Thesis Ref. No _____

OUTBREAK INVESTIGATION AND MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS IN POULTRY FARMS AT MODJO AND BISHOFTU TOWNS, CENTRAL ETHIOPIA

MVSc THESIS

BY

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SEPTEMBER, 2020

BISHOFTU, ETHIOPIA

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SIGNED DECLARATION SHEET

First, I declare that this thesis is my original work and that all sources of materials used for this thesis have been duly acknowledged. This has been submitted in partial fulfilment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University or College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for award of any academic degree, diploma, or certificate.

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ACKNOWLEDGEMENTS

First of all I would like to praise the Almighty God for being with me throughout my life!

Secondly my special gratitude and appreciation goes to my respectful advisors: Dr. Gezahegne Mamo, Dr. Teshale Sori, and Dr. Mattia Cecchinato (from University of Padova, Italy) for their unreserved interest to help me to understand the scientific background of the study and friendly approach, and devotion of time for the accomplishment of this thesis. In addition, I am also grateful for Dr. Matteo Legnardi (PhD student, University of Padova, Italy) for his molecular analysis of the samplesand providing all the available informations about the whole laboratory process.

Thirdly, my family (my lovely wife & son, my mother, brothers and sisters), who show much care for me, deserve my gratitude and respect.

I am also grateful for professionals and farm owners for reporting outbreak cases and letting me to take representative samples.

The last, but not the least I am grateful to Dr. Dereje Shegu from NAHDIC for his kind support in cull tulture of the samples, my close and classmate friends for their support and encouragement in compiling this thesis.

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LIST OF ABBREVIATIONS

AC-ELISA	Antigen Capture- Enzyme-linked Immunosorbent Assay
AGID	Agar Gel Immunodiffusion
AGPT	Agar Gel Precipitin Test
BF	Bursa of Fabricius
bp	base pair
CAM	Chorioallantoic Membrane
cDNA	Complementary Deoxyribonucleic acid
CEF	Chicken Embryo Fibroblast
CPE	Cytopathic Effect
CSA	Central Statistical Agency
cv	Classical Virulent
DNA	Deoxyribonucleic acid
dpi	days post infection
dpv	days post vaccination
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FP	Fowl Pox
FT	Fowl Typhoid
FTA	Flinders Technology Associates
DMEM	Dulbeccos modified Eagle‟s medium
HVR	Hypervariable region

HVR	Hypervariable region
HVT	Herpes Virus of Turkey
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
IFN-γ	Interferon gamma
IL	Interleukin
Mabs	Monoclonal antibodies
MD	Marek's Disease
MDA	Maternally derived anti bodies
MEGA	Molecular Evolutionary Genetics Analysis
MUSCLE	MUltiple Sequence Comparison by Log- Expectation
NAHDIC	National Animal Health Diagnostic and Investigation Center
ND	Newcastle Disease
NVI	National Veterinary Institute
OIE	World Organization for Animal Health
ORF	Open Reading Frames
PAs	Peasant Associations
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PI	Post Infection
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction

SPF	Specific Pathogen-Free
TNF	Tumor Necrosis Factor
UAE	United Arab Emirates
USA	United States of America
VNT	Virus Neutralization Test
VP	Viral Protein
vv	Very virulent

