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MOLECULAR DETECTION AND VIRUS ISOLATION OF INFECTIOUS BURSAL DISEASE VIRUS FROM OUTBREAK CASES IN SELECTED DISTRICTS OF CENTRAL ETHIOPIA

MSc THESIS

BY

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ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

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BISHOFTU, ETHIOPIA

Molecular Detection and Virus Isolation of Infectious Bursal Disease Virus from Outbreak Cases in Selected Districts of Central Ethiopia

A Thesis Submitted to the College of Veterinary Medicine and Agriculture at Addis Ababa University in Partial Fulfilment of the Requirements for the Degree of Master of Science in Veterinary Microbiology

By

Endeshaw Mulu Addis

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BISHOFTU, ETHIOPIA

Molecular Detection and Virus Isolation of Infectious Bursal Disease Virus from Outbreak Cases in Selected Districts of Central Ethiopia

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SIGNED DECLARATION SHEET

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LIST OF ABBREVIATIONS

Ab	Antibody
AGIDA	Agar Gel Immuno-diffusion
AHY	Animal Health Year
AtIBDV	Attenuated Infectious Bursal Disease Virus
BF	Bursa of Fabricius
CAM	Chorioanallotic Membrane
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CEF	Chicken Embryo Fibroblast
CEK	Chicken Embryo Kidney
clIBDV	Classical Infectious Bursal Disease Virus
CMI	Cell Mediated Immunity
CPE	Cytopathic Effect
CSA	Central Statistical Agency
dsRNA	Double Stranded Ribonucleic Acid
ELISA	Enzaymlinked Immunosorbent Assay
GALT	Gut-associated Lymphoid Tissues
GMEM	Glasgow Minimum Essential Medium
GILZ	Glucocorticoid-Induced Leucine Zipper
HVT	Herpes Virus of Turkey
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
IGM	Immunoglobulin M
IL	Interleukin
INF	Interferon
MAb	Maternal Antibody
MDA	Maternal Derived Antibody
MHC	Major Histocompatibility Complex
NVI	National Veterinary Institute

OIE	Office International des Epizooties
ORF	Open reading frames
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PKR	Protein Kinase R
QGIS	Quantum Geographic Information System
qRT-PCR	Real-Time Quantitative RT-PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribose Nucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SPF	Specific Pathogen Free
USAID	United States Agency for InternationalDevelopment
VarIBDV	Variant Infectious Bursal Disease Virus
VLP	Virus like Particle
VNT	Virus Neutralization Test
VP	Viral Protein
VTM	Virus Transport Medium
VvIBDV	Very Virulent Infectious Bursal Disease Virus

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ABSTRACT

Infectious bursal disease also called Gumboro is a highly contagious viral disease of young chickens that causes significant economic losses in the poultry industry worldwide. The disease is caused by the *Infectious bursal disease virus* (IBDV), which belongs to the genus *Avibirnavirus* of the family *Birnaviridae*, double strand RNA virus with a non-enveloped, icosahedral capsid with bi-segmented genome. An outbreak based cross sectional study was conducted in poultry farms located in Bishoftu, Dukem, Modjo, Gelan and Tulu-Dimtu, central Ethiopia, from December 2022 to May 2023 for molecular detection and isolation of infectious bursal disease virus (IBDV) from outbreak cases. Purposive sampling technique and a semi-structured questionnaire was employed on selected farm owner and attendants to assess management and vaccination practices of the farms. Pools of bursal samples were collected, and virus isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) were performed to confirm the outbreak cases. From the total nineteen farms surveyed 47.37% vaccinate their chickens against IBDV. Only 21.05% vaccinate according to manufacturer's protocol and 78.95% revealed lack of knowledge about biosecurity practice, and 63.16% had poor hygienic condition, while only 26.32% practice regular cleaning and disinfectants. Out of a total of 4 bursal suspensions collected and prepared from 19 poultry farm outbreaks, all were showed characteristic cytopathic effects starting on day two post infection after second passage on differentiated fibroblasts (CEF) cell lines. Of the seven pooled samples analyzed by RT-PCR, six of them were positive results. The results indicated that there is continuous presence of IBD in poultry farms in the country suggesting that current vaccine procedures and protocols as well as management practices may not be working properly. In conclusion, IBD has become the most devastating disease of chicken in central Ethiopia, with high economic lose. Hence, sequencing of the positive samples is much needed to know the circulating virus strains in the areas.

Key words: *Detection, Ethiopia, IBD, Isolation, Poultry farm, PCR, Questionnaire*

1. INTRODUCTION

The poultry sector is one segment of the livestock sector in Ethiopia that can be characterized into three major production systems: large commercial, small commercial, and village or backyard poultry production systems. These production systems have their own specific chicken breeds, inputs and production properties. Each can sustainably coexist and contribute to solving the socio-economic problems of different target societies (Mekuriaw *et al.,* 2017). In Ethiopia, among the total of 228 million livestock populations, 56 million are chickens (CSA, 2021). The poultry sector plays a role by providing the needed animal protein that contributes to the improvement of the nutritional status of the people (USAID, 2013).

Ethiopian poultry production has a long traditional practice which is characterized by low input and low output. Attempts are underway to enhance chicken productivity and their contribution via importing and distributing improved breeds to farmers living in different parts of Ethiopia (Zeleke *et al.,* 2005; Mulugeta and Tebkew, 2013).With the intensification of poultry farming, there is an occurrence of epidemics of newly introduced diseases and/or epidemics of endemic diseases. Among those diseases, infectious bursal disease is the one that has become a serious threat, causing frequent outbreaks and a challenge to young, growing poultry farms (Mazengia, 2012; Mekuriaw *et al.,* 2017).

Infectious bursal disease is a highly contagious viral disease of young chickens that causes significant economic losses in the poultry industry worldwide. The disease is caused by the Infectious bursal disease virus (IBDV), which belongs to the genus *Avibirnavirus* of the family *Birnaviridae*. *Birnaviridae* is a family of viruses with bi-segmented dsRNA genomes with a total of about 6 kb forming icosahedral, non-enveloped virions (Delmas *et al.,* 2019). Based on the virus neutralization test (VNT), IBDV is classified into serotypes I and II (Jackwood, 2004). Serotype I viruses are pathogenic to chickens. There have been four phenotypes: classical IBDV (clIBDV), vvIBDV, attenuated IBDV (atIBDV), and variation IBDV (varIBDV), while serotype II

viruses (isolated from turkeys) are non-pathogenic to chickens. Several studies have reported the evolution of IBDV in some geographical locations worldwide with the emergence of an antigenic variant and recombinant, reassortant, and distinct strains of the virus (Aliyu *et al.,* 2021). Young chicks between the ages of 3 and 6 weeks are most commonly affected by the disease, which is characterized by an enlarged Fabricius bursa, watery diarrhoea, urate buildup in the urinary system, and severe depression (WOAH, 2012; Jackwood, 2017).

Infectious bursal disease virus strains are identified and characterized using genome sequencing studies, polymerase chain reaction, and serological methods (Banda *et al*., 2003). Since it identifies sequences typical of classical IBDV, variant IBDV, or vvIBDV, nucleotide sequencing of the hyper-variable region of the VP2 gene has been a preferred method (van den Berg, 2000). Gumboro disease (IBD) was first reported in 2002 in Ethiopia at a privately owned commercial poultry farm, where a 45–50% mortality rate was documented (Zeleke *et al*., 2003). Subsequently, it is becoming one of the most important diseases for the juvenile poultry industry in the country (Mazengia, 2012; Jenberie *et al.,* 2014). In addition to sociological studies, molecular characterization of the Ethiopian IBD virus isolates collected for the first time in 2005 from the samples from Kombolcha Poultry Multiplication Centre and in commercial and breeding poultry farms in Ethiopia between 2009 and 2011 (Jenbreie *et al*., 2012).

In Ethiopia, research finding indicates that, from a total of 25 tissue samples processed for virus isolation, 95% (18/20) of the bursa and 80% (4/5) of the spleen samples showed visible cytopathic effects (CPE). The positive samples were confirmed by PCR and 19 of them had the expected band (643 bp). Furthermore, 11 representative samples were sequenced which confirmed that the circulating virus among the poultry population in the country is vvIBDV (Mekuriaw *et al.,* 2017). Another reported prevalence of IBD in Ethiopia is: Mekele region, 90.3% (Shiferaw *et al.,* 2012), Southwest Showa of Ethiopia, 76.64% (Hailu *et al.,* 2010), Mekelle town, 45.05% (Sindu *et al.,* 2015), Debre-Zeit, 82.2% (Tesfaheywet and Getnet, 2012), Andassa poultry farm, 100% (Solomon and Abebe, 2007), Eastern Ethiopia, 83% (Tadesse and Jenbere, 2014), and Debre Brehan, 94.7% (AHY, 2011) respectively.

In recent years, IBD has become the most divesting disease of chickens in Ethiopia, with mortality rates that exceed 50% even in vaccinated flocks. Frequent outbreaks and the occurrence of new strains of IBD became a challenge to the juvenile poultry industry in Ethiopia (Mazengia, 2012). The IBDV is now more virulent, and the disease is now seen as being more serious than the earlier outbreaks. Therefore, under various production systems, IBD is becoming a serious concern for chickens. Finding out the disease's epidemiology on the ground is important since it will provide information that may be used to create practical controls. The case history, clinical signs, and postmortem (PM) examinations are frequently used to make the diagnosis of IBD. A differential diagnosis of acute IBD should consider other diseases that can induce sudden death, severe depressions in young chickens, hemorrhages, nephritis, or bursal lesions. These include infectious diseases such as coccidiosis, Newcastle disease, Marek's disease, chicken anemia and infections by infectious bronchitis viruses with nephron-pathogenic tendencies (WOAH, 2008). However, a screening test is necessary because the disease symptoms might be mistaken for a number of viral infections. Isolation and molecular detection of pathogens are considered standard tests for diagnosis. Therefore, the main objective of this study is conducts on:

1.1. General Objective

- ✓ Molecular Detection and Virus Isolation of Infectious Bursal Disease from outbreak cases in selected districts of central Ethiopia

1.2. Specific Objectives

- To assess the status of outbreak, biosecurity and management practice of poultry farms in current study area.
- To isolate infectious bursal disease virus using primary chicken fibroblast cell culture technique.
- To detect infectious bursal disease virus by Reverse Transcriptase Polymerase Chain Reaction from outbreak cases.

2. LITERATURE REVIEW

2.1. Historical Background

Infectious bursal disease is a specific disease that affects the bursa of Fabricius in young chickens and has lymphoid tissue as its primary target with special predilections. It was referred to as "avian nephrosis" because of the extreme kidney damage found in birds that succumbed to infection. The disease, also named ‘‘Gumboro disease’’ according to the first outbreak of infectious bursal disease (IBD) that occurred in 1957 in a broiler farm near Gumboro, the Delaware area in the USA, was caused by the classical serotype 1 IBDV (Cosgrove, 1962), but was later designated infectious bursal disease (IBD) according to varying morphologic and histological changes observed in the bursa of Fabricius (Hitchner, 1970). In the years 1960 and 1964, the disease was observed in most parts of the USA and became a devastating disease in Europe in the years of 1962 to 1971.With its pandemic movement from 1966 to 1974, the disease was reported in southern and western Africa, the Far East, the Middle East, India and Australia (Lasher and Davis 1997).

Infectious bursal disease has currently become an international issue. 95% of the 65 countries that responded to a survey conducted by the OIE in 1995 announced the presence of infection, including New Zealand, which had been free of disease until 1993. Only chickens develop IBD after infection by serotype 1 viruses. 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 (WOAH, 1995).

Gumboro disease (IBD) was first reported in 2002 in Ethiopia at a privately owned commercial poultry farm, where a 45–50% mortality rate was documented (Zeleke *et al*., 2003). Subsequently, it is becoming one of the most important diseases for the juvenile poultry industry in the country (Mazengia, 2012; Jenberie *et al.,* 2014; Mekuriaw *et al.,* 2017; Shegu *et al.,* 2020). In addition to sociological studies, molecular characterization

of the Ethiopian IBD virus isolates collected for the first time in 2005 from the samples from Kombolcha Poultry Multiplication Centre and in commercial and breeding poultry farms in Ethiopia between 2009 and 2011 (Jenbreie *et al*., 2012).

2.2. Etiology of the Disease

Infectious bursal disease virus (IBDV) is the aetiology of infectious bursal disease "Gumboro disease", which belongs to the genus *Avibirnavirus*, family Birnaviridae, and is a double-stranded RNA virus (dsRNA) with a non-enveloped, icosahedral capsid with a bi-segmented genome (Mahgoub, 2012; Muller *et al.*, 2012). The family includes three genera: *Aquabirnavirus*, whose type species is infectious pancreatic necrosis virus (IPNV), which infects fish, mollusks, and crustaceans; *Avibirnavirus*, whose type species is infectious bursal disease virus (IBDV), which infects birds; and *Entomobirnavirus*, whose type species is Drosophila X virus (DXV), which infects insects (Eterradossi and Saif, 2008).

2.3. Taxonomy Morphology and Structural Protein of the Virus

Infectious bursal disease (IBD) is a highly contagious viral disease of young chickens that causes significant economic losses in the poultry industry worldwide. The disease is caused by the infectious bursal disease virus (IBDV), which belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (Delmas *et al.,* 2019). Based on the virus neutralization test (VNT), IBDV is classified into serotypes I and II (Jackwood, 2004). Serotype I viruses are pathogenic to chickens and have four phenotypes: classical IBDV (clIBDV), vvIBDV, attenuated IBDV (atIBDV), and variation IBDV (varIBDV), while serotype II viruses (isolated from turkeys) are non-pathogenic to chickens. Several studies have reported the evolution of IBDV in some geographical locations worldwide with the emergence of an antigenic variant and recombinant, reassortant, and distinct strains of the virus (Aliyu *et al.,* 2021).

The capsid shell exhibits icosahedral symmetry and is composed of 32 cashmeres with a diameter ranging from 55 to 65 nm. Non enveloped, single-shelled T=13 icosahedral symmetry capsid of about 70 nm in diameter, composed of 260 trimers of VP2 that form

spikes projecting radially from the capsid. The peptides derived from pre-VP2 C-terminal cleavages remain associated within the virion. VP3 forms a ribonucleoprotein complex with the genomic RNA. Minor amounts of VP1 are also incorporated in the virion (Abdel *et al.,* 2001) (Figure 1 below).

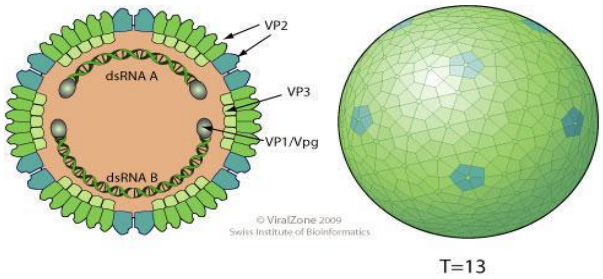


Figure 1: Morphology of IBD Virus

Source *:*(Viral zone, 2009).

Its genome consists of two segments of linear double-stranded RNA, designated A and B, each 6 kb in length. Segment A is 3.2 kb in length and contains two partly overlapping open reading frames (ORF) (Figure 2 below). The largest ORF encodes a poly-protein that is auto catalytically cleaved into two structural proteins, VP2 and VP3, and a serine protease, VP4 (Birghan *et al.,* 2000; Lejal *et al.,* 2000). VP2 is considered to be the major host-protective antigen and contains the major antigenic site responsible for eliciting neutralizing antibodies (Abs) (Fahey *et al*., 1989). At least two neutralizing epitopes are located on this polypeptide. VP2 induces virus-neutralizing antibodies that protect susceptible chickens from vIBDV. It is responsible for antigenic variation, tissue-culture adaptation and viral virulence (Brandt *et al*., 2001).

Segment A also encodes a 17-kD non-structural protein, VP5, from the small ORF (Mundt *et al*., 1995). VP5 is a class II membrane protein with a cytoplasmic N-terminus and an extracellular C-terminal domain (Lombardo *et al.,* 2000). It is highly basic, cysteine-rich, and semi-conserved among all serotype I IBDV strains (Mundt *et al*., 1995). VP5 accumulates within the cell membrane, resulting in its disruption and decreasing cellular viability. VP2 and VP5 have been shown to induce apoptosis in in vitro cultures (Rodriguez-Lecompte *et al.,* 2005). Segment B is 2.8 kb in length and encodes VP1, a 97-kD protein with polymerase activity (Murphy *et al.,* 1999). VP1 exists

as a genome-linked protein, circularizing segments A and B. DNA vector-based RNA interference, directed towards VP1, prevents IBDV replication in Vero cells (Gao *et al*., 2008).

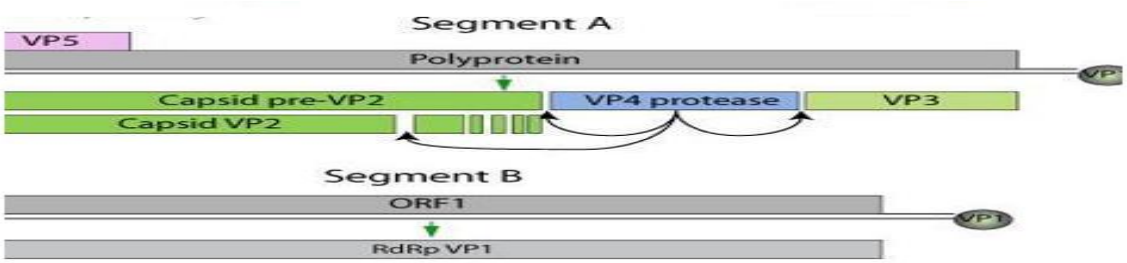


Figure 2: The structure of the segmented linear dsRNA genome.

