinary Institute, Ethiopia**

Minutes No. and date of review: **VM/ERC/02/14/022, 01/03/2022**

The Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University has reviewed the above research project and unanimously approved the application of **Dr Debebe Ashenafi.**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University College of Veterinary Medicine and Agriculture

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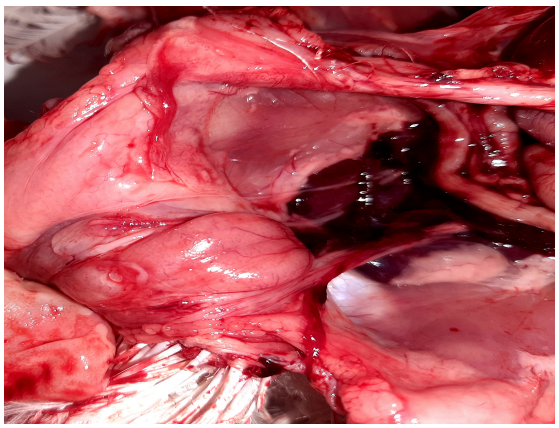
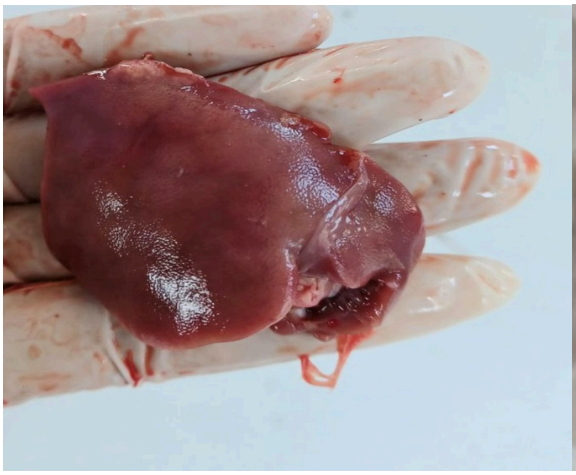
Annex IV: Different pictures at field and work

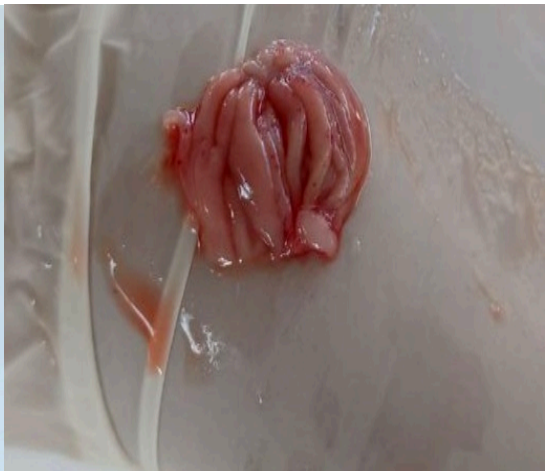
Bursa and spleen of affected chicken



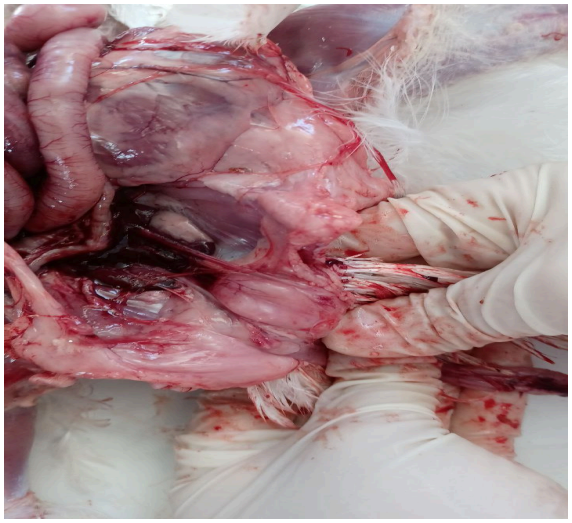
grossly enlarged bursa



Liver of IBDV infected chicken



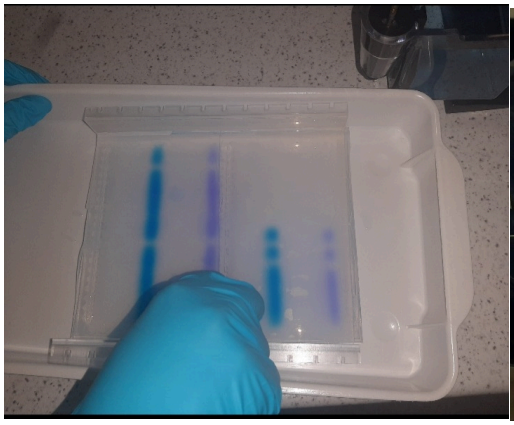
hemorrhagic bursa



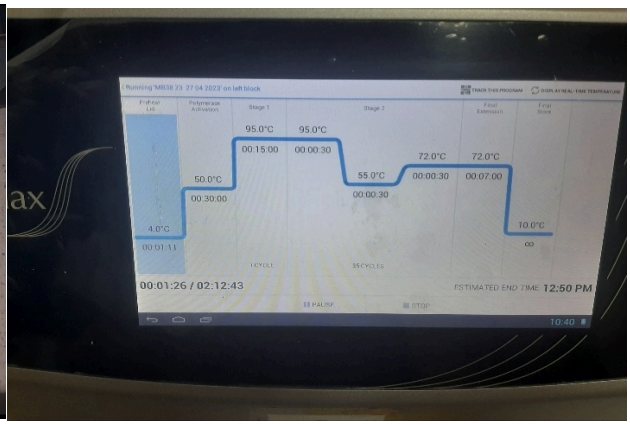
Pictures from post mortem examination

During post mortem examination

farm at Mojo



Making gel for PCR



conventional PCR machine