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ABSTRACT

Infectious Bursal Disease (Gumboro disease) is a highly contagious viral disease of chickens with a worldwide prevalence, which is caused by infectious bursal disease virus, (genus *Avibirnavirus*, family *Birnaviridae*), infects chickens and is becoming serious threat and challenging to the emerging poultry industry in Ethiopia. Molecular characterization targeting the major capsid protein, viral protein 2, plays an important role in the identification of the various strains of infectious bursal disease virus, tracing back of the origin of the virus and in vaccine matching studies. An outbreak based cross sectional study was carried out from October 2019 to May 2020, with the main objectives of characterizing field strains of the virus from outbreak cases in poultry farms at Bishoftu and Modjo towns, Central Ethiopia. A total of six farms were addressed during the study time and four to five chickens were opened per outbreak from each farm. Smear samples for molecular analysis and pooled samples for culture were collected on Flanders Technology Associates cards and in universal bottles containing viral transport medium, respectively. Virus isolation and reverse transcriptase-polymerase chain reaction and sequencing were performed to confirm the outbreak cases. Bursal suspensions were prepared for both cell culture and molecular analysis. Up on visualizations of the DF-1 cell lines used for cell culture after two passages, cytopathic effects: cell swelling, cell rounding, detachment and floating, were observed. Up on characterization of viral protein 2 region of the virus using reverse transcriptase polymerase chain reaction and sequencing, both vaccinal (six IBDV Genogroup 1) and field viral strains (five IBDV Genogroup 3) were detected. The current isolates made three clusters, namely, isolates identical to vaccinal strains ((Winterfield 2512-like) - CEVAC® IBD L)) and (Faragher 52/70 used in HVT+IBD vaccines), field strains identical to very virulent strains deposited in the Genbank, and isolates identical to vaccine strain manufactured and marketed by National Veterinary Institute, Ethiopia. The results indicate that, in addition to the continuous circulation of vvIBDV strains in the country, vaccinal strains are also reverting to their virulence being one of the causes for the investigated outbreaks. Therefore, further and sustained molecular characterization of the vaccinal and field IBDV strains is crucial as the virus is resistant and prone to change in its nucleotide sequences.

Key words: *Bishoftu, IBDV, Infectious bursal disease, Modjo, Molecular characterization, Outbreak investigation*

1. INTRODUCTION

Chicken production is an important activity in the long journey to attainment of protein demand for the vulnerable member of the society (Natnael, 2015). The poultry population is, however, challenged by several constraints including infectious diseases. Infectious bursal disease (IBD) also called Gumboro, is one of the major constraints of chicken production posing enormous economical and societal impact to farmers (Mack *et al*., 2005). IBD is caused by a double-stranded RNA virus, infectious bursal disease virus (IBDV), belonging to the *Birnaviridae* family. It is featured by high mortality in its acute clinical form and immunosuppression induced by subclinical infection (Eterradossi and Saif 2013). Due to its immunosuppressive effect, affected flocks show increased susceptibility to infection with opportunistic pathogens, often leading to chronic disease situations and vaccine failures (Lukert and Saif, 2003; Ingrao *et al*., 2013). It causes considerable economic losses in poultry industries worldwide due to immunosuppression (Jackwood and Sommer-Wagner 2010) and high morbidity and mortality (Jackwood *et al*., 2009). The disease can also jeopardize public health since it enhances infection of chicken with zoonotic pathogens such as *Salmonella*, *Campylobacter* and Avian influenza (Ingrao *et al*., 2013).

Infectious Bursal Disease Virus consists of two serotypes, 1 and 2. Serotype 1 viruses are infectious for chickens, differing in their pathogenicity and are classified as avirulent, classical, variant and very virulent (vv) strains (Muller *et al*., 2003; Sapats and Ignjatovic, 2000). Variant and vvIBDV strains have been isolated from disease outbreaks despite the presence of high levels of maternal antibody to classic strains of IBDV (Jackwood and Saif, 1987). The use of an appropriate vaccine is vital for effective protection and hence differentiation and identification of local IBDV isolates is crucial for selection of appropriate vaccine strain. Amplification of IBD virus protein 2 (VP2) genes and linking genetic variation found in this region with antigenic variation has been the major focus for strain identification in recent years (Bayliss *et al.*, 1990; Brown *et al*., 1994; Wu *et al*., 2007). The IBDV VP2 hypervariable region (HVR) is commonly used to differentiate IBDV strains (Jackwood, 2004).