Source :(Viral zone, 2009)

Table 1: Functions of IBDV proteins

Proteins	**Function**	**Reference**
VP1	Viral polymerase	Saugar *et al.*, 2010
	Virulence determinant	lenouen *et al.*, 2012
VP2	Host receptor binding	Ogawa *et al.*, 1998
	Contains neutralizing epitopes	Azad *et al.*, 1987
	Virulence determinant	Brandt *et al.*, 2001
	Cell culture adaptation	Mundt, 1999
	Apoptosis	Fernandezarias *et al.*, 1997
	Endopeptidase activity	Irigoyen *et al.*, 2009
VP3	Chaperone activity	Chevalier *et al.*, 2004
	Anti-apoptosis by interacting with PKR	Busnadiego *et al.*, 2012
	Suppresses hosts RNA silencing mechanism	Valli *et al.*, 2012
	Transcriptional activator	Tacken *et al.*, 2002
	Forms ribonucleo-protein complex	Luque *et al.*, 2009
VP4	Viral protein processing (viral protease)	Birghan *et al.*, 2000
	Trans-activate VP1 synthesis	Birghan *et al.*, 2000
	Suppresses type I IFN by interacting with GILZ	Li *et al.*, 2013b
VP5	Early anti-apoptotic effects	Liu u. Vakharia, 2006
	Late apoptotic effects	Li *et al.*, 2012

Keys: VP-Viral protein

2.3.1. Physico-Chemical Nature of the Virus

The most interesting feature of IBDV is its ability to remain infectious for a very long period of time and its resistance to commonly used disinfectants (Jackwood, 2014). The virus has been shown to remain infectious for 122 days in a chicken house, for 52 days in feed, water and faces in the chicken houses, and for up to 4 weeks in the bone marrow of infected chickens (Elankumaran *et al.,* 2002). The virus is non-enveloped and quite resistant to physical and chemical agents. It is susceptible to mutation, highly stable and resistant to a variety of chemicals and disinfectants like phenolic derivatives and quaternary ammonium compounds, but the iodine complex has a toxic effect on viruses. It is also resistant to treatment with chloroform and ether, remains viable from pH 2 to 12 and is inactivated only at 70°C for 30 minutes. The virus is unaffected by exposure for 1 hour at 0.5% to 30% phenol but virus infectivity was markedly reduced when exposed to 0.5% formalin for 6 hours. IBDV is also heat stable and viable after treatment at 56 °C for 5 hours (Dwight *et al.*, 2005; Eterradossi and Saif, 2008).

2.3.2. Antigenic Variation of IBDV Strains

Historically, mutations in the IBDV genome have impacted antibody recognition and led to variations in antigenicity, immunogenicity, virulence, and tropism of circulating infectious bursal disease virus strains (Zierenberg, 2004). Therefore, continuous surveillance, along with rapid identification and characterization of new IBDV isolates and comparison with previously described viruses, is of vital importance (Van den Berg, 2000). The molecular basis for these emerging antigenic differences was traced to the antigenic domains of the VP2 protein of IBDV (Fahey, 1989). The viral capsid protein, VP2, is the major host protective immunogene, as it is the only viral protein responsible for the induction of neutralizing antibodies and for serotype specificity (Van den Berg, 2000).

In Ethiopia, a recent countrywide study reported IBDV Sero-positivity rates in backyard chickens to be close to 92% (Chaka *et al.*, 2012; Jenbreie *et al.*, 2012) and IBDV isolates appear clonal and are very virulent. How the vvIBDV strains evolved in Ethiopia remains

unclear. Literature suggests that international trade in live poultry and poultry products may facilitate the global spread of IBDV (Cobb, 2011). Almost all acute disease outbreaks in backyard chickens in developing countries remain undiagnosed. vvIBDV isolates from wild birds and backyard chickens were shown to be highly pathogenic for SPF chickens under experimental conditions and maintain virulence marker amino acid residues across their VP2 and VP1 genes (Hernandez-Divers *et al.,* 2008).

2.3.3. Re-assortment and Recombination of IBDV

In the case of RNA viruses, such as IBDV, biological events, including genetic re-assortment or recombination, alter the phenotypes and genotypes of circulating viruses and compromise their genetic stability. Genetic re-assortment might be accountable for the emergence of vvIBDV in the late 1980s in Europe (Hon *et al.,* 2006). The most common re-assortants of IBDVs contain segment A of vvIBDV and segment B from attenuated strains, indicating the drawbacks of extensive application of live IBDV vaccines. The process of re-assortment may be more complex in the field than expected and may involve the interactions of several factors: time, environment and vaccine pressure (Wei *et al.,* 2008). The risk of live vaccines recombining to generate virulent natural recombinants has been well described, and disease outbreaks associated with these viruses have recently been described for infectious laryngotracheitis virus (ILTV) infections in chickens (Lee *et al.,* 2012). Natural homologous intragenic recombination apart from re-assortment may lead to new variants of IBDV and recombination may also lead to antignetically and genetically diverse IBDV populations and the emergence of novel vvIBDV. It has the potential to alter the interactions of IBDV proteins and the orientation of the capsid domains, preventing neutralization by pre-existing Abs, which leads to vaccine failure groups (He *et al.,* 2009a).

The existence of RNA virus quasi-species may have a paramount contribution to virus evolution. An RNA virus population is made up of heterogeneous viruses that share the consensus sequence but differ from each other by one or many mutations (Domingo *et al.,* 1985). In IBDV vaccine and field strains, the quasi-species phenomenon has been described by real-time RT-PCR and melting curve analysis (Jackwood, 2002; Hernandez

et al., 2006). Attenuated live IBDV vaccines are most frequently used to vaccinate commercial chickens. Reversion of these attenuated vaccine strains to more virulent phenotypes under field and experimental conditions has been frequently reported (Yamaguchi *et al.,* 2000; Jackwood *et al.,* 2008) possibly due to a lack of IBDV polymerase fidelity during vaccine viral genome replication in the host cells. A tissue culture-adapted IBDV generated by reverse genetics from a vvIBDV strain reverted phenotypically and genotypically to the vvIBDV pathotype after inoculation into SPF chickens and maintained this pathotype afterwards (Raue *et al.,* 2004).

2.3.4. Immune Response of IBD

IBDV infection in chickens activates all branches of the immune system. However, the level of activation varies depending on the virulence of infecting strains, age, immune status and genetic background of affected chickens. The immune response can be altered by maternal antibodies, and the more virulent vaccine strains can override higher levels of antibodies. Progeny of parent flocks vaccinated with classical strains of the IBD virus may have poor maternal immunity against strains of the virus (Ignjatovic *et al.,* 2001). A high level of maternal antibodies will protect most young chickens against challenge by the vvIBD virus for up to 3 weeks after hatching. This is borne out by the excellent passive protection provided by maternal antibodies against immune suppression, bursal lesions, or mortality (Van den Berg, 2000).

The influx of macrophages, heterophils and mast cells in the bursa of Fabricius constitutes the early innate immune response to IBDV. Nitric oxide released by macrophages may constitute an early host defense against IBDV and promote the killing of IBDV-infected and possibly virus-free cells (Palmquist *et al.,* 2006; Khatri and Sharma, 2009a). Humoral immunity plays a significant role in protection against IBDV. All classes of Igs can be produced, but the Ab response may not protect chickens from antignetically different IBDV strains. Neutralizing Abs is directed against the conformation dependent neutralizing epitopes of VP2. MAb positive chickens developed significantly fewer bursal lesions than Ab negative chickens (Aricibasi *et al.,* 2010).

2.4. Epidemiology of the Disease

2.4.1. Distribution

Infectious bursal disease (IBD) is an acute, highly contagious viral infection in young chickens. The first outbreak of infectious bursal disease (IBD) occurred in 1957 on a broiler farm near Gumboro, Delaware, in the USA, caused by the classical serotype 1 IBDV (Cosgrove, 1962). Infections with serotype 1 IBDV are of worldwide distribution, occurring in all major poultry-producing areas. Strains of vvIBDV have rapidly disseminated to every poultry-producing country, such as the Middle East, Asia, and Africa, South and Central America in 1999, and the USA in 2009 (Jackwood *et al.*, 2009), but there has been no report that shows the existence of infectious bursal disease in Canada, Mexico, Australia and New Zealand (Aregitu, 2015). Gumboro disease (IBD) was first reported in 2002 in Ethiopia (Zeleke *et al*., 2003).

2.4.2. Host Range

The infectious bursal disease virus (IBDV) is host-specific. Although serologic evidence of natural infection with the virus has been reported in turkeys, ducks, guinea fowl and ostriches, clinical disease occurs solely in chickens (WOAH, 2008). It is strongly believed that the serotype IBDV-1 is highly host-specific for chickens, which develop IBD after infection by serotype 1 viruses. Reports have shown that serotype 2 of IBDV is more prevalent in many species of wild birds, with the natural host considered to be turkeys (Okoyo and Uzoukwu, 2005). There is no evidence that the IBD virus can infect other animals, including humans. An infectious bursal disease virus has recently been isolated from a sparrow in China, suggesting that wild birds could act as carriers (Wang *et al.,* 2007).

2.4.3. Route of excretion and Transmission of the virus

Infected birds excrete virus in their droppings for at least 14 days (Baxendale, 2002). It is excreted in the faces and then contaminates water, feed and litter, where it persists and from where it commonly spreads. The most common mode of infection is through the

oral route, conjuctival and respiratory routes may also be involved (Sharma *et al.,* 2000), but the virus is highly contagious, so disease is transmitted by direct contact with excreting subjects or by indirect contact with any inanimate or animate (farm staff, animals) contaminated vectors between infected and susceptible flocks (WOAH, 2008). The high tenacity of the virus and its resistance to several disinfections and virucidal procedures may contribute to the rapid distribution of the virus (Garriga *et al.,* 2006). IBDV may spread through contaminated equipment (Jackwood and Sommer-Wagner, 2010). There is no evidence to suggest that IBDV is spread via transovarial transmission. No specific vectors or reservoirs of IBDV have been established, but the virus has been isolated from mosquitoes (Aedes vexans), rats, and lesser mealworms (Alphitobius diaperinus) (Eterradossi and Saif, 2008). A viable vvIBD virus was recovered after 2 days from the faces of a dog that had been fed tissues from experimentally infected chickens, indicating that dogs may act as mechanical vectors for the virus (Pages-Mante *et al.,* 2004).

2.4.4. Molecular Epidemiology and Field Evolution

The molecular epidemiology of IBDV has been studied in many geographical areas and IBDV evolution has been well documented. Particularly, serotype 1 IBDV strains have been circulating in many poultry operations in North and South America, Europe, Asia and African countries (Kasanga *et al.,* 2012). The high mutation rate of the RNA polymerase of RNA viruses generates a genetic diversification that could lead to their emergence in the field of viruses, with new properties allowing them to persist in immune populations. In the case of IBDV, these mutations lead to antigenic variation and modification of virulence in vivo and attenuation in vitro. In several field studies, IBDV strains isolated from different geographic areas showed amino acid substitutions at the minor hydrophilic domains. The VP2 protein has a high mutation rate and it contains the antigenic region responsible for the induction of neutralizing antibodies and for serotype specificity (Jackwood, 2011).

The first outbreak of infectious bursal disease (IBD) that occurred in 1957 in the USA was caused by the classical serotype 1 IBDV (Cosgrove 1962). The variant IBDV strains

then emerged in the 1980s on IBDV-vaccinated farms in the Delmarva area and in the late 1980s, vvIBDV emerged in Europe (Chettle *et al.,* 1989) and rapidly spread across continental Europe and Asia (Shcherbakova *et al.,* 1998), Middle East (Pitcovski *et al.,* 1998), South America (Difabio *et al.,* 1999), and Africa (Zierenberg *et al.,* 2000). However, there are significant differences between the African, European, and Asian vvIBDV strains, suggesting independent evolution (Van den Berg, 2000). Based on a recent study, it is expected that, worldwide, about 60 to 76% of IBDV isolates are of the vvIBDV genotype. Most of these viruses have been identified in areas where they have been circulating for a long period of time. The rest of the isolates are classical and variant strains based on their VP2 characteristics (Jackwood, 2007; He *et al.,* 2012b).

2.4.5. *Morbidity and Mortality*

Infectious bursal disease is extremely contagious and in infected flocks, morbidity is high, with up to 100% serological conversion after infection, while 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. At the same time, in Europe, Africa and subsequently in Japan, high mortality rates of 50% to 60% in laying chickens and 25% to 30% in broilers were observed. These hyper virulent field strains caused up to 100% mortality in specific pathogen-free (SPF) chickens (Nunoya *et al.,* 1992).

2.5. Pathogenesis

The most common route of infection is oral, but conjunctival and respiratory routes may also be important. Following host entry via oral ingestion or inhalation, IBDV may bind to host cell proteins such as N-glycosylated polypeptide(s) expressed on the cell membrane of immature IgM+ B-cells during the viral entry process. Due to its short incubation periods, which range from 2 to 3 days, a pore-forming peptide of the virus (pep46), which is associated with the outer capsid of the IBDV particle, may facilitate viral entry into the cytoplasm of infected cells. A lipid-mediated endocytic mechanism

was suggested based on the results of an in vitro study to support the entry of attenuated IBDV into the cells (Jordan *et al.*, 2002; Yip *et al.*, 2012).

IBDV initiates infection and replication in lymphocytes and macrophages of the gut-associated lymphoid tissues (GALT). This stage of viral replication marks primary viremia. Infected macrophages transport the virus to the bursa of Fabricius (BF), the prime target organ for extensive IBDV replication in the cytoplasm of intra bursal IgM+ B-cells. After 16 hours post-infection, a second viremia occurs, leading to disease and death, or the virus destroys the lymphoid follicles in the bursa of Fabricius as well as the circulating B-cells in the secondary lymphoid tissues such as GALT (gut-associated lymphoid tissue), CALT (conjunctiva), BALT (bronchial) and caecal tonsils. Virus dissemination to other lymphoid organs such as the thymus, bone marrow, spleen, payer's patches, cecal tonsils and Harderian glands may take place mainly during vvIBDV infection of susceptible chickens. As early as 48 hours post infection, IBDV infection induces prominent inflammation in the BF. By days 3 to 4, all bursal IgM+ B-cells are infected and show cytolytic changes. Clinical signs and death may result from the acute phase (7–10 days) of IBD (Eterradossi and Saif, 2008).

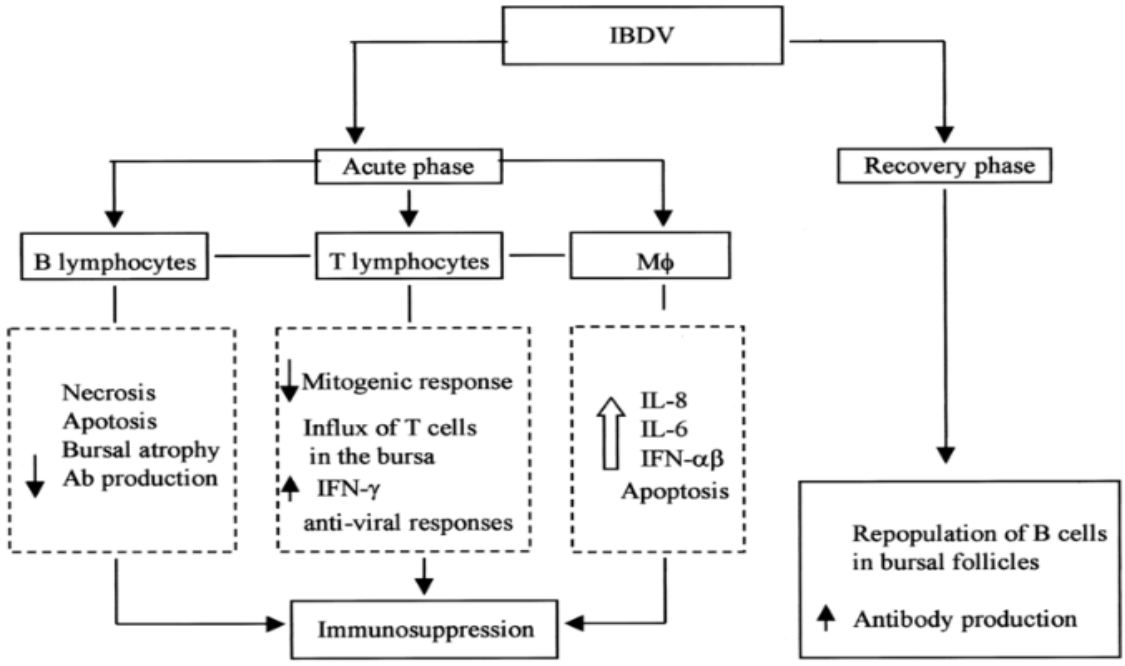
2.5.1. Immuno-Suppression Mechanism

Viral replication during the acute lytic phase causes a rapid reduction in the number of circulating IgM+ cells and prolonged suppression of the primary antibody response (Sharma *et al.*, 2000). The necrotizing action of these viruses on the host tissues causes acute illness and death. Even if the bird survives and recovers from this stage of the illness, it still has a depressed immune system, making chickens more vulnerable to opportunistic infections and inhibiting the protective effects of commonly prescribed vaccines against other viruses (Van den Berg, 2004). The virus predominantly targets B lymphocytes that are actively differentiating and multiplying, which results in immunological reduction with advancing age. The bursa of Fabricius becomes infected with the virus, which then kills IgM-bearing B cells that are actively dividing (Kim *et al.*, 2000). This has shown that IBDV replication in the bursa is accompanied by an influx of T cells during infection. According to reports, macrophages and monocytes may also be

susceptible to contracting the virus (Khatri *et al.*, 2005), in addition to B cells. IBDV-infected macrophages may act as virus transporters from the bursa and other peripheral tissues to the infection site in the gut (Van den Berg *et al.* 2000).