Infectious bursal disease was first reported in Ethiopia in 2002 in privately owned commercial poultry farm in Bishoftu (Zeleke *et al*., 2003). Subsequently, it became among the most important diseases affecting poultry industry throughout the country (Mazengia, 2012; Jenbreie *et al*., 2013; Jenberie *et al*., 2014). Research findings and case reports from various regions of the country showed the occurrence of outbreaks of IBD in commercial farms, breeding and multiplication centers despite regular vaccination practices and improved biosecurity measures (Zeleke *et al*., 2005). Mortality ranging from 25 to 75% has been observed in exotic and cross-bred chickens due to IBD (Zeleke *et al*., 2002; Zeleke *et al*., 2005; Woldemariam and Wossene 2007). Besides, it has been reported in significant proportion of village chicken (Zeleke *et al*., 2005; Mazengia *et al*., 2009; Hailu *et al*., 2010; Kassa and Molla, 2012; Shiferaw *et al*., 2012; Tesfaheywet and Getnet, 2012). IBD is mainly disease of young stock affecting chicken aged less than six weeks (Tesfaheywet and Getnet, 2012; Hailu *et al.*, 2010; Shegu *et al*., 2020; Mekuriaw *et al*., 2017).

The distribution of improved breed of chickens from infected poultry breeding and multiplication centers, mainly from Central Ethiopia, to the village is suspected of disseminating diseases to village chicken. Previous molecular characterization carried out elsewhere using both monoclonal antibody and direct sequencing of a genome fragment encoding the immunodominant VP2 gene showed that isolates from France, Britain, Dutch and Belgium had close relationships among them, which differed from one another by no more 3 amino acids, whereas they differed from African isolates by six amino acids (Eterradossi *et al*., 1999). The phylogenetic analysis revealed that the African IBD viruses may belong to a genetically distinct lineage of vvIBDVs. Molecular characterization targeting the major capsid protein VP2 plays an important role in the identification of the various strains of IBDV, tracing back of the origin of the virus and in vaccine matching studies. Such molecular investigations are crucial for effective national and/or regional mitigation strategies, including improved diagnostics and vaccination. However, with the exception of recent vaccine mismatch study that revealed the occurrence disparity between field and vaccine strains (Shegu *et al*., 2020); molecular data on IBDV are extremely scarce in Ethiopia.

Therefore, this study was performed with the following general and specific objectives:

General objectives

- To characterize the field isolates of IBDV from outbreaks from poultry farms in Bishoftu and Modjo towns, Central Ethiopia.

Specific objectives

- To isolate IBDV from outbreak cases and confirm with reverse transcriptase polymerase chain reaction (RT-PCR) and
- To explore their genetic relationship with strains deposited in the Gene Bank using reverse transcriptase-PCR and sequencing of VP2 gene.

2. LITERATURE REVIEW

2.1. Description of the disease

Infectious bursal disease (IBD) is an immunosuppressive disease of young chickens of worldwide prevalence. It is caused by IBD virus (IBDV), a double-stranded RNA birnavirus which is highly resistant to harsh environmental conditions. Two serotypes of the virus (serotypes 1 and 2) have been described and antigenic variants of both serotypes have been recognized. However, only viruses of serotype 1 are pathogenic and multiple serotype 1 pathotypes have been described. The disease could result in high morbidity and mortality. In addition, the immunosuppressive effect of the disease lowers the bird's resistance to other infections and reduces responsiveness to commonly used vaccines (Eterradossi and Saif, 2008).

The infectious bursal disease (IBD) caused by infectious bursal disease virus (IBDV), is also known as "Gumboro disease." It is a highly contagious viral disease of chickens with a worldwide prevalence (Ganguly and Rastogi, 2018). The clinical disease in birds is referred by various synonyms like infectious bursal disease, infectious bursitis, and infectious avian nephrosis (Castón *et al*., 2008). The virus targets the immune system, particularly the bursa of Fabricius. It destroys B lymphocytes resulting in immunosuppression, which further facilitates secondary infection and decreases efficacy of other vaccinations (Eterradossi and Saif 2013). The disease is usually associated with high mortality, especially in young chicks less than 3 weeks of age. It was first detected in 1962, in a small town in the Unites States-Gumboro (southern Delaware) (Khan *et al*., 2017).