IBDV disease is possible in other cells, such as bone marrow-derived mesenchymal stem cells (Khatri and Sharma, 2009b). IBDV induces apoptosis, or programmed cell death, in chicken peripheral bursal lymphocytes, chicken embryos, chicken embryo fibroblasts, and Vero cells (Tham and Moon, 1996). Infection with IBDV causes the production of pro-inflammatory mediators and cytokines in macrophages, which peaks during the early phase of active virus replication (Palmquist *et al.,* 2006). The role of T cells in IBDV-induced immune pathogenesis and tissue recovery or depletion of B-cells in the bursa is accompanied by an influx of activated both CD4+ and CD8+ T cells post-infection (Kim *et al.,* 2000; Sharma *et al.*, 2000). T-cells are resistant to infection and the replication of IBDV. However, IBDV infection can severely decrease the in vitro proliferative response of T cells to mitogens, indicating that cellular immune responses are also compromised (Sharma *et al.,* 2000).

Evidence suggests that T cells may modulate IBDV immune-pathogenesis by limiting viral replication in the bursa in the early phase of the disease, but they may also enhance bursal tissue destruction, suppress immunity, and delay recovery of bursa follicles through their release of cytokines and cytotoxic effects (Rautenschlein *et al.,* 2002a). Generally, the sequelae of IBDV infections, such as severity of clinical signs, organ lesions and immune suppression, correlate with the status of immunity, age and genetic background of affected chickens and with the virulence of the infecting virus strain (Kim *et al.*, 2000).



An outline of the pathogenic and immunosuppressive aspects of IBDV

Source: (Sharma *et al*., 2000).

2.5.2. Incubation Period and Clinical Signs

The incubation period of the infectious bursal disease virus is very short and clinical signs of the disease are seen within 2-3 days after exposure, following subclinical or clinical IBD, depending on the age at which chickens are infected (Tsegaye and Mersha, 2014). The subclinical form of the disease occurs in chickens less than 3 weeks of age. Chickens present no clinical signs of disease but experience permanent and severe immune suppression. The reason young chickens exhibit no clinical signs of disease are not known. However, immune suppression occurs due to damage to the bursa of the Fabricius (Jordan *et al.*, 2002). The disease is clinically seen only in chickens older than 3 weeks. While the clinical symptoms are described as acute, including depression, trembling, white and watery diarrhea, anorexia, prostration, ruffled feathers, and vent feather solids with urates, in severe cases, affected birds became dehydrated, and in terminal stages of the disease, they had a subnormal temperature and died (Zeleke *et al.*, 2005; Ahmed *et al.*, 2009).

2.5.3. Pathological Lesion

According to the virus's virulence and pathogenicity, IBDV causes more or less severe lesions on the bursa of Fabricius and other organs such as the spleen, thymus and kidneys and may induce immune suppression and mortality in birds (Sharma, 2000; Van den Berg, 2004; Eterradossi and Saif, 2008). Macroscopic lesions are observed principally in the bursa, which presents all stages of inflammation following acute infection (Muller, 2003). In autopsies performed on birds that died during the acute phase (three to four days following infection), the bursa was initially hypertrophic, edematous and hemorrhagic; its color turned from white to cream; and a yellow transudate covered its serosa early in infection (Figures A and B). 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, often accompanied by petechiae and hemorrhages. By the fifth day, the bursa reverts to normal size and by the eighth day, it has atrophied to less than a third of its normal size. Moreover, in the acute form of the disease caused by hyper virulent strains, macroscopic lesions may also be observed in other lymphoid organs (thymus, spleen, caecal tonsils, Harderian glands, and Peyer's patches) (Eterradossi and Saif, 2008).

On postmortem examination, the affected animals have hypertrophic and whitish kidneys containing deposits of urate crystals (Figure C) and cell debris, severely dehydrated carcasses, often darkened pectoral muscles, and many petechiae hemorrhages. Masses in the thigh and pectoral muscles may be present (Figure D) and are frequently observed, probably due to a coagulation disorder (Skeeles *et al.*, 1980). Mucus may also be present in the intestines. The liver appears pale, bile stained and grey foci may also be present on an enlarged spleen (Figure E).

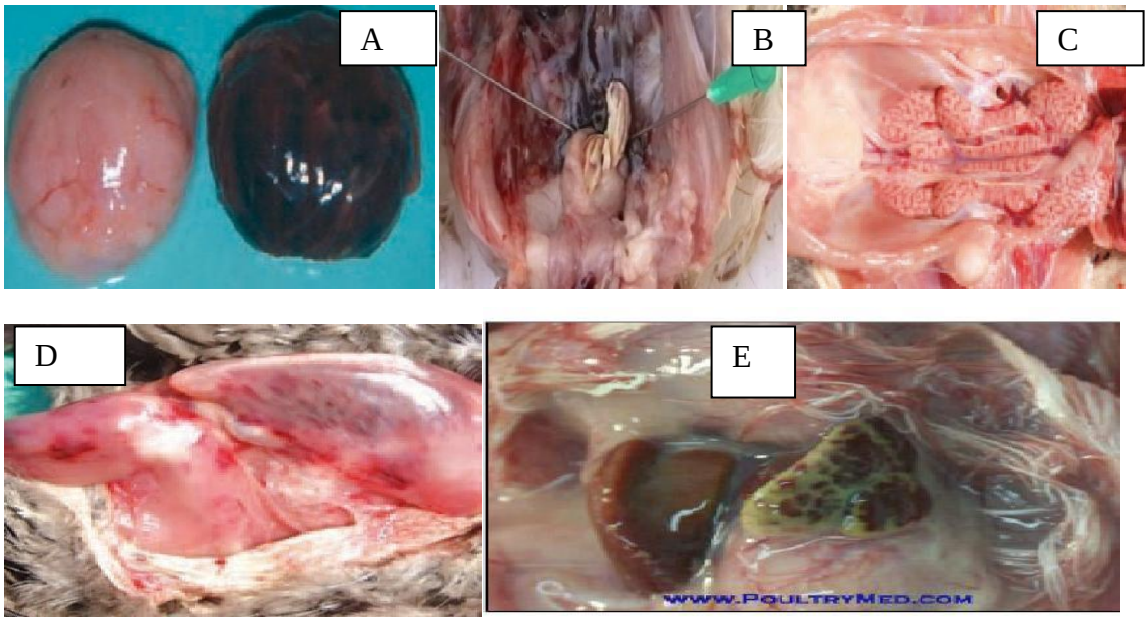


Figure 3: Different post-mortem pathological lesion on IBD infection.

Source*:* (Lucien Mahin, 2008).

2.6. Diagnosis

The diagnosis of IBD involves consideration of the flocks' history and of the clinical signs and lesions. Obviously, chickens less than 3 weeks of age present no clinical signs of disease, while chickens greater than 3 weeks of age present clinical signs as described. The severity of the clinical signs will depend on the factors described. while the clinical diagnosis of the acute forms of IBD is based on the 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, in particular of the bursa of Fabricius (Rajaonarison *et al.*, 2006). A necropsy examination will usually show changes in the bursa of Fabricius such as swelling, edema, hemorrhage, the presence of a jelly serosa transudate and eventually, bursal atrophy. Pathological changes, especially hemorrhages, may also be seen in the skeletal muscle, intestines, kidney and spleen; however, definitive diagnosis can only be achieved by the isolation and/or specific detection and characterization of IBDV (WOAH, 2012).

2.6.1. Symptomatology and Gross Lesions

Severe outbreaks are characterized by the sudden onset of depression in susceptible flocks (WOAH, 2008). Chickens in the acute phase of the disease are prostrate and reluctant to move, with ruffled feathers and frequently watery or white diarrhea. The age susceptibility is extended, covering the entire growing period in broilers, and the peaks of mortality show a sharp death curve followed by rapid recovery. Clinical IBD has clearly characteristic signs and post-mortem lesions. A flock will show very high morbidity, with severe depression lasting in most cases for 5-7 days. Mortality rises sharply for 2 days, and then declines rapidly over the next 2-3 days. Usually between 5% and 10% of birds die, but morbidity can reach 30–40% (Van den Berg *et al.,* 2000). On postmortem examination of birds that died during the acute phase of vvIBD, the bursa of Fabricius is the principal diagnostic organ; it is turgid, edematous, and sometimes hemorrhagic and turns atrophic within 7 to 10 days. This atrophy might be more rapid, even 3 to 4 days after inoculation (Tsukamoto *et al.,* 1992).

In addition, dehydration and nephrosis with swollen kidneys are common, and ecchymotic hemorrhages in the muscle and the mucosa of the proventriculus are observed in the majority of the affected birds. Severe depletion of lymphoid cells is observed not only in the bursa of Fabricius but also in the non bursal lymphoid tissues. The pathogenicity of IBDV has been associated with virus distribution in non-bursal lymphopoietic and haematopoietic organs. Indeed, using various immune staining methods, a higher frequency of antigen-positive cells could be demonstrated after infection of birds with vvIBDV compared with other strains in the thymus (Sharma *et al.,* 2000), the spleen and the bone marrow. In particular, atrophy of the thymus has been associated with the acute phase of the disease and might be indicative of the virulence of the isolate, although it is not associated with extensive viral replication in thymic cells. An increased number of macrophages is found in various organs (Tanimura *et al.,* 1997). Thrombocytes also represent a target for IBDV, and acute disease is characterized by disseminated hemorrhages probably related to an impairment of the clotting mechanism (WOAH, 2008).

Confirmation of a diagnosis of clinical IBD can be made at necropsy by examining the BF during the early stages of disease for characteristic gross lesions. During later stages of disease, it is difficult to confirm a diagnosis of IBD by examining only shrunken, atrophied BF, as other diseases like Marek's disease and mycotoxicosis produce similar changes. In birds less than 3 weeks of age or in young chickens with maternal antibodies, IBD virus infections are usually subclinical. Thus, typical clinical signs are not present, and diagnosis should be supported by histopathologic study of the suspect bursa of Fabricius, serologic studies, or virus isolation (Singh *et al.,* 2015).

2.6.2. Serological Diagnosis

Serological tests such as the agar gel immune-diffusion (AGID), enzyme-linked immunosorbent assay (ELISA) and virus neutralization test (VNT) are carried out in serum samples to detect antibodies used for monitoring vaccine responses and might be additional information for the diagnosis of infection in unvaccinated flocks (WOAH, 2012). ELISA is the most commonly used test for the detection and quantification of IBDV antibodies to check the response to vaccination in the natural field. It is economical, simple, and quick to test a large number of samples at the same time (Lukert and Saif, 2003). It is likewise essential to confirm the disease-free status of flocks. Viral antigens in the bursa of Fabricius can be demonstrated based on plates coated with IBDV specific antibodies by the antigen-capture ELISA or by the agar-gel precipitin assay (Islam *et al.,* 2001a). The AGID test can be used to detect viral antigen in the bursa of Fabricius. A portion of the bursa is removed, homogenized and used as antigen in a test against a known positive antiserum. The VN titers accurately correlate with the protection of chickens against IBDV (Knoblich *et al.,* 2000). Differentiation of classic and variant strains has been made by using ELISA and monoclonal antibodies (Sapats *et al.,* 2005). However, these methods may not be as rapid and sensitive as molecular methods (Jackwood, 2004).

2.6.3. Virological Diagnosis

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 (WOAH, 2012). The viral antigens specific to IBDV may be detected by direct and indirect immunofluorescence (Abdel-Alim and Saif, 2001) or by immune-peroxidase staining (Cho *et al.*, 1987). In the bursal follicles of infected chickens between the fourth and sixth days after infection, immunofluorescence and electron microscopy of the infected cell culture or embryonated eggs are valuable tools for monitoring the growth of IBDV, particularly those strains lacking a pronounced cytopathic effect (CPE) (McNulty *et al.*, 1979; Macdonald, 1980).

2.6.3.1. Embryo Inoculation

The inoculation of bursal homogenates from IBDV infected chickens per the chorioallantoic membrane of 9-10 days old embryonated SPF (specific-pathogen-free) chicken eggs is the most sensitive diagnostic method for virus isolation. The most sensitive route of inoculation is the CAM; the yolk sac route is also practicable (WOAH, 2012). It is important, especially for wild-type IBDV, which usually does not replicate in conventional cell culture but can also be regenerated by the reverse genetics approach and grow in embryonated chicken eggs. Some strains grow well in embryos (Brandt *et al.*, 2001; Islam *et al.*, 2001c) but are not readily adapted to grow in CEF (chicken embryo fibroblasts) or CEK (chicken embryo kidney) (Lee and Lukert, 1986). Variant viruses, however, do not kill the embryos but cause embryo stunting, discoloration, splenomegaly and hepatic necrosis (Lukert and Saif, 2003).

2.6.3.2. Cell Culture

A filtered homogenate of the bursa of Fabricius is inoculated into 9 to 11 day-old embryonated eggs originating from hens free of anti-IBDV antibodies. Primary cell cultures of CEF, bursa (CEB) and CEK have been used to propagate the virus (Boot *et al.*, 2000). The cloacal bursa and spleen are used for the isolation of the virus (Lukert and Saif, 2003). The virus can be found in other organs such as the thymus, liver and bone

marrow, but in significantly lower quantities than in the bursa (Eterradossi and Saif, 2008). The inoculum for virus isolation is prepared by homogenizing the tissue sample in antibiotic-containing buffer (PBS) that is centrifuged to remove larger tissue particles and is used for inoculating embryonated eggs and tissue culture (Lukert and Saif, 2003).

2.6.4. Molecular Identification

The classical methods for molecular characterization and differentiation of IBDV field isolates include reverse transcriptase polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP), nucleotide sequence analysis, and quantitative real-time RT-PCR (qRT-PCR) (Jackwood, 2004; Wu *et al.,* 2007a). Nowadays, reverse transcription-polymerase chain reaction (RT-PCR) is a molecular tool frequently applied in IBDV diagnosis. RT-PCR in combination with restriction enzyme analysis allows the rapid identification of vvIBDV (Zierenberg *et al.,* 2001). Nucleotide sequencing of RT-PCR products is widely used for further characterization of IBDV strains (Zierenberg *et al.,* 2000; Islam *et al.,* 2001a). The VP2 gene of IBDV contains a variable region, which suggests the potential of this region for differentiation of IBDV strains (Jackwood, 2004). RT-PCR followed by digestion with multiple restriction enzymes, or RFLP (Zierenberg *et al.,* 2001), and nucleotide sequencing of the VP2 gene have been used for the differentiation of IBDV strains (Lin *et al.,* 1993).

The molecular differentiation of IBDV strains using VP2 has been improved by the use of labeled probes in real-time RT-PCR (Jackwood and Sommer, 2005). In recent years, detection of nucleotide variation has been facilitated by the application of melt curve analysis. A TaqMan qRT-PCR and melting curve analysis can be used to trace mutations in the hVP2 region (Jackwood *et al.,* 2003). This method allows comparing sequences between field and vaccine strains (Gao *et al.,* 2007). It determines a single nucleotide polymorphism in VP2 (Wu *et al.,* 2007a). Genetic typing according to the VP2 sequence of IBDV has been widely used as a means of tracing the spread of IBDV and virulence change (Jackwood and Sommer-Wagner, 2007).

2.6.5. Histological Diagnosis

Histological diagnosis is based on the detection of modifications occurring in the bursa. The ability to cause histological lesions in non-bursal lymphoid organs, such as the thymus, 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 the diagnosis of both acute and chronic or subclinical, forms of the disease. Detection of viral antigens: thin sections of the bursa of fabricious chickens are prepared to detect viral antigens specific to IBDV 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 after 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 (WOAH, 2008).

2.6.6. Differential Diagnosis

Coccidiosis lesions and symptoms are quite comparable; they exhibit sudden onset, ruffled feathers, and bloody excretions but no bursal lesion, similar to IBD (Kaufer and Weiss, 1980). IBD can be distinguished from coccodiosis by muscle haemorrhages and bursal edema. Infectious bronchitis virus, hemorrhagic syndrome, and Marek's disease are further diseases that resemble IBD (Lukert, 1986). According to Quinn, larger muscle hemorrhages and edematous or hemorrhagic cloacal bursas would suggest IBD, but the involvement of the cloacal bursa will often distinguish IBD from other conditions that cause nephrosis. Marek's disease causes tumours and bursal atrophy, although the nerve lesion is extremely apparent. The typical symptom of the illness is hemorrhage syndrome, which can produce bursal muscular mucosal haemorrhage without a bursal lesion (Kaufer and Weiss, 1980). Bursal atrophy is brought on by non-infectious conditions like mycotoxins (Swayne, 1998). Trichothecenes generally inhibit lymphocytopoesis, and aflatoxins cause the thymus and bursa to degenerate. Zearalenon at high doses also reduces bursal weight. In animals who are undernourished or stressed for other reasons, steroidal anti-inflammatory drugs like corticosteroids can induce apoptosis and reduce the

generation of lymphocytes and smaller bursas may appear (Farner *et al.,* 1983; Ridell, 1996).

2.7. Treatment, Control and Preventions

2.7.1. Treatment

There is no specific therapy for the disease. Facilitate access to water to prevent dehydration. As with every disease, optimize the climate and reduce stress to a minimum. The use of antibiotics can sometimes be advisable to limit the impact of secondary infections (Austic and Nesheim, 2000).

2.7.2. Control and Prevention

2.7.2.1. Management and Hygiene Procedures

Infectious bursal disease virus is both highly contagious and very resistant to inactivation, which accounts for its persistent survival on poultry farms despite disinfection (Van den Berg *et al.,* 2000; Eterradossi and Saif, 2008), so that it requires strict hygienic and managemental practice. Therefore, even with strict biosecurity program (e.g., 'down time' between broods, all-in/all-out production, cleaning and disinfection of the premises and equipment), it is vital for the prevention of IBDV infection, but vaccination is also especially important to reduce the incidence and impact of IBD in the poultry industry (Van den Berg *et al.,* 2000; Eterradossi and Saif, 2008).

2.7.2.2. Vaccine and Vaccination

Immunization of chickens with high-quality vaccines is the primary method of controlling many poultry infectious diseases. However, IBDV is resistant to a large variety of disinfectants and is environmentally very stable, but is mainly controlled by vaccination (Van den Berg, 2000; Muller *et al.,* 2003; Dacic *et al.,* 2008) with a proper vaccination schedule. Vaccines developed against the Gumboro disease were effective for about 25 years. The first vaccines to prevent IBD in broilers and replacement pullets were prepared by adaptation of field isolates in embryonated eggs (Edgar and Cho 1973).

Rational vaccination schedules and strict biosecurity measures were indicated in many reports as essential tools for the control of IBD (Farooq *et al.,* 2003).

Vaccines and vaccination programs vary widely, depending on several local factors such as type of production, level of biosecurity, local pattern of disease, status of maternally derived antibodies (MD Abs), vaccines available, costs and potential losses. Many previous studies proved the role of the MD Abs in protection against IBDV in chicks (AI-Natour *et al.,* 2004). In vivo cross-protection studies, vaccination-challenge studies, and progeny challenge studies are frequently performed for the assessment of IBDV vaccine efficacy and to determine the pathogenicity and antigenic phenotypes of IBDV strains (Dormitorio *et al.,* 2007).

More recently, an IBDV reverse genetics system was implemented to introduce selected amino acid changes into the VP2 encoding region of the classic IBDV strain D78 in order to assess antigenic determinants of IBDV (Letzel *et al.,* 2007). This process, combined with nucleotide and amino acid sequencing and MAb reactivity patterns, may provide a more comprehensive analysis of IBDV strains for better diagnosis and vaccination program design (Mundt *et al.,* 2009).

Traditionally, breeder flocks are hyper immunized by priming with live vaccines and boosting with killed vaccine prior to laying in order to confer high titers of MAb to their progeny (Muller *et al.,* 2012) which is applied in some countries. This passive immunity protects chicks against early immunosuppressive infections for 1 to 3 weeks; however, protection may be extended to 4 or 5 weeks by boosting the immunity in breeders with oil-adjuvant vaccines (Eterradossi and Saif, 2008). Serological monitoring of the antibody level in a breeder flock or its progeny can aid in determining the right time to vaccinate (Eterradossi and Saif, 2008; WOAH, 2012). Oral, nasal or ocular mild vaccines were effective only in immunizing chicks that had passively acquired neutralizing antibody titers lower than 100 (Van den Berg, 2000).

2.7.2.3. Current Used Vaccines

Live Attenuated Vaccines: Live vaccines are produced from classical and variant IBDV strains by passaging these viruses in tissue cultures or embryonated chicken eggs (Jackwood and Sommer-Wagner, 2011). Several live-attenuated virus vaccines that differ according to their virulence and antigenic characteristics are available commercially. With regard to virulence or residual virulence for SPF chickens, and the level of attenuation, vaccine strains are classified as mild, mild intermediate, intermediate, intermediate plus, or "hot" (WOAH, 2012). Live-attenuated vaccines are administered via drinking water application or nebulization between the ages of 7 days and 2 or 3 weeks (Van den Berg *et al.,* 2000; Eterradossi and Saif, 2008).

Live vaccines are administered to achieve active immunity, but the interference of MDA is the crucial problem in determining a successful live IBDV vaccination schedule. Vaccinating chickens in the presence of high levels of MDA results in vaccine virus neutralization and no immunity (Moraes *et al.,* 2005). Live vaccines are favorable for mass application through drinking water and can induce strong humoral and cellular immunity (Muller *et al.,* 2012). The proven reversion to virulence (Yamaguchi *et al.,* 2000) and their residual immunosuppressive effects are major safety concerns in their extensive field applications (Rautenschlein *et al.,* 2007).

Inactivated Killed Vaccines: Killed vaccines contain either inactivated viruses, viral subunits or recombinant viral antigens and are less immunogenic compared to live attenuated vaccines unless they are administered repeatedly with adjuvants or as a booster dose after live attenuated vaccine administration (Lawal *et al.,* 2018). Killed vaccines are labour-intensive and costly in terms of administration, thus limiting their usage only to birds of high economic value, such as breeders, where point-of-lay vaccination passively confers immunity to their progenies in ovo (Alkie and Rautenschlein, 2016). Nevertheless, their administration in 1 to 10 day-old chicks in heavily IBDV contaminated environments has been reported (Wyeth and Chettle, 1990). Killed IBD vaccines are administered via subcutaneous or intramuscular injection at sixteen to twenty weeks of age (Van den Berg *et al.,* 2000) as water-inoil emulsion and induce

strong T-cell activation and inflammatory responses but must have a high antigenic content for them to effectively protect progeny chickens from vvIBDV strains (Rautenschlein *et al.,* 2002).

Genetically Engineered Vaccines: Reverse genetics has been employed to modify IBDV, thus making it attenuated for live vaccine agents (Mundt & Vakharia, 1996), since the successful establishment of a reverse genetic system for the entire nucleotide sequences of the IBDV RNA genome segments. As the viral capsid protein, VP2 carries immunodominant epitopes responsible for the induction of a protective humoral immune response. The genes encoding the polyprotein or mature VP2, or immunogenic/neutralizing domains of VP2 are targeted to produce new generation candidate vaccines. Unfortunately, the mutated viruses easily revert to virulence following a few passages in chickens (Raue *et al.,* 2004; Noor, 2009).

Some studies showed that generated re-assortants of serotypes 1 and 2 IBDV induced high levels of serotype specific neutralizing antibodies (Oberlander, 2004; Zierenberg *et al.,* 2004), but they could also revert to virulence. IBDV-VP2 subunit vaccines produced in yeast and Escherichia coli expression systems have been licensed for commercial use (Rong *et al.,* 2007), in addition to the development of virus-like particles (VLP) of IBDV (Jackwood, 2013) using genetic engineering, but they all have the same limitations as killed inactivated vaccines. However, these vaccines can be used as DIVA technology to differentiate between naturally infected and vaccinated flocks (Muller *et al.,* 2012).

Immune Complex Vaccine: A new concept of vaccines was the establishment of immune complex vaccines (Icx). These vaccines consist of a mixture of a certain number of IBDV-specific antibodies obtained from the sera of hyper immunized chickens and the infectious IBD vaccine virus (Whitfill *et al.,* 1995). The benefits of this technology are the quality and strength of the protection coming from the IBD virus's capability to colonize the bursa without harming the bird, resulting in full IBD protection against clinical signs, complete resistance against IBDV infection, a high reduction of shedding and diminishing the risk of virus changes after continuous re-infection. The vaccine adapts to the passive immunity levels of each individual chicken and replicates at the

optimum time when MDA levels are at a lower level. Their major advantage is that they are suitable for in ovo vaccination at day 18 of incubation with commercial egg-injection machines. In addition, it has been shown that these vaccines are effective in the presence of maternally derived antibodies (Haddad *et al.*, 1997; Giambrone *et al.*, 2001).

2.7.2.4. Vaccination Failures and Potential Causes

The occurrence of vvIBDVs has increased the economic importance of the disease. Until 1987, the strains of the virus were of low virulence, causing less than 2% mortality, and vaccination was able to satisfactorily control the disease. However, the occurrence of vvIBDV has led to vaccination failures and increased mortality and morbidity (van den Berg, 2000). In 80% of the WOAH member countries, acute clinical disease due to IBDV has been reported (van den Berg, 2000).

In general, vaccine efficacy highly depends on the dose and strains of the vaccine and challenge viruses, as well as the route of administration, the appropriate vaccination time, and the levels of maternal antibodies (WOAH, 2012). The potential causes that affect the outcome of an IBDV vaccine are largely based on the gap in correlation between strains of the vaccine and pathogenicity and antigenicity types of the circulated virus, the appropriate vaccination time, the age and breed of the bird, the presence or absence of neutralizing antibodies (MAB) and the vaccination history of the progeny of parent flocks, which determine the efficacy of IBD vaccination. In addition to this, vaccination is not usual practice in smallholder poultry and control is further complicated by the regular emergence of new strains that may not be covered by existing vaccines. On top of this, most control strategies designed in the country do not take into consideration the local chickens, and this may lead to the failure of most strategies (Hailemariam *et al.*, 2006).

The causes of failure in live-virus vaccinations are numerous. The most trivial cause is interference from MAB (AI-Natour *et al.*, 2004), which is one of the most frequent causes of failure. The duration and uniformity of this immunity may be influenced by the concentration and antigenic specificity of the vaccine strain (Van den Berg *et al.*, 2000).

Therefore, it requires continuous monitoring of the antibody level in a breeder flock or its progeny to aid in determining the right time to vaccinate (Eterradossi and Saif, 2008). Classical live attenuated vaccines may induce broad, lifelong protection, but they also carry residual pathogenicity and the potential to revert to virulence (Van den Berg *et al.*, 2000). Inactivated vaccine failure is rare but may occur, either due to the absence of previous contact of some of the birds with a live virus (vaccine virus) or to the existence of antigenic variants not present in the vaccine. These vaccines are not ideal for stimulating a primary antibody response; therefore, they tend to be most effective in chicks that have been "primed" with a live virus vaccine or are naturally infected through field exposure to IBDV (Eterradossi and Saif, 2008).

2.8. Economic Impacts of IBD

The economic impact of IBD in poultry is serious and influenced by strain of virus, susceptibility and breed of flock; current primary and secondary pathogens; and environmental and managemental factors. Clinical IBDV leads to direct losses due to high mortality, in addition, condemnation of carcasses due to skeletal muscle, thigh and pectoral muscle hemorrhages can be an important cause of economic losses (van den Berg, 2004). Indirect losses in Gumboro disease arise due to the severe immune suppression of broilers and egg-laying chickens and their increased predisposition for other diseases and vaccination failure (Van den berg, 2004). Thereby, as a consequence, they result in delayed growth, reduced weight gain, greater food conversion, longer fattening, lesser production values, increased mortality and lower quality of products observed. The occurrence of vvIBDVs has increased the economic importance of the disease (Sharma, 2000).

The presence of disease may also limit opportunities in the market, either locally or internationally, and hinder the adoption of improved technologies, be they improved breeds, better management systems, or more efficient processing and marketing methodologies. There would be further loss of income for an extended period because of the stamping-out policy. The disruption to the flow of product and decreased production may cause job losses on farms and in service and associated industries, depending on the

time it takes to bring the outbreak under control. Even a small outbreak would result in the dislocation of the industry and its normal marketing patterns. An uncontrolled outbreak would markedly increase production costs because of the impact of the disease and the need for continuing control measures (Aregitu, 2015).

2.9. The Status of IBD in Ethiopia

Various studies have been conducted with the main objective of determining the prevalence and associated risk factors of infectious bursal disease in different poultry production systems of Ethiopia (Natnael, 2015). Gumboro disease was first reported in 2002 in Ethiopia at a privately owned commercial poultry farm, in which a 45–50% mortality rate was documented (Zeleke *et al.,* 2003). It was diagnosed first in commercial poultry and thereafter in a government-owned poultry multiplication center and a commercial broiler farm (Chanie *et al.,* 2009) with serological tests. Infectious Bursal Disease is a newly emerging disease of chicken in Ethiopia. As described by Zeleke *et al.,* (2005), the disease has been considered to have been introduced concurrently with the increased number of commercial state and private poultry farms flourishing in the country. Different researcher's reports from various regions of the country indicated that viral diseases are posing a growing threat to the young poultry industry (Hailu *et al.,* 2009; AHY, 2011; Shiferaw *et al.,* 2012; Natnael, 2015).

In a study conducted by Sindu *et al. (*2015), an overall seroprevalence of 45.05% (173/384) of infectious bursal disease (IBD) in chickens reared under backyard poultry production systems around Mekele town; out of 552 serum samples tested, 458 (83%) were in backyard chickens at selected woredas of Eastern Ethiopia by Tadesse and Jenbere (2014); and 27.8% of IBD in chickens owned by 775 households in the Amhara region of Bahir Dar and Farta district, respectively, by Hailu *et al.* (2009) were indicated. Hailu *et al.* (2010) also reveal an overall Sero-prevalence of 76.64% of IBD in local chicken (269/351) in a study that was conducted in three selected districts of the south and west Showa zones of the Oromia region, namely: Waliso, Welemera and Ambo, which has a prevalence of 89.78% in Waliso and a significant difference with sero-prevalence in Ambo (70.69%) and in Welemera (40.81%).

Table 2: Different studies conducted on prevalence of IBD in Ethiopia

Study area	Prevalence (%)	Authors
Gondar and west Gojjam	72 and 75	Kassa and Molla, 2012;Terefe, 2018
Southwest showa	76.6	Hailu *et al.,* 2009
Mekelle town	45.1	Zegeye *et al.,* 2015
Central Ethiopia	82.2	Tesfaheywet and Getnet, 2012
Andassa poultry farm	98.9	Solomon and Abebe, 2007
Eastern Ethiopia	83	Tadesse and Jenbere, 2014
Debre Brehan	94.7	AHY, 2011
Bishoftu	82.2	Zeryehun and Fekadu, 2012
Bahir Dar	29.4	Mazengia *et al.,* 2010
Farta	21.7	Mazengia *et al.,* 2010
Bishoftu	93.3	Zeleke *et al.,* 2005

In addition, molecular characterization of the Ethiopian IBD virus isolates was done for the first time in 2005 from the samples collected from Kombolcha Poultry Multiplication Center and on commercial and breeding poultry farms in Ethiopia between 2009 and 2011 (Shiferaw *et al.,* 2012). In both cases, for virus isolation using chicken fibroblast cell culture, the positive isolates were processed for further antigenic and genomic characterization and were identified as virulent classical viruses and very virulent IBD viruses. According to the importance of the disease as reflected by the high mortality and reduced productivity, chicken traders suffer huge financial losses due to IBDV mortality in chickens (Zeleke *et al.,* 2005). Therefore, infectious diseases like IBD are becoming real threats to chicken under different production systems (Aregitu, 2015).

3. MATERIALS AND METHODS

3.1. Study Areas

An outbreak based cross sectional study was conducted in Bishoftu, Dukem, Modjo, Gelan and Tulu Dimtu in central Ethiopia, which are known to be potential sources of different poultry production systems in areas where frequent IBD outbreaks occur.

Bishoftu is located 47 km from Addis Ababa. In Bishoftu town, several commercial poultry farms, hatcheries and breeding farms are found, from which pullets of various ages are distributed to different parts of the country (http://www.mwud.gov.et/web/bishoftu/home). Gelan is a town located in the Akaki district of the Shegar city and 25 km south-east of Addis Ababa. Dukem is a town in central Ethiopia located 37 km southeast of Addis Ababa. Tulu Dimtu is located in Akaki Kaliti sub-city of Addis Ababa. In Tulu Dimtu, they have a lot of mixed livestock populations in the area (*https://ethiopia.places-in-the-world.com/327229-place-tulu-dimtu.html*). Modjo is located in the East Shewa zone of the Oromia region of Ethiopia, 66 km far from Addis Ababa. In Modjo town, there are different poultry production systems (CSA, 2005).

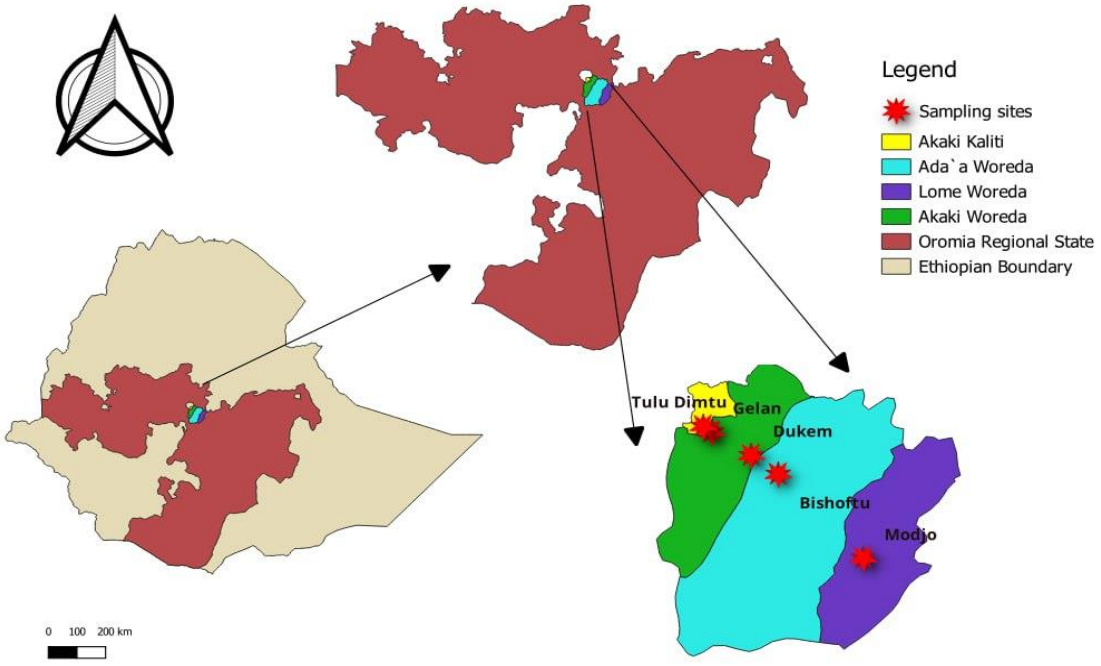


Figure 4: Map of the Study Areas

Source: (Q-GIS Version 3.30)

3.2. Study Animals

The study animals were chickens of Cobb-500, Lowmas traditions, Bovans brown and Saso breed types. Chickens kept between 23 and 48 days in commercial poultry production systems were used. Moribund or recently dead chickens were examined and used for the study.