2.2. Etiology

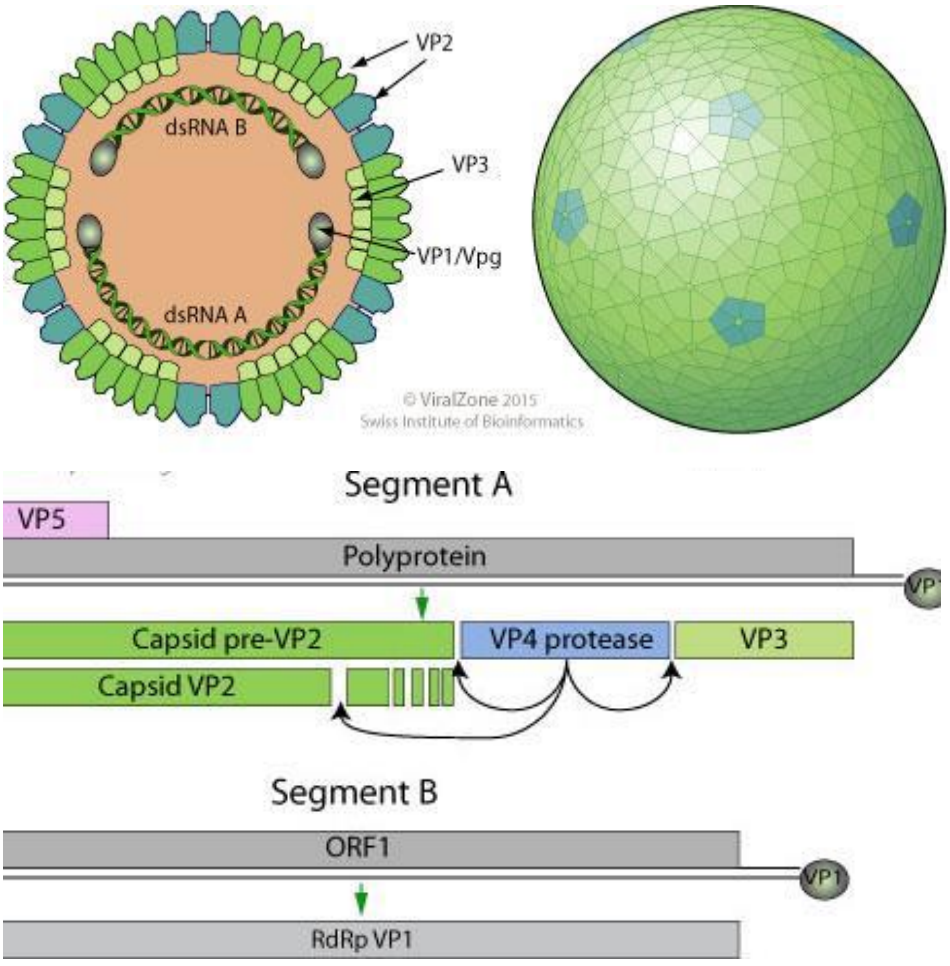
The etiological agent of infectious bursal disease is infectious bursal disease virus (IBDV) which belongs to the family *Birnaviridae*, whose primary target is the lymphoid tissue of the bursa of Fabricius (Van den Berg *et al*., 2000; Quinn *et al*., 2003). Characterization of the viral genome as bi-segmented double-strand RNA (Van den Berg, 2007) allowed placing IBDV into a new family of virus the Birnaviridae contains the genera which affect chicken, fish, and insect (Quinn *et al*., 2003). Among the family includes three genera: (*Aquabirnavirus*, *Entomobirnavirus*, and *Avibirnavirus*); *Avibirnavirus* whose type-species is infectious bursal disease virus (IBDV), which infects birds (Van den Berg, 2007).

2.2.1 Genome Structure and Organization of IBDV

The IBD virus belongs to the family Birnaviridae, genus *Avibirnavirus*. It is non-enveloped, hexagonal, 50-60 nm in diameter, with single-shelled icosahedral virus. The simple structure of the virus confers on its high resistance to environmental conditions, which adversely affects disease control (Van der Berg, 2008). The viral genome is composed of two unrelated segments, A and B, encoding five major viral proteins (VP 1-5) (Maclachlan *et al*., 2017) (Figure 2b). Segment A, the larger segment, approximately 3.2 Kb, contains two largely overlapping open reading frames (ORF), A1 and A2. Larger ORF, A2, encodes VP2, VP3, and VP4, while A1 encodes VP5. Segment B, the smaller segment, encodes VP1. VP1 is an RNA dependent polymerase responsible for viral replication and transcription. It exists as a genome-linked protein (VPg) that circularizes segments A and B by tightly binding their ends (Maclachlan *et al*., 2017). VP2 and VP3 are structural viral proteins (Figure 2a). VP2, an external capsid protein, is responsible for eliciting neutralizing antibodies, binding to cellular receptors, and determining the cellular tropism of the virus. In addition, it represents the molecular basis for antigenic variations. VP2 is folded into three main domains (basal, shell, and projections domains) (Mahgoub *et al*., 2012). VP3, an internal capsid protein, contains group specific antigenic determinates and is associated with the genomic RNA. VP4, a viral protease, cleaves N-pVP2-VP4-VP3-C to VP2, VP3, and VP4 (Khan et al. 2017; Maclachlan *et al*., 2017). VP5 is a class II membrane, highly basic, cysteine rich protein that possesses regulatory functions. Resembling

other RNA viruses like reoviruses and influenza viruses, the genome terminals, 5′ and 3′, share a high degree of sequence identity between the segments (Ganguly and Rastogi, 2018).

(a)



(b)

Figure 1: IBDV physical structure (a), IBDV genome structure (b).

Source: Viral Zone (Swiss Institute of Bioinformatics, 2015)

The functions of the IBDV-encoded proteins are summarized below (Table 1). IBD viruses have the ability to replicate in both chicken and mammalian cells; however, highly pathogenic strains cannot be easily cultivated. In chicken embryo cells, the multiplication cycle is approximately

10–36 h, whereas in Vero and BGM-70 cells, it takes a longer time, approximately 48 h (Eterradossi and Saif, 2013). Two serotypes of IBD virus are described in literatures. Serotype 1 shows variation in pathogenicity, while serotype 2 is not associated with any diseases (Van der Berg, 2008). There is no obvious cross-neutralization between the two strains in vitro, neither is there cross-protection in vivo (Van den Berg *et al*., 2000). Regarding the virulence, serotype 1 has three antigenic groups: classic, variant, and very virulent strains. Classic (standard) viruses show worldwide distribution and can cause up to 10-50% mortalities in young chickens. Variant viruses were first discovered in the USA in broiler flocks that were properly vaccinated. They were characterized as antigenic drift of serotype 1. They did not induce any mortality but are reported to be associated with immunosuppression. vvIBDV have started to emerge in Europe in well-managed and vaccinated farms. They are highly virulent and are associated with mortalities ranging from 50% to 100%. They also occur in Asia, Africa, Caribbean islands, and South America and have been reported in California, USA (Van der Berg, 2008; Maclachlan *et al*., 2017).

Table 1: The IBDV-encoded proteins and their functions

S.N.	Protein	Encoded by	Size (KDa)	Function	References
1	VP1	Segment B	97	RNA-dependent RNA polymerase	Macreadie and Azad (1993))
2	VP2	Segment A	54.4	Induces neutralizing antibodies Responsible for antigenic variations Virus virulence factor	Qi et al. (2013)
3	VP3	Segment A	32	Suppresses innate immune response Scaffold protein binds to the viral dsRNA and VP1	Mertens et al. (2015)
4	VP4	Segment A	28	Viral protease cleaves polyprotein Suppresses innate immune response Suppresses IFN-I expression	Li et al. (2013)
5	VP5	Segment A	17	Apoptosis inducer	Lombardo et al. (2000)