3.3. Study Design and Sampling Methods

An outbreak based cross-sectional study design was carried out from December 2022 to May 2023 under chickens of all breeds reared in commercial poultry farms in selected districts (Bishoftu, Dukum, Modjo and Tulu Dimtu), central Ethiopia. A purposive sampling technique was used based on the occurrence of outbreaks. Explanation of the characteristic features of IBD was communicated to farm attendants and owners so that they could report up on recognizing those features of the disease. From purposively selected nineteen poultry farms, a total of 32 pooled bursa samples (five bursal tissues per

pool) were collected. Two to three pooled samples were collected from a single farm based on the number of flocks found during farm visits. Accordingly, 12 bursal tissue samples from Bishoftu in six poultry farms, 10 bursal tissue samples from Dukem in five poultry farms, 3 bursal tissue samples from Tulu-Dimtu in three farms, 3 bursal tissue samples from Gelan in three farm and 4 bursal tissue samples from Modjo in two poultry farms were collected.

3.4. Sample Collection and Transportation

3.4.1. Biological Sample Collection and Transportation

Bursa tissue samples were collected from IBD-suspected clinically sick and recently deceased chickens after postmortem examination. For both virus isolation and molecular detection, bursa samples were collected aseptically and placed in sterile and labeled universal bottles with additions of appropriate transport media (VTM). Then the samples were transported using an icebox with an icepack to National Veterinary Institute (NVI) and kept at -80^{o}C until processed (WOAH, 2012).

3.4.2. Questionnaire Survey

A questionnaire survey was implemented on each poultry farm owners and the information includes farm category, location, contact with other farms (same owner, sharing equipment, trucks, personnel, origin of the chicks…), applied vaccination program (type of vaccine and time of vaccination), symptomatology, lesions, morbidity, and mortality. Personal observation was used to assess the general status of the poultry farm and one questionnaire was administered per farm.

3.5. Laboratory Investigation of IBD Suspected Samples

3.5.1. Sample Preparation

Because of the limitation of resources, only seven pool bursal samples were processed for molecular detection by RT-PCR and were randomly selected after being systematically

grouped depending on study areas. Further, four RT-PCR positive samples were cultured on primary chicken fibroblast medium to appreciate the growth and CPE of the virus. The viral antigen was prepared directly during the collection of suspected bursa of Fabricius samples and frozen bursal samples were thawed and taken out of the labeled sample bottle in the class II biosafety cabinet and 1 gram of tissue was taken 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 buffered saline solution supplemented with penicillin and streptomycin (1000 µg/ml each). The suspension was transferred into a 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.

3.5.2. RNA Extraction and Reverse Transcription

The field sample was subjected to processing prior to a molecular biology analysis, and 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 suspensionsample using the RNeasy® Mini Kit manufactured by QIAGEN, Germany, in accordance with the manufacturer's instructions for RNA extraction (Appendix II).

3.5.3. Master Mix preparation

The formulation of Master Mix was executed in a designated location (Master Mix room). PCR amplification of the resulting cDNA was performed based on the partial sequence of the VP2 gene of IBD virus by using reverse transcriptase enzyme and IBDV specific designed primers, forward primer IBDF3 design (5’TCT TGG GTA TGT GAG GCT TG -3’) and reverse primer IBDR3 design (5’CCC GGA TTA TGT CTT TGA -3') with a 400 bp amplification capacity, with negative and positive control reactions. The targeted amplicon size was approximately 400 bp. A reaction mixture with a total volume of 25 µl was prepared by combining 4µl of RNase free water, 5µl of 5X RT-PCR buffer,

5µl of Q-solution, and 1µl of 10 mM dNTPs mix, 1µl of one step RT-PCR enzyme mix and 5µl of template RNA extracts.

3.5.4. Polymerase Chain Reaction (PCR) Conditions

The viral suspensions were subjected to VP2 gene amplification. The PCR reaction was done one cycle at 50°C for 30 minutes for the cDNA template, followed by initial denaturation of the PCR reactions for one cycle at 95 °C for 15 minutes; subsequent cycles 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. Subsequently, the amplified fragment was subjected to visualization and subsequent comparison with bands derived from molecular markers.

3.5.5. Agarose gel Electrophoresis of PCR Products

PCR products were analyzed by 2% (w/v) Agarose electrophoresis gel stained with 4 µl gel red and 100 mL TAE buffer. Briefly, 10 µl each PCR product was mixed with 1 µl 10X loading buffer and loaded into a separate well of the pre-prepared gel and a 100 bp DNA molecular marker was also added onto the first lane and run at 120 volts for about 1:20 hours in the electrophoresis apparatus. The PCR product band was visualized by gel documentation under a UV-lamp camera and the size of the PCR products was estimated by comparing it with the band size of the molecular marker 100 bp ladder (marker) that was loaded on a separate lane (WOAH, 2012).

3.5.6. Cell Culture Preparation

Using 11-day-old viable, specified pathogen-free (SPF) embryonated eggs, primary chicken embryo fibroblast (CEF) cells were prepared inside a biosafety cabinet class II. In line with this, ethanol at a 70% concentration was used to clean and de-embryo SPF eggs. The body was washed three times in sterile phosphate buffered saline (PBS) to eliminate any remaining red blood cells (RBCs) after the viscera and extremities (the head and legs) were removed. Sterile scissors were also used to cut the carcass. The chopped material (tissue fragments) was mixed with 10 ml of a 37°C pre-warmed 0.25%

trypsin solution, and then the mixture was slowly agitated for 15 minutes in a 37°C incubator. The remaining tissue fragments were retrypsinized for 5 minutes in 10 ml of 2x trypsin after suspension was collected in a mesh-filtered flask containing 5% foetal bovine serum. The collected cell suspensions were put in sterile centrifuge tubes and centrifuged for 10 minutes at 1000 rpm. Pelleted cells were re-suspended in Glasgow minimal essential medium (GMEM), which was treated with antibiotics (Penicillin G 100 IU/ml, Streptomycin Sulphate 100 g/ml, and 10% calf serum). The trypsin solution's supernatant was discarded. The cell suspension was transferred into a 25 cm2 flask made of sterile plastic for tissue culture, and it was kept at 37°C with 5% CO2.

3.5.7. Virus Isolation

A 10% tissue suspension was inoculated onto primary chicken fibroblast cells (CFC) that had already been confirmed using in adsorption techniques (WOAH, 2012). After an hour of adsorption, the inoculated cultures were maintained in GMEM containing 2% bovine calf serum and kept at 37°C. Cultures were observed under a microscope for up to seven days to look for the specific to IBDV cytopathic effect (CPE). Samples without CPE were blindly passed a further three times after seven days following two cycles of freeze-thawing. The samples that displayed diagnostic CPE, however, were considered positive and stored at -20°C for subsequent molecular examination. After the third blind transit, samples were deemed negative if no CPE was visible (WOAH, 2012).

3.6. Ethical Statement

Ethical clearance for this research was obtained from Addis Ababa University College of Veterinary Medicine and Agriculture Animal Research Ethical Review Committee (Certificate Reference Number: VM/ERC/28/02/14/2023). All animal work was conducted according to animal research ethics (Appendix VII).

4. RESULTS

4.1. Questionnaire Survey Result

During the current study period, 19 commercial poultry farms (where 68.42% of them were broilers and 31.58% were layers) were visited and questionnaires were collected from farm attendants and owners regarding their farm management, including vaccination and biosecurity measures. Based on this, of the total farms visited, 47.37% have vaccinated their chickens against IBD, whereas 21.05% follow the manufacturer's vaccination protocol. On top of this, 78.95% and 63.16% of the respondents lack knowledge about biosecurity practices and implement poor hygienic conditions (Table 4 below). Total average morbidity rate 82.51 and total average mortality 31.85 with the total average case fatality rate 38.60 also recorded (Table 3 below).

Table 3 : Mortality and Morbidity rate result

Areas	No of chicken at risk	Morbidity	Morbidity (%)	Mortality	Mortality (%)	Case fatality
Bishoftu	8800	1500	17.04	896	10.18	59.73
Dukem	9800	1290	13.16	234	2.39	18.14
Tulu Dimtu	2800	760	27.14	242	8.64	31.84
Modjo	2000	320	16	100	5	31.25
Gelan	1700	156	9.17	96	5.64	61.54
Total	25100	4026	82.51	1568	31.85	38.60

Table 4: Questionnaire survey result

Factors	Category	Frequency	Percent
Area	Bishoftu	6	31.58
	Dukem	5	26.32
	Tulu Dimtu	3	15.79
	Modjo	2	10.53
	Gelan	3	15.79
Breed	Cobb-500	3	15.79
	Bovans brown	6	31.58
	Saso	6	31.58
	Lowman tradition	4	21.05
Vaccine against IBD	No	4	21.05
	Yes	9	47.37
	Unknown	6	31.58
Vaccination according to the manufacturer protocol	No	6	31.58
	Yes	4	21.05
	Unknown	9	47.37
Farm type	Broiler	13	68.42
	Layer	6	31.58
Received bio-security training	No	15	78.95
	Yes	4	21.05
Regular cleaning and disinfection used	No	14	73.68
	Yes	5	26.32
Equipment exchange	No	11	57.89
	Yes	8	42.11
Informed if an outbreak occurred in the area	No	9	47.37
	Yes	10	52.63
Hygienic condition	Poor	12	63.16
	Good	7	36.84
Special room for sick chickens	No	12	63.16
	Yes	7	36.84
	Total	19	100.00

4.2. Clinical Findings and Postmortem Examinations

A total of 25,100 chickens were observed, which were reared in nineteen commercial poultry farms and 160 chickens were examined postmortem level. The diseased chickens showed clinical signs of severe depression, emaciation, watery diahorrea (Figure 5A, B, and C), ruffled feathers (Figure 5B), an enlarged hemorrhagic bursa (Figure 5E, F), and accumulation of urate in the kidney, haemorrhage in the thigh and pictorial muscle during postmortem examinations. The age of the chickens varied from 23 to 48 days and the mortality rate varied in all flocks.

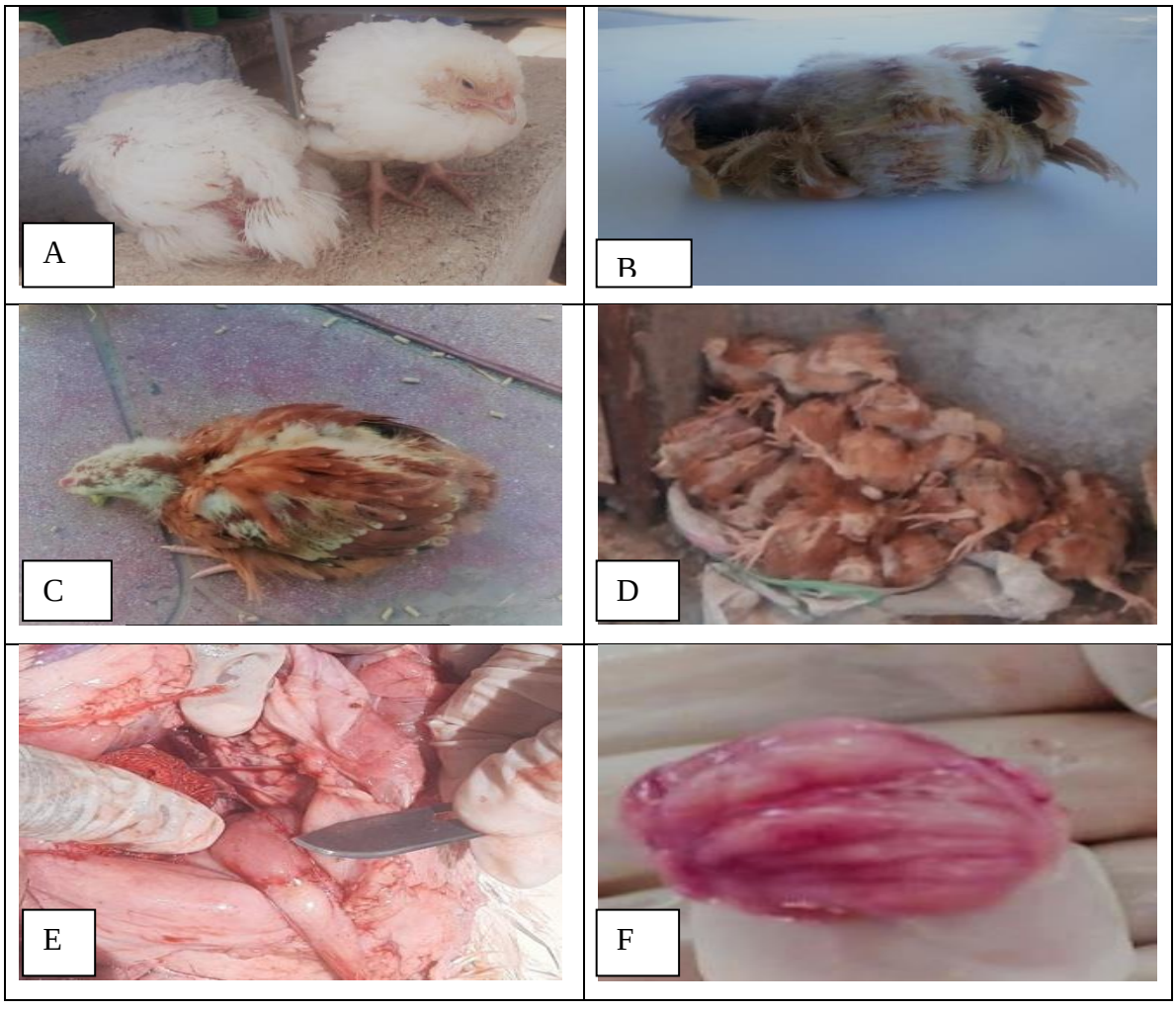


Figure 5: Clinically Diseased Chicken and Post mortem Lesion.

4.3. IBDV Molecular Detection by RT- PCR Results

The present findings result indicated that among the processed for molecular detection by RT-PCR technique, six (85.71%) of were found PCR positive with the required amplicon size, 400 bp (Figure 6 below).

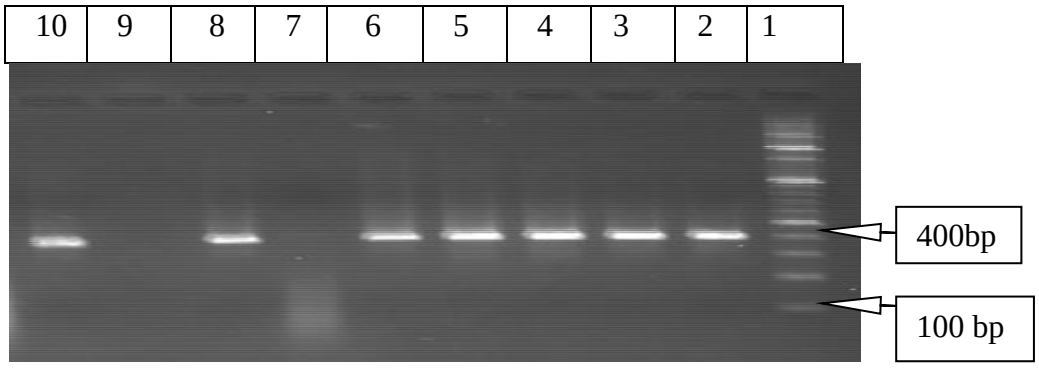


Figure 6: IBDV PCR agarose gel documentation result

Description: Lane -1. 100 bp Marker, Lane -2 to 8 suspected bursa sample, (lane 2, 3, 4, from Bishoftu, 5, 6 from Dukem, 7 from Modjo and 8 from Tulu Dimtu), Lane -9 negative control and Lane -10 was positive control.

4.4. Virus Isolation

Out of four samples inoculated into the primary chicken fibroblast cell line, all of them have shown characteristic cytopathic effect (CPE) such as cell swelling, cell rounding, detachment and floating starting on day two post infection after the second passage (Figure 7 below).

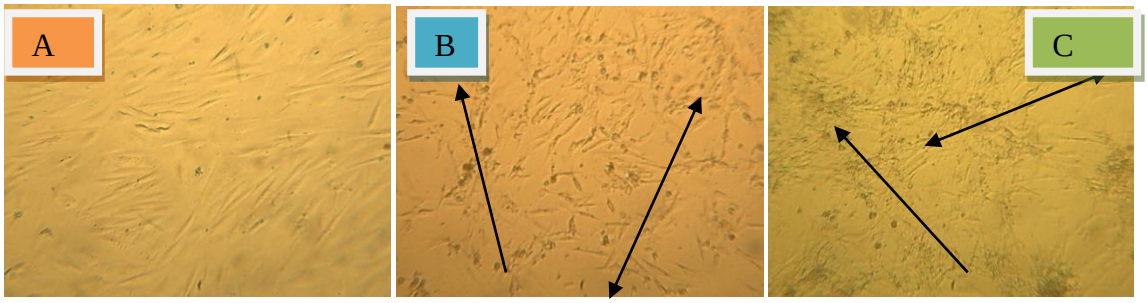


Figure 7: IBDV on primary chicken embryo fibroblasts (CEF) cell line with a visible CPE.

A. Positive control, B and C indicates visible cytopathic effect (CPE).

Table 5: Suspected IBDV Samples, Molecular Detection Results by PCR Method

Factors	**Category**	**No .of examined samples**	**No. of positive samples**	**F–value**	**P–value**
Area	Bishoftu	3	3	7.00	0.0719
	Dukem	2	2		
	Modjo	1	0		
	Tuludemitu	1	1		
Breed	Cobb -500	2	2	2.92	0.4047
	Bovans brown	2	2		
	Saso	2	1		
	Lowman's tradition	1	1		
Vaccine against IBD	Yes	3	2	1.56	0.4594
	No	1	1		
	Unknown	3	3		
Farm type	Broiler	4	3	0.88	0.3496
	Layer	3	3		
Received bio-security training	No	5	5	2.92	0.0877
	Yes	2	1		
Using regular disinfectant and cleaning	No	6	5	0.19	0.6592
	Yes	1	1		
Hygienic condition	Poor	6	5	0.19	0.6592
	Good	1	1		
	Summer	5	5		
	Total	7	6 (85.71%)		

Keys: F- Fisher square, IBD-Infectious Bursal Disease, No.-Number, P- Pearson square

5. DISCUSSION

Infectious bursal disease virus (IBDV), also known as "Gumboro disease," belongs to the genus *Avibirnavirus*, family *Birnaviridae*, is highly contagious and very resistant to inactivation, and persists on poultry farms despite disinfection, so that it requires strict hygienic and managemental practise (Eterradossi and Saif, 2008). Immunization of chickens with high-quality vaccines is the primary method of control of many poultry infectious diseases through vaccination following a proper vaccination schedule (Muller *et al.,* 2003; Dacic *et al.,* 2008).

The present study indicated an overall variability in the application of biosecurity measures. For instance, of the total number of farms visited, 47.37% vaccinated their chickens against IBD, and only 21.05% followed the manufacturer's vaccination protocol. Besides, 78.95% lack knowledge about biosecurity practices, 63.16% had poor hygienic practices, 36.84% had a special room for sick chickens, and 57.89% did not exchange equipment with other farms and only 26.32% practice regular cleaning and disinfection. And the average mortality and morbidity rates of 31.85% and 82.51% were recorded with 38.60% being the average case fatality rates. This high percent of poor hygienic conditions and lack of knowledge and training about biosecurity is in line with the study conducted by Ismael *et al.* (2021) on commercial chicken farms established in Bishoftu town, Ethiopia, where 93.18% of participants did not receive training on biosecurity and 75% of the farms had poor hygienic conditions. And also in line with the study conducted by Alemneh and Getabalew (2019) in and around Bahir Dar Ethiopia, who reported (88.64%) did not exchange equipment with other farms and only (20.45%) had isolation rooms for diseased chickens.

On the contrary, the present study finding is inconsistent with the study conducted by Alemneh and Getabalew (2019) in Bahir Dar, Ethiopia, on small-scale farmers who implemented above 75% of the biosecurity aspects, which include wild bird proofing, availability of clean water, vaccination against economically important diseases, regular cleaning and disinfection of feeders and drinkers and cleaning and disinfection of chicken

houses between batches. The improved biosecurity practice might be due to the better veterinary service and poultry production experience of farmers living in Bahir Dar.

The present study revealed the presence of a moderate mortality rate in chickens. The finding is in line with the study conducted by Shegu *et al.* (2020) in different parts of Ethiopia, who reported a 48% average mortality rate. And also, the present study's findings line up with those of a study conducted by Mohammed and Abdelmalik (2022) in Khartoum, Sudan, who reported a 51% average mortality rate. The present study's findings disagreed with the report conducted by Nunoya *et al.* (1992) in Japan, which reported 100% mortality. The lower mortality rate recorded during the current study could probably be attributable to the viral strain difference that caused the outbreak and agro-ecological variability.

The incubation period and clinical signs like depression, ruffled feathers, anorexia, and white diarrhoea tinged with blood, sudden death, emaciation, and dropping of the wing were among the major manifestations. These clinical manifestations were similar to the findings of Zeleke *et al.* (2005), Mekuriaw *et al.* (2017), Shegu *et al.* (2020) in Ethiopia and Dey *et al.* (2019) in India.

There are many reports on the existence of new strains of infectious bursal disease virus in Ethiopia (Tesfaheywet *et al.*, 2012; Jenbreie *et al.*, 2012; Mekuriaw *et al.*, 2017; Shegu *et al*., 2020). Most of those reports were supported by virus isolation and molecular analysis, and serological data. The present study findings indicated that 6 (85.71%) of the samples were found positive from a total of 7 pool bursal samples processed for molecular detection by reverse-transcription polymerase chain reaction (RT-PCR). Further, four PCR-positive samples were processed for viral isolation on DF-1 cells and all samples showed visible cytopathic effect (CPE).

These results of PCR detection and viral isolation in the present study are in agreement with the recent studies in Ethiopia conducted by Mekuriaw *et al.* (2017), who reported that from a total of 25 tissue samples processed for virus isolation, 95% (18/20) of the bursa and 80% (4/5) of the spleen samples showed visible cytopathic effects (CPE). The

positive samples were confirmed by PCR and 19 of them had the expected band of 643 bp. The present virus isolation result is similar to the study conducted by Shegu *et al.* (2020) in Ethiopia. Who processed nine outbreak samples on the DF-1 cell line and all of the pooled samples produced CPE.

The present study findings are much more in line with the study conducted by Islam *et al.* (2012) in Bangladesh. He processed 35 bursal samples. From 35 bursal tissue samples, 34 (97.1%) were positive and generated 254 bp products by RT-PCR. The number of positive samples from RT-PCR in the present study is higher (in terms of detection rate) than a similar study conducted by Mawgod *et al.* (2014) in Egypt, who reported 20 positives from 52 field samples suspected of infectious bursal disease virus using the same technique. This variation could be due to simple size differences that were processed for RT-PCR genomic amplifications of the virus.

6. CONCLUSION AND RECOMMENDATIONS

The poultry sector is one segment of the livestock sector in Ethiopia, characterized into large commercial, small-scale commercial and village or backyard poultry production systems. These production systems have their own specific chicken breeds, inputs and production properties and are challenged by the occurrence of many infectious diseases. Among these, infectious bursal disease is the one that has become a serious threat, causing frequent outbreaks and a challenge to young, growing poultry farms. In the present study, it was observed that IBD has become the most devasting disease of chickens in central Ethiopia, with high mortality rates. In the present study finding indicate the presence of IBDV in commercial poultry farms, confirmed by both viral isolation in cell culture and molecular detection by RT-PCR. The continuous presence of this disease may be due to a lack of appropriate biosecurity, a poor vaccination schedule, hygienic conditions, an uncertain vaccination history of flocks from the sources, and other factors.

In line with the above concluding remarks, the following recommendations are forwarded:

- Management factors like farm management, including vaccines and vaccination schedules, and proper biosecurity measures should be implemented to reduce the magnitude of IBDV infection in the investigation area.
- On top of molecular detections and virus isolation, sequencing of the positive samples is mandatory to know the circulating strains of the virus in the study areas.
- The efficacy of current vaccines against IBD should be tested and vaccine matching studies should be conducted to understand the deviation between vaccine and field strains.

7. LIMITATION OF THE STUDY

Field investigation to follow up on an outbreak was difficult because the cases weren't reported promptly (due to ignorance or delayed reporting of the cases). One of the main challenged issues during the study period was the limitation of resources (Time and Logistics), because of this, couldn’t process further test like sequencing.

8. REFERENCES

Abdel-A G. A. and Saif Y.M., (2001): Pathogenicity of cell culture-derived and bursa-derived infectious bursal disease viruses in specific-pathogen-free chickens. *Avian Dis.,* **45**: 844 - 52.

Ahmed M. S., Sarker A. and Rahman M. M., (2009): Prevalence of infectious diseases of broiler chickens in Gazipur district. *Bangladesh Journal of Veterinary Medicine*, **7**(2): 326-331.

Alemneh T. and Getabalew M., (2019): Exotic chicken production performance, status and challenges in Ethiopia. *Int J Vet Sci Res*, **5**(2), 039-045.

Aliyu H. B., Hair-Bejo, M., Omar, A.R. and Ideris, A., (2021): Genetic Diversity of Recent Infectious Bursal Disease Viruses Isolated from Vaccinated Poultry Flocks in Malaysia. Front. *Vet. Sci.* **8**: 643-976.

AL-natour M., Ward. L., Saif. Y., Stewart-brown u. B., and Keck L., (2004): Effect of different levels of maternally derived antibodies on protection against infectious bursal disease virus. *Avian Dis.* **48**: 177-182.

Animal Health Year book (AHY) (2011): Veterinary epidemiology, Disease prevention, control and eradication. Ministry of Agriculture animal and plant health Regulatory Directorate, Ethiopia. pp- 22.

Aregitu M., (2018): Review on infectious Bursal disease: Threat for Ethiopian Poultry industry. *International Journal of Advanced Life Sciences,* **11**:60-61.

Aregitu M., (2015): Outbreak Investigation and Molecular Characterization of Infectious Bursal Disease Virus and Vaccine Immunogenicity trial in Ethiopia. MSc thesis: Addis Ababa University, College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia.

Aricibasi M., Jung, A., Helleru, E., and Rautenschlein S., (2010): Differences in genetic background influence the induction of innate and acquired immune responses in chickens depending on the virulence of the infecting infectious bursal disease virus (IBDV) strain. *Vet I Immunopathol***135**, 79-92.

Austic E.R., and Nesheim, M.C., (2000): Poultry production. **13**th ed: Infectious bursal disease. pp. 58-60.

Azad A.A, Jagadish M.N., Brown m.A. and Hudson P.J., (1987): Deletion mapping and expression inEscherichia coliof the large genomic segment of a birnavirus. *Virology*, **161**: 145-152.

Banda A., Villegas, P. and El-Attrache, J., (2003): Molecular characterization of infectious bursal disease virus from commercial poultry in the United States and Latin America. *Avian diseases*, **47**: 87-95.

Baxendale W., (2002): Birnaviridae. In: Jordan, F., Pattison, M., Alexander, D., and Faragher, T. *Poultry Diseases*, **5th** Edition, W.B. Saunders. London, pp. 320-324.

Becht H., (1994): Birnaviruses - Animal. In: Webster, R.G., Garnoff, A, Eds. *Encyclopedia of virology Vol.***1**. London, UK: *Academic Press*, Harcourt Brace & Company. pp. 143 - 149.

Birghan C., Mundt, E., and Gorbalenya A. E., (2000): A non-canonica l Lon proteinase lacking the ATPase domain employs the Ser-Lys catalytic dyad to exercise broad control over the life cycle of a double-stranded RNA virus. *EMBO Journal*, **4**, 114–123.

Biro E., K. Kocsis, Nagy .N. Molnar. D., Kabell. S., Palya u. V., and Olah. I., (2011): Origin of the chicken splenic reticular cells influences the effect of the infectious bursal disease virus on the extracellular matrix. *Avian Pathol*, **40**, 199-206.

Brandt M, Yao K, Liu M, Heckert RA, Vakharia VN., (2001): Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. *J Virol.* **75**:11974–11982.

Busnadiego I., A. M. Maestre, D. Rodriguez u. and J. F. Rodriguez, (2012): The Infectious Bursal Disease Virus RNA-Binding VP3 Polypeptide Inhibits PKR-Mediated Apoptosis. PLoS One **7**th Ed 467- 468

Central Statistical Agency of Ethiopia (CSA) (2021): Original data from Accessed via https://public.knoema.com/pbwfnlf/livestock-statistics-of-Ethiopia

Chaka, H., F. Goutard, S. P. Bisschop u. and P. N. Thompson (2012): Sero-prevalence of Newcastle disease and other infectious diseases in backyard chickens at markets in Eastern Shewa zone, Ethiopia. *Poult Sci*. **91**: 862-869.

Chanie M., Negash, T. and Tilahun, S.B., (2009): Occurrence of concurrent infectious diseases in broiler chickens is a threat to commercial poultry farms in Central Ethiopia. *Tropical animal health and production*, **41**(7): 1309-1317.

Chettle N., Stuart, J.C., Wyeth, P.J., (1989): Outbreak of virulent infectious bursal disease in East Anglia. *Vet. Rec.***125**, 271–272.

Cho B., Snyder, D., Lana, D. and Marquardt, W. (1987): Infectious bursal disease: Rapid diagnosis by immune peroxidase monoclonal antibody stain, In Proc. **36**th *Western Poultry Disease Conference, 3-5 March, Davis, California. University of California Davis*, p. 112.

Cobb, S. P. (2011): The spread of pathogens through trade in poultry meat: overview and recent developments. *Rev Sci Tech.*, **30**, 149-164.

Cosgrove, A.S. (1962): An apparently new disease of chickens: avian nephrosis. *Avian diseases*, **6**(3): 385-389.

CSA (2005): report on livestock and livestock characteristics, production constraints survey, Ethiopia; Pp, 12 -16.

Dacic, M., Zugic, G., and Petkovic, J., (2008): The effect of a gumboro disease control program on the reduction of economic losses. *Acta. Vet.* (Beogard), **58**(1):53-62.

Delmas, B., Attoui, H., Ghosh, S., Malik, Y.S., Mundt, E. and Vakharia, V.N., (2019): ICTV virus taxonomy profile: *Birnaviridae. J. Gen. Virol.***100** (1): 5-6.

Dey, S., Pathak, D.C., Ramamurthy, N., Maity, H.K. and Chellappa, M.M. (2019): Infectious bursal disease virus in chickens: prevalence, impact, and management strategies. *Vet. Med.* (Auckl), **10**, 85–97.

Di Fabio, J., Rossini, L.I., Eterradossi, N., Toquin D. and Gardin, Y., (1999): European-like pathogenic infectious bursal disease viruses in Brazil. *Veterinary Record*, **145:** 203-204.

Dormitorio. T., Giambrone. J., Guo K. and Jackwood D., (2007): Molecular and phenotypic characterization of infectious bursal disease virus isolates. *Avian Diseases.***51**:597–600.

Dwight, C.H., James, N.M. and Richard, L.W., (2005): *Veterinary microbiology* 2nd ed. Asia: Blackwell. pp. 407-419.

Edgar S. A., and Cho, Y., (1973): Immunization of chickens for the control of infectious bursal disease. *Poult. Sci.*, **52**: 492-497.

Elankumaran S., Heckert, R., and Moura, L., (2002): Pathogenesis and tissue distribution of a variant strain of infectious bursal disease virus in commercial broiler chickens. *Avian Diseases,* 46:169–76.

Eterradossi and Saif, Y.M. (2008): Infectious Bursal Disease. In*: Diseases of Poultry*, **12**th Edition (Eds. Saif, Y.M., Fadly A.M., Glissen J.R., McDougald L.R., Nolan L.K., Swayne D. E.) *Wiley Black well*, pp. 185-208.

Eterradossi N., and Saif, Y. M., (2013): Infectious bursal disease: *Diseases' of poultry*, 219-246.

Eterradossi, N., (2000): Progress in the diagnosis and prophylaxis of infectious bursal disease in poultry. *Journal of poultry science**,* **42**: 36-6.

Fahey, K.J., Erny, K. and Crooks, J., (1989): A conformational immune-gene on VP2 of infectious bursal disease virus that induces virus-neutralizing antibodies that passively protect chickens. *Journal of General Virology*, **70**: 1473–1481.

Farner D. S, King JR. Parkes K. C., (1983): Avian Biology. Cambridge, Massachusetts, USA: Academic *Press Inc*.7.

Farooq M., Durrani F., Imran N., Durrani Z. and Chand N., (2003): Prevalence and economic losses due to infectious bursal disease in broilers in Mirpur and Kolti districts of Kashmir. *International Journal of Poultry Science*, **2** (4): 267-270.

Gao Y, Liu W, Gao H, Qi X, Lin H, Wang X, Shen R., (2008): Effective inhibition of infectious bursal disease virus replication in vitro by DNA vector-based RNA interference. *Antiviral Res.***79**:87–94.

Gao H., Wang. X., Gao u. C. and Fu. Y., (2007): Direct Evidence of Reassortment and Mutant Spectrum Analysis of a Very Virulent Infectious Bursal Disease Virus. *Avian Dis.***51**: 893-899.

Garriga D. Querol-Audi. J., Abaitua. F., Saugar. I., Pous. J., Verdaguer. N., Caston. J. and Rodriguez. J., (2006): The 2.6-Angstrom structure of infectious bursal disease virusderived T=1 particles reveals new stabilizing elements of the virus capsid. *Journal of Virology*, **80**:6895–6905.

Giambrone, J.J., Dormitorio, T. and Brown, T., (2001): Safety and efficacy of in ovo administration of infectious bursal disease viral vaccines. *Avian Diseases,***45**:144-148.

Haddad E.E., Whitfill, C.E., Avakian, A.P., Ricks, C.A., Andrews, P.D., Thoma, J.A. and Wakenell, P.S., (1997): Efficacy of a novel infectious bursal disease virus (IBDV) immune complex vaccine in broiler chickens. *Avian Diseases*, **41**, 882-889.

Hailemariam T. Legesse. D. Alemu Y. and Nigussie D., (2006): Adopting poultry breeds in the high lands of Ethiopia, Addis Ababa, Ethiopia. *Res. Rep.,* pp.65-70.

Hailu, D., Melese, B., Moti, Y., and Mekedes, G., (2010): Seroprevalece of Infectious Bursal Disease in backyard chickens of Oromia regional state, Ethiopia. *Veterinary Research,***3**(4**)**, pp.89-93.

Hailu M., Tilahun, S. B. and Negash, T.,(2009): Incidence of infectious bursal disease in village chickens in two districts of Amhara Region, Northwest Ethiopia. *Livestock Research for Rural Development,* **21**, 12p.

He C. Q., L. Y. Ma, D. Wang, G. R. LI u. and N. Z. Ding (2009a): Homologous recombination is apparent in infectious bursal disease virus. *Virology*. **384**:51-58.