2.2.2 Classification of the IBDV

Three main parameters are used to classify the IBDV: pathology, antigenicity, and pathogenicity. According to pathogenicity, IBDV is classified into two main serotypes. Serotype 1 induces high mortality rates as well as bursal lesions in the affected chickens. It is further classified according to virulence into four main groups: the very virulent, the virulent, the attenuated, and the variant strains. However, serotype 2 does not produce bursal lesions or cause mortality among the affected chickens (Lim *et al*., 1999). According to the antigenic properties of serotype 1, it is classified into six subtypes (Li *et al*., 2006). Meanwhile, the phylogenetic analysis based on the hv-VP2 revealed seven genotypes of this serotype (Michel and Jackwood, 2017). In the Arabian Peninsula, both genotypes 4 and 6 were detected in the UAE and Saudi Arabia, respectively (Lupini *et al*., 2016). Interestingly, genotype 4 isolated from UAE was quite similar to that isolated from chicken from Italy (Lupini *et al*., 2016).

2.2.3 Characteristics of IBDV

The most interesting feature of IBDV is its ability to remain infective for extended period and to overcome the effects of commonly used disinfectants (Jackwood and Sommer-Wagner, 2005). It is susceptible to mutation, highly stable and resistant to a variety of chemical and disinfectant like phenolic derivative and a quaternary ammonium compound, but iodine complex has a deleterious effect on virus; and can persist in faces, bedding, contaminated feed and water for up to four months in certain conditions. It is also resistant for treatment with chloroform and ether, remains viable from pH 2-12 and is inactivated only in 70°C for 30 minutes. The virus is unaffected by exposure for one hour at 0.5% to 30% phenol but virus infectivity was markedly reduced when exposed to 0.5% formalin for six hours. Infectious bursal disease virus (IBDV) is also heat stable, viable after treatment at 56°C for 5 hours (Dwight *et al*., 2005; Fragher, 2001; Elankumaran *et al*., 2002).

2.3. Epidemiology

2.3.1 Host range

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 may be infected, the clinical disease occurs only in chickens (OIE, 2008). It is strongly believed that the serotype IBDV one is highly hosted specific to 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). 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). The duck can also be an asymptomatic carrier of serotype 1 is no confirmation that the IBD virus could infect other animals, as well as humans.

2.3.2 Transmission of IBD Virus

Chickens are the sole well-known avian species to develop clinical disease and distinct lesions once exposed to IBDV. The IBD transmits with a horizontal way only, with healthy subjects being infected by the oral or respiratory pathway. The most common mode of infection is through the oral route. Conjunctiva and respiratory routes may also be involved (Sharma *et al*., 2000). There is no evidence of egg transmission of the virus and no carrier state has been detected in chickens. The lesser mealworm (*Alphitobius diaperinus*) is recognized as a carrier and the virus has been isolated from mosquitoes (*Aedes vexans*) and evidence of infection in rats has been reported but there is no indication that either species is a reservoir for the virus. Infected subjects excrete the virus in faces as early as 48 hours after infection, and may transmit the disease by contact over a sixteen-day period. The possibility of persistent infection in recovered animals has not been studied. The IBD is very contagious and will be transmission by the movement of poultry product, equipment, feed bags, vehicles, and people. In lesser extent the disease also spread through aerosols of the dust (Sharma *et al*., 2000; Eterradossi and Saif, 2008).

There is no data that suggest IBDV is transmitted by wild birds, however direct or indirect transmission of the virus between wild birds and domestic chickens probably occurs (Motohiko *et al*., 2005). The extreme resistance of the virus to the outside environment and its viral incubation period is about 2-3 days and can be shed as soon as 24 hours following infection and may last up to 2 weeks. In the absence of effective cleaning, disinfection, and insect control can enhance the potential for transmission when they are scavenging of dead chickens