He X., P. Wei, X. Yang, D. Guan, G. Wang u. and A. Qin (2012b): Molecular epidemiology of infectious bursal disease viruses isolated from Southern China during the years 2000-2010. *Virus Genes.* **45**:246-255.

Hernandez-divers, S. M., P. Villegas, C. Jimenez, S. J. Hernandez-Divers, M. Garcia, S. M. Riblet, C. R. Carroll, B. M. O'connor, J. L. Webb, M. J. Yabsley, S. M. Williams u. and S. Sanchez, (2008): Backyard chicken flocks pose a disease risk for neotropic birds in Costa Rica. *Avian Dis***52**, 558-566.

Hitchner, S.B., (1970): Infectivity of infectious bursal disease virus for embryonating eggs. *Poultry Science*, **49**:511-516.

Hon C., C., T.-Y. Lam, A. Drummond, A. Rambaut, Y.-F. Lee, C.-W. Yip, F. ZENG, P.-Y. Lam, P. T. W. Ng u. and F. C. C. Leung, (2006): Phylogenetic analysis reveals a correlation between the expansion of very virulent infectious bursal disease virus and reassortment of its genome segment B. *J Virol.* **80**:8503-8509.

Inoue M., Fujita, A. and Maeda, K., (1999): Lysis of myelocytes in chickens infect with infectious bursal disease virus. *Veterinary Pathology*, **36**: 146-151.

Irigoyen N., D. Garriga, A. Navarro, N. Verdaguer, J. F. Rodríguez, and J. R. Castón., (2009): Autoproteolytic Activity Derived from the Infectious Bursal Disease Virus Capsid Protein. *The Journal of Biological Chemistry*, **284**:8064-8072.

Islam M. Zierenberg K. and Muller H., (2001c): Regeneration and rescue of "very virulent" infectious bursal disease virus from cloned cDNA using cell culture and chicken embryo host systems in combination, Bangladesh J. *Microbiol.* **18**, in press.

Islam MR., Zierenberg K. and Muller H., (2001a): The genome segment B encoding the RNAdependent RNA polymerase protein VP1 of very virulent infectious bursal disease virus (IBDV) is phylogenetically distinct from that of all other IBDV strains. *Archives of Virology,* **146** (12): 2481-2492.

Islam M.R., Zierenberg, K., Eterradossi, N., Toquin, D., Rivallan, G. and Müller, H., (2001a): Molecular and antigenic characterization of Bangladeshi isolates of infectious bursal disease virus demonstrate their similarities with recent European, Asian and African very virulent strains. *Journal of Veterinary Medicine, Series B,* **48**(**3**): 211-221.

Jackwood D. J., (2017): Advances in vaccine research against economically important viral diseases of food animals: Infectious bursal disease virus. *Veterinary microbiology*, **206***:*121-125.

Jackwood D. J., B. Sreedevi, L. J. Lefever U. and S. E. Sommer-Wagner, (2008): Studies on naturally occurring infectious bursal disease viruses suggest that a single amino acid substitution at position 253 in VP2 increases pathogenicity. *Virology*.**377**:110-116.

Jackwood D. J., Sommer-Wagner, S. E., Stoute, S. T., Woolcock, P. R., Crossley, B. M., Hietala, S. K., and Charlton, B. R. (2009): Characteristics of a very virulent infectious bursal disease virus from California. *Avian diseases,* **53**(4):592-600.

Jackwood J. D., (2014): The Mark veterinary manual: Overview of Infectious Bursal Disease (Gumboro ease) in Poultry, *http://www.merckvetmanual.com.*

Jackwood D., (2004): Recent trends in the molecular diagnosis of infectious bursal disease viruses. *Animal Health Res Rev.* **5**:313-316.

Jackwood D. and Sommer-Wagner E., (2010): Detection and characterization of infectious bursal disease viruses in broilers at processing. *Preven Vet Med*, **97**: 45-50.

Jackwood D. and Sommer S., (2005): Molecular studies on suspect very virulent infectious bursal disease virus genomic RNA samples. *Avian Dis.* **49**:246-251

Jackwood D. and Sommer u. S., (2002): Identification of infectious bursal disease virus quasispecies in commercial vaccines and field isolates of this double-stranded RNA virus. *Virology.* **304**:105-113.

Jackwood D. and Sommer-Wagner u. S., (2007): Genetic characteristics of infectious bursal disease viruses from four continents. *Virology* **365:**369-375

Jenberie S., Lynch, S. E., Kebede, F., Christley, R. M., Gelaye, E., Negussie, H. and Ayelet, G., (2014): Genetic characterisation of infectious bursal disease virus isolates in Ethiopia. *Actatropica*, **130:**39-43.

Jenbreie, S., Ayelet, G., Gelaye, E., Kebede, F., Lynch, S. E., and Negussie, H., (2012): Infectious bursal disease: seroprevalence and associated risk factors in major poultry rearing areas of Ethiopia. *Tropical animal health and production*, **45**(1):75-79.

Jordan, F., Pattison, M., Alexander, D. and Fragher, T., (2002): *Poultry disease,***5**th ed. London: Black well Saunders, pp.319-322.

Kabell. S., Igyarto B., Magyar.A., Hajdu. Z., Biro E., Bisgaard u. M., and Olah. I., (2006): Impact of heterophil granulocyte depletion caused by 5-fluorouracil on infectious bursal disease virus infection in specific pathogen free chickens. *Avian Pathol*, **35**:341-348.

Kasanga, C. J., T. Yamaguchi, H. M. Munang'andu, K. Ohya u. and H. Fukushi., (2012): Genomic sequence of an infectious bursal disease virus isolate from Zambia: classical attenuated segment B reassortment in nature with existing very virulent segment A. Arch Viro. 59–167.

Kassa S. and Molla W., (2012): Seroprevalence of infectious bursal disease in backyard chickens of North West Ethiopia. *Sci. J. Crop Sci.*,**1**(1): 20-25.

Kaufer I. and Weiss E., (1980): Significance of bursa of Fabricius as target organ in infectious bursal disease of chickens. *Infect. Immun.*, **27**: 364-367.

Khatri M. and Sharma u. J., (2009): Susceptibility of chicken mesenchymal stem cells to infectious bursal disease virus. *J Virol Methods*. **160**:197-199.

Khatri M. and Sharma J., (2009a): Response of embryonic chicken lymphoid cells to infectious bursal disease virus. *Vet Immuno Immunopathol.* **127**:316-324.

Khatri M., Palmquist. J., Cha.R., and Sharma. J., (2005): Infection and activation of bursalmacrophages by virulent infectious bursal disease virus. *Virus Res.***113**:44-50.

Kim I., You S., Kim H., Yeh H. and Sharma J., (2000): Characteristics of bursal T lymphocytes induced by infectious bursal disease virus. *J. Virol*. **74**:8884-8892.

Knoblich H., Sommer u. E. and Jackwood D., (2000): Antibody titers to infectious bursal disease virus in broiler chicks after vaccination at one day of age with infectious bursal disease virus and Marek's disease virus. *Avian Disease,***44**: 874-884.

Lasher H. N. and Davis V.S., (1997): History of infectious bursal disease in the USA. The first two decades. *Avian Dis.*, **41**: 11-19.

Lawal N, Hair-bejo M, Arshad SS, Omar AR and Ideris A., (2018): Propagation and molecular characterization of bioreactor adapted very virulent infectious bursal disease virus isolates of Malaysia. *Journal of Pathogens*,

Le Nouen, C. L., D. Toquin, H. Muller, R. Raue, K. M. Kean, P. Langlois, M. CherbonneL u. and N. Eterradossi (2012): Different domains of the RNA polymerase of infectious bursal disease virus contribute to virulence.

Lee, S. W., P. F. Markham, M. J. Coppo, A. R. Legione, J. F. Markham, A. H. Noormohammadi, G. F. Browning, N. Ficorilli, C. A. Hartley u. and J. M. Devlin (2012): Attenuated vaccines can recombine to form virulent field viruses. *J. Science.* 337, 188.

Lee L. and Lukert. P., (1986): Adaptation and antigenic variations of infectious bursal disease virus. *J Chin Soc Vet Sci.***12**:297-304.

Lejal N, Da Costa B, Huet JC, Delmas B., (2000): Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *J Gen Virol*.**81**:983–992.

Letzel, T., F. Coulibaly, F. A. Rey, B. Delmas, E. Jagt, A. A. Van loon u. and E. Mundt, (2007): Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. *J Virol.***81**, 12827-12835.

Li, Z. H., Y. Q. Wang, Y. F. Xue, X. Q. Li, H. Cao u. and S. J. J. Zheng (2012): Critical Role for Voltage-Dependent Anion Channel 2 in Infectious Bursal Disease Virus-Induced Apoptosis in Host Cells via Interaction with VP5. *Journal of Virology*. **86**:1328-1338.

Li, Z., Y. Wang, X. Li, H. Cao u. and S. J. Zheng, (2013b): Critical roles of glucocorticoidinduced leucine zipper in infectious bursal disease virus (IBDV)-induced suppression of type I Interferon expression and enhancement of IBDV growth in host cells via interaction with VP4. *J Virol.***87**:1221-1231.

Lin, Z., A. Kato, Y. Otaki, T. Nakamura, E. Sasmaz u. and S. Ueda, (1993): Sequence Comparisons of a Highly Virulent Infectious Bursal Disease Virus Prevalent in Japan. *Avian Dis*. **37**:315-323.

Liu, M. H. u. and V. N. Vakharia, (2006): Nonstructural protein of infectious bursal disease virus inhibits apoptosis at the early stage of virus infection. *J Virol.***80**:3369-3377.

Lombardo E, Maraver A, Espinosa I, Fernandez-Arias A, Rodriguez JF., (2000): VP5, the nonstructural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. *Virology.***277**:345–357.

Lucien Mahin, (2008): Different progressive infectious bursal disease pathological lesion pictures. *The Poultry Site Quick Disease Guide*.

Lukert PD., (1986): Serotyping recent isolates of infectious bursal disease virus. Paper presented at: 123rd Annual meeting of the American veterinary medical association; Kearney, NE, USA.

Lukert, P.D. and Saif, Y.M., (1991): Infectious bursal disease. In: Diseases of Poultry, Eds. B.W. Calnek, *et. al., **9** th* Edition, *Wolfe Publishing Ltd., London.* pp. 648 - 63.

Lukert P.D. and Saif, Y.M., (2003): Infectious bursal disease. In: Diseases of Poultry. **11**th Eds. Pages 161-180. Iowa State University Press, Ames.

Luque D., I. Saugar, M. T. Rejas, J. L. Carrascosa, J. F. Rodriguez u. and J. R. Caston, (2009): Infectious Bursal Disease Virus: Ribonucleoprotein Complexes of a Double-Stranded RNA Virus. *J Mol Biol.***386**:891-901.

Mahgoub H., Bailey. M. and Kaiser P., (2012): An overview of infectious Bursal disease. *Arch. Virol.*, **157**(11): 2047-57.

Mawgod SA., Arafa AS., and Hussein HA., (2014): Molecular genotyping of the infectious bursal disease virus (IBDV) isolated from broiler flocks in Egypt. International Journal of Veterinary Science and Medicine, **2**(1): 46-52.

Mazengia H., (2012): Review on major viral diseases of chickens reported in Ethiopia. *Journal of Infectious Diseases and Immunity,* **4**(1): 1- 9.

Mc. Nulty, M. S., G. M. Allan, and J. B. McFerran, (1979): Isolation of infectious bursal disease virus from turkeys. *Avian Pathol.* **8**: 205-212.

Mekuriaw A., Bitew, M., Gelaye, E., Mamo, B. and Ayelet, G., (2017): Infectious bursal disease: outbreak investigation, molecular characterization, and vaccine immunogenicity trial in Ethiopia. *Tropical Animal Health and Production*, **49** (6), pp.1295-1302.

Mohammed and Abdelmalik (2022): Epidemiology and laboratory diagnosis of very virulent infectious bursal disease virus in vaccinated chickens in Khartoum, Sudan. Open *Jan-Feb*; **12**(1):33-43.

Moraes, H.L.S., Salle, C.T.P., Nascimento, VP., Rocha, ACGT. and Souzak, G.F., (2005): Infectious bursal disease: Evaluation of maternal immunity and protection by vaccination of one-day old chicks against challenge with very virulent virus isolate. *Brazil Journal of Poultry Science,***7**: 51-57.

Muller H, Schnitzler D, Bernstein F, Becht H, Cornelissen D & Lutticken DH., (1992): Infectious bursal disease of poultry: Antigenic structure of the virus and control. *Veterinary Microbiology,* **33** (1-4): 175-183.

Müller H., Islam, M. R., and Raue, R., (2003): Research on infectious bursal disease, the past, the present and the future. *Veterinary microbiology*, **97**(1, 2), 153-165.

Muller H., Mundt, E., Eterradossi, N. and Islam, M., (2012): Current status of vaccines against infectious Bursal disease. *Avian. Pathol.* **41**: 133 - 39.

Mulugeta A. and Tebkew A., (2013): Evaluation of indigenous chicken productivity by using a questioner survey, in selected Chagni town, Awi-administrative zone, Amhara Region, Ethiopia. *World Journal of Agricultural Sciences*, **1** (1): 26-35.

Mundt E. and Vakharia VN., (1996): Synthetic transcripts of double-stranded Birnavirus genome are infectious. *Proceedings of the National Academy of Sciences of the United States of America,* **93**(20): 11131–11136.

Mundt E. Beyer J. Muller H., (1995): Identification of a novel viral protein in infectious bursal disease virus-infected cells. *J Gen Virol.***76** (2):437–443.

Mundt E., (2009): "Reverse Genetics and IBDV." The Poultry Informed Professional Mar-Apr: 1-6.

Mundt E., (1999): Tissue culture infectivity of different strains of infectious bursal disease virus is determined by distinct amino acids in VP2. *J. Gen. Virol.***80**:2067–2076.

Murphy FA., Gibbs EPJ, Horzinek MC, Studdert MJ (1999): Veterinary virology, **3**rd edn. *Academic Press,* London, pp 405-409.

Natnael T., (2015): Pathological And Seroprevalence Studies on Infectious Bursal Disease in chickens in and around Bahir Dar, North West,Ethiopia. An Msc Thesis presented to Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Pathology and Parasitology. pp. 1-54.

Noor M., (2009): Development of Infectious Bursal Disease Virus Vaccine Candidates by ReverseGenetics. PhD Thesis, *Department of Pathology, Faculty of Veterinary Science*, Bangladesh Agricultural University, Mymensingh, Bangladesh. Pp 580.

Nunoya T, Otaki Y, Tajima M, Hiraga M, Saito T., (1992): Occurrence of acuteinfectious bursal disease with high mortality in Japan and pathogenicityof field isolates in SPF chickens. *Avian Dis*, **36**: 597-609.

Oberlander Y., (2004): Generation and Characterization of Serotype 1-/ Serotype 2Recombinants of Infectious Bursal Disease Virus (IBDV). PhD Thesis, *Institute of Virology*, University of Leipzig, Leipzig, Germany. Pp 707.

Ogawa M., Yamaguchi, T., Setiyono, A., Ho, T., Matsuda, H., Furusawa, S., Fukushi, H., and Hirai, K., (1998): Some characteristics of a cellular receptor for virulent

infectious bursal disease virus by using flow cytometry. *Arch. Virol.* **143**:2327–2341.

WOAH, (1995): Office International des Epizooties Resolution No. XVIII. Progress in the diagnosis and control of serious poultry diseases: Salmonellosis and Gumboro disease. *Bull*. OIE, **107** (5):363-364.

WOAH, (2004): Infectious bursal disease (Gumboro disease): Manual of diagnostic test and vaccine for terrestrial animals.

WOAH, (2018): Office International des Epizootics. Infectious Bursal Disease (Gumboro disease). In Manual of diagnostic tests and vaccines for terrestrial animals. **3.3.12**: 931-932.

WOAH Terrestrial Manual, (2008): Infectious bursal disease (Gumboro disease). pp. 549-563.

WOAH, (2012): Office of International des Epizooties. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. *Newcastle Disease*: (Chap) **2.3.14**:576-89 Infectious Bursal Disease.

Okoyo, J.O. and Uzoukwu M., (2005): Infectious bursal disease among chickens. *Avian Disease,* **25**(4): 1034-103.

Pages-Mante A., Torrents D., Maldonado J. and Saubi N., (2004): Dogs as potential carriers of infectious bursal disease virus. *Avian Pathology*, **33(**2):205–209.

Palmquist J., Khatri M., Cha R., Goddeeris B., Walcheck. B., and Sharma, J., (2006): In vivo activation of chicken macrophages by infectious bursal disease virus. *Viral Immunol*. **19**, 305–315.

Pitcovski, J., Goldberg, D., Levi, B.Z., Di-Castro, D., Azriel, A., Krispel, S., Maray, T., and Shaaltiel, Y. (1998): Coding region of segment A sequence of a very virulent isolate of IBDV-comparison with isolates from different countries and virulence. *Avian Dis*. **42**:497–506.

Rajaonarison, J.J., Rakotonindrina, S.M., Rakotondramary, E.K. and Razafimanjary, S., 2006. Gumboro Disease (Infectious bursitis) in Madagascar. *Revue d'élevage et de medicine vétérinaire des pays tropicaux*, **47** (1): 15-17.

Raue, R., M. R. Islam, M. N. Islam, K. M. Islam, S. C. Badhy, P. M. Das u. and H. Muller (2004): Reversion of molecularly engineered, partially attenuated, very

virulent infectious bursal disease virus during infection of commercial chickens. *Avian Pathol.***33**:181-189.

Rauf A., (2011): *Persistence, distribution and immunopathogenesis of infectious bursal disease virus in chickens* (Doctoral dissertation, The Ohio State University).

Rautenschlein, S., C. Kraemer, J. Vanmarcke u. E. Montiel (2005b): Protective efficacy of intermediate and intermediate plus infectious bursal disease virus (IBDV) vaccines against very virulent IBDV in commercial broilers. *Avian Dis.***49**:231-237.

Rautenschlein, S., H.Y. Yeh, M.K. Njenga, and J.M. Sharma, (2002): Role of intrabursal T cells in infectious bursal disease virus (IBDV) infection: T cells promote viral clearance but delay follicular recovery. *Archives of Virology,* **147**:285–304.

Rautenschlein. S., Samson-Himmelstjerna. G.,and Haase. C. (2007): A com-parison of immune response to infection with virulent infectious bursal disease virus (IBDV) between specific-pathogen-free chickens infected at 12 and 28 days of age. *Vet. Immunol. Immunopathol.* **115**, 251–260.

Rauw. F., LambrechtU. B., and Van den berg. T. (2007): Pivotal role of ChIFN gamma in the pathogenesis and immunosuppression of infectious bursal disease. *Avian Pathol.* **36**, 367-374.

Ridell C. (1996): Avian Histopathology. 2nd (edn). Philadelphia, PA, USA: *American Association of Avian Pathologists.* University of Pennsylvania. 1996.

Rodriguez-Lecompte JC, Nino-Fong R, Lopez A, Frederick Markham RJ, Kibenge FS (2005): Infectious bursal disease virus (IBDV) induces apoptosis in chicken B cells. *Comp Immunol Microbiol Infect Dis* **28**:321–337.

Rong J, Jiang T, Cheng T, Shen M, Du Y, Li S, Wang S, Xu B and Fan G (2007). Large-scale manufacture and use of recombinant VP2 vaccine against infectious bursal

Rosenberger J.K. and Cloud S.S (1986): Isolation and characterization of variant infectious bursal disease viruses disease in chickens. *Vaccine*, **25** (46): 7900-7908.

Sapats, S., Gould, G., Trinidad, Parede, L. H. and David Ignjatovic, J., (2005): An ELISA fordetection of infectious bursal disease virus and differentiation of very virulent

strains based on single chain recombinant chicken antibodies. *Avian Pathology*, **34**: 449-455.

Saugar I., N. Irigoyen, D. Luque, J. L. Carrascosa, J. F. Rodriguez u. J. R. Caston, (2010): Electrostatic Interactions between Capsid and Scaffolding Proteins Mediate the StructuralPolymorphism of a Double-stranded RNA Virus. *J Biol Chem*. **285**:3643-3650.

Sharma, (2000): Developmental and Comparative Immunology, **24.**Pp 228

Shcherbakova, L. O., A. I. Lomakin, A. V. Borisov, V. V. Drygin u. and A. A. Gusev (1998): [Comparative analysis of the VP2 variable region of the gene from infectious bursal diseasevirus isolates]. *Mol Gen Mikrobiol Virusol* 35-40.

Shegu D., Teshale S., Asaminew T., Alebachew B., Hawa M., Teferi D., Belayneh G., Takele A. and Esayas G., (2020): Sequence-based comparison of field and vaccine strains of infectious bursal disease virus in Ethiopia reveals an amino acid mismatch in the immunodominant VP2 protein. *Archives Virol.,***165**: 1367-1375.

Shiferaw J., Ayelet, G., Esayas, G., Fekadu, K., Stacey Lynch, E. and Haileleul, N., 2012. Infectious bursal disease: seroprevalence and associated risk factors in major poultry rearing areas of Ethiopia. Tropical Animal Health and Production, **45**: 75-79.

Sindu, Z., Yisehak, T., Haftay, A. and Nesibu, A., (2015): Sero-prevalence of infectious bursal disease in backyard chickens around Mekelle, Northern Ethiopia. *African Journal of Biotechnology*.**14**(5): 434-437.

Singh, J., Banga, H. S., Brar, R. S., Singh, N. D., Sodhi, S., & Leishangthem, G. D. (2015): Histopathological and immunohistochemical diagnosis of infectious bursal disease in poultry birds. *Veterinary World*, **8** (11), 1331.

Skeeles, J.K., Slavik, M., Beasley, J.N., Brown, A.H., Meinecke, C.F., Maruca, S. and Welch, S. (1980): An age-related coagulation disorder associated with experimental infection with infectious bursal disease virus. *American Journal of Veterinary Research*, **41**:1458– 1461.

Solomon,W. and Abebe, W., (2007): Infectious bursal disease (Gumboro disease) case report of Andasa poultry farm Amahara region. *Ethiopian Veterinary Journal*, **11**: 151.

Tacken M. G. J., B. P. H. Peeters, A. A. M. Thomas, P. J. M. Rottier u. H. J. Boot, (2002): Infectious bursal disease virus capsid protein VP3 interacts both with VP1, the RNAdependent RNA polymerase, and with viral double-stranded RNA. *J Virol.***76**, 11301-11311.

Tadesse B. and Jenbere, S., (2014): Sero-Prevalence of Infectious Bursal Disease in Backyard Chickens at Selected Woredas of Eastern Ethiopia. *Journal of Biology, Agriculture and Healthcare,* **4** (17).

Tanimura N. and Sharma, J. M., (1997): Appearance of T cells in the bursa of Fabricius and cecal tonsils during the acute phase of infectious bursal disease virus infection in chickens. *Avian Disease,* **41**:638-45.

Terefe S., (2018): Infectious bursal disease: an important challenge in transforming the Ethiopian poultry sector.*Greener Journal of Agricultural Sciences*, **8**, 277-285.

Tesfaheywet, Z. and Getnet, F., (2012): Seroprevalence of infectious bursal disease in chickens managed under backyard production system in Central Oromia, Ethiopia. *African Journal of Microbiology Research,* **6**(38): 6736-6741.

Tham K. and Moon. C., (1996): Apoptosis in cell cultures induced by infectious bursal disease virus following in vitro infection. *Avian Diseases*. **40**:109–113.

Tsukamoto K, Saito S, Saeki S, Sato T, Tanimura N, Isobe T, Mase M, Imada T, Yuasa N and Yamaguchi S., (2002): Complete, long-lasting protection against lethal infectious bursal disease virus challenge by a single vaccination with an avian herpesvirus expressing VP2 antigens. *Journal of Virology*, **76**(11): 5637-5645.

Tsukamoto K., Tanimura, N., Mase, M. and Imai, K., (1992): Comparison of virus replication efficiency in lymphoid tissues among three infectious bursal disease virus strains. *Avian Diseases,***39** (4), 844-852.

Tulu D., (2019): Epidemiology, Status and Economic Importance of Infectious Bursal Disease in Poultry Production, Ethiopia. *Epidemol Int J.*, **3** (3): 000129.

USAID (United states Agency for International Development), (2013): Livestock Market Development Project. Addis Ababa, Ethiopia.

Valli A., I. Busnadiego, V. Maliogka, D. Ferrero, J. R. Caston, J. F. Rodriguez u. and J. A. Garcia, (2012): The VP3 Factor from Viruses of Birnaviridae Family Suppresses RNA Silencing by Binding Both Long and Small RNA Duplexes.

Van den Berg TP., (2000): Acute infectious bursal disease in poultry: a review. *Avian Pathology*. **29**(1):175-194.

Van den Berg, T., Morales, D., Eterradossi, N., Rivallan, G., Toquin, D., Raue, R., Zierenberg, K., Zhang, M., Zhu, Y., Wang, C., Zheng, H., Wang, X., Chen, G., Lim, B. and Muller, H., (2004): Assessment of genetic, antigenic, and pathotypic criteria for the characterization of IBDV strains. *Avian Pathol*., **33**: 470 - 476.

Viral zone, (2009): http://viralzone.expasy.org/all_by, species/572.html. Accessed on 17 March (2016).

Wei, Y. W., X. P. Yu, J. T. Zheng, W. Y. Chu, H. Xu, X. M. YU u. and L. YU (2008): Reassortant infectious bursal disease virus isolated in China. *Virus Res.***131**:279-282.

Whitfill, C.E., Haddad, E.E., Ricks, C.A., Skeeles, J.K., Newberry, L.A., Beasley, J.N., Andrews, P.D., Thoma, J.A. and Wakenell, P.S. (1995): Determination of optimum formulation of a novel infectious bursal disease virus (IBDV) vaccine constructed by mixing bursal disease antibody with IBDV. *Avian Diseases,* **39**:687-699.

Wu C., Rubinelli u. P., and Lin. T., (2007a): Molecular detection and differentiation of infectious bursal disease virus. *Avian Dis*. **51**, 515-526.

Wyeth P. J. and Chettle N., (1990). Use of infectious bursal disease vaccines in chicks with maternally derived antibodies. *Veterinary Record*, **126** (23): 577-578.

Yamaguchi, T.; Setiyono, A.; Kobayashi, M.; Takigami, S.; Fukushi, H. and Hirai, K. (2000): Infectious bursal disease live vaccines: changes in the virus population during serial passage in chickens and chicken embryo fibroblast cells. *Avian Dis.***44**: 284-290.

Yao K, Goodwin MA, and Vakharia VN., (1998): Generation of a mutant infectious bursal disease virus that does not cause bursal lesions. *J. Virol.***72**:2647–2654.

Yip C.W., C.C. Hon, F. Zeng and F.C.C. Leung, (2012): Cell culture-adapted IBDV uses endocytosis for entry in DF-1 chicken embryonic fibroblasts. *Virus. Research*, **165**(1): 9-16.

Zegeye S, Yisehak T, Haftay A. and Nesibu A. (2015): Sero-prevalence of infectious bursal disease in backyard chickens around Mekelle, Northern Ethiopia. *Afri. J. Biotechnol.,***14**(5): 434-437.

Zeleke A, Yami, M, Kebede, F, Melese, N and Senait, B. (2003): Gumboroo: An emerging disease threat to poultry farms in Debre Zeit. Proceedings of the **17**th Annual Conference of Ethiopian Veterinary Association, Addis Ababa, Ethiopia.

Zeleke A., Gelaye, E., Sori, T., Ayelet, G., Sirak, A. and Zekarias, B., (2005): Investigation on Infectious Bursal Disease outbreak in Debre zeit, Ethiopia. *International Journal Poultry Science, **7**: 504-506*.

Zeryehun T. and Fekadu, G., (2012): Seroprevalence of infectious bursal disease in chickens managed under backyard production system in central Oromia, Ethiopia. *Afri. J. Micro Research,* **6**(38):6736-6741.

Zierenberg K., R. Raue, H. Nieper, M. R. Islam, N. Eterradossi, D. Toquin u. and H. Muller, (2004): Generation of serotype 1/serotype 2 reassortant viruses of the infectious bursal disease virus and their investigation in vitro and in vivo. *Virus Res.***105**, 23-34

Zierenberg, K., Raue, R., and Müller, H. (2001): Rapid identification of "very virulent" strains of infectious bursal disease virus by reverse transcription-polymerase chain reaction combined with restriction enzyme analysis. *Avian Pathol.* **30**:55–62.

Zierenberg K., Nieper. H., Van den berg.T., Ezeokoli .C., Voss u. M., and Muller H., (2000): The VP2 variable region of African and German isolates of infectious bursal disease virus: comparison with very virulent, "classical" virulent, and attenuated tissue cultureadapted strains. *Arch Virol.***145**:113-125.

9. APPENDEXS

Appendix 1: Sample Preparation for molecular detections

After rearrange all the necessary materials that have used to chopping tissue samples,

- Take small pieces of sample (1gm) and wash 2 t0 3 times with PBSA.
- Chop the sample with sterile scissors and grind by using sterile mortar and pestle.
- Mince in Tryptose Phosphate Broth (TPB) or phosphate buffer saline solution (PBSA) with antibiotics.
- Transfer sample in centrifuge tubes and Centrifuge at 1500 rpm for 10 min.
- Collect the supernatant in appropriate screw cupped test tube, Label and use to molecular detections of the suspected out break cases.

Appendix 2: RNA Extraction and Reverse Transcriptase

The extraction step was as follows:

- A volume 350 µl of the homogenized sample suspension were transferred to eppendorf tube
- Added 350 µl of RLT buffer 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.

Appendix 3: Conventional PCR for IBDV identity test procedure

One step RT-PCR master mix preparation			
Type of reagent		For one reaction	Total 8 reaction
RNase free water		4 µl	32 µl
PCR buffer 5X		5 µl	40 µl
Q solution 5X		5 µl	40 µl
Primer-IBDVP2 PANVAC-4fow-5pm/ µl5'TCTTGGGTATGTGAGGCTTG -3'		2 µl	16 µl
Primer-IBDVP2 PANVAC-5Rev-5pm/ µl5'CCCGGATTATGTCTTTGA -3'		2 µl	16 µl
10mM dNTPs		1 µl	8 µl
One step RT-PCR enzyme		1 µl	8 µl
Add template (RNA)		5 µl	
Total volume		25 µl	
RT-PCR condition			
	Temperature	Time	Cycle
cDNA synthesis	50^{o}c	30 minute	1 cycle
Initial Denaturation	95 ^{o}c	15 minute	1 cycle
Denaturation	95 ^{o}c	30 second	35 cycle
Annealing	55 ^{o}c	30 second	
Elongation	72 ^{o}c	30 second	
Final elongation	72 ^{o}c	7 minute	1 cycle
Put at	4 ^{o}c	Until machine off	

Appendix 4: Agarose gel preparation

- ✓ Prepare 2% Agarose gel
- ✓ Add 4 µl Gel red with loading dye into 10 µl PCR product,

- ✓ Then loading PCR product 10 µl in each well
- ✓ And add 10 µl molecular marker (ladder) started 100bp.
- ✓ Run electrophoresis for 1:20 hour at 120 V.
- ✓ Read the result by using UV-light
- ✓ It is around 400bp positive for IBDV

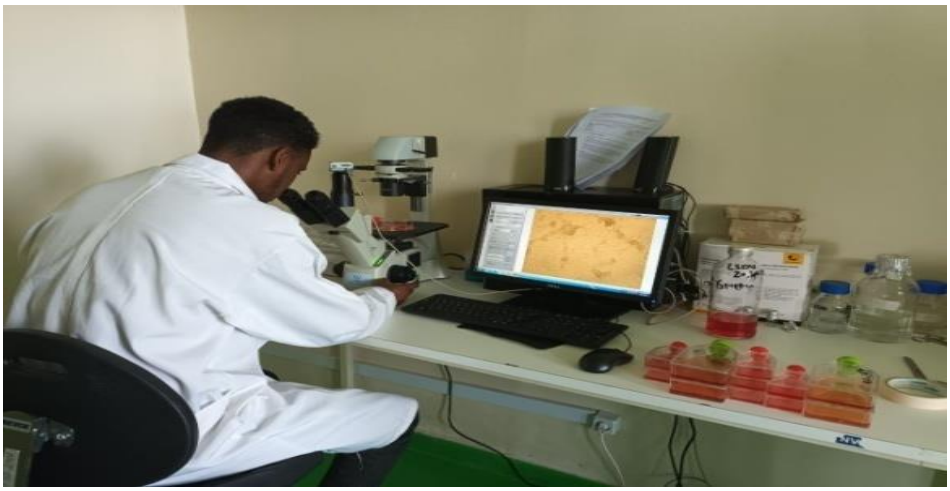
Appendix 5: Preparation of Chicken Embryo Fibroblast (CEF) Cell Cultures

- Use 9-11 day old embryos. The technique described here is for 3-5 embryos.
- Place all media, solution and trypsin in 37°c water bath.
- Spray eggs with Bio guard disinfectant (70% ethyl alcohol) place in hood. Using sterile technique, open shell and remove embryo with blunt ended curved forceps.
- Place embryos in Petri dish and cut off heads. Removal of limbs and viscera.
- Transfer bodies to new Petri dish or beaker containing PBS with antibiotics.
- Wash with PBS 3-4 times to remove red blood cells and extra debris
- In the beaker, the bodies can be fragmented by carefully chopping them with sterile scissors.
- Pour tissue fragments into trypsinization flask containing magnetic stirring bar.
- Add about 50 ml pre-warmed (37^{o}c) trypsin solution (0.25%) and put on stir plate at slow speed into 37^{o}c incubator for 10-15 minutes.
- Pour off supernatant into centrifuge tube with calf serum.
- Add 50 ml Trypsin solution and stir slowly in 37^{o}c incubator for 8 minutes. This may be repeated 1 more time for a total of 2 trypsinization.
- Centrifuge 10 min. at 1500 rpm. Note the amount of Pelleted cells obtained.
- Pour off Trypsin solution and resuspend cells complete cell culture medium (10% calf serum and tryptose phosphate broth contained GMEM or MEM. The cells may be counted or diluted 1:200and dispensed with in tissue culture flask and incubate at Co2 connected 37^{o}c incubator.

Prepared CEF Culture Plate

Appendix 6: Procedure for Inoculating Preformed Monolayer's

- Place all media and solution in 37°c water bath.
- Swirl T.C flask to re-suspend as many RBC's and debris as possible and then decant and discard growth medium.
- Wash monolayer gently with 2-3times of pre warmed PBS and discard. And add 1 ml sample inoculums to the small T C flask (25 cm2)or 2 ml for the larger size
- Rock each plate gently to distribute inoculums evenly over the cell monolayer.
- Incubate inoculated cultures in 37°c incubator for 45 minutes to 1 hour to allow virus to adsorb. And Rock tray once or twice during incubation if possible.
- Add 20 ml maintenance medium (2% calf serum) to each small T C flask
- Incubate at 37°c. Check plates daily for cytopathogenic effect (CPE) and condition of cells. To harvest samples, freeze-thaw 2-3 times and collects.



CPE Observation

Appendix 7: Ethical Clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
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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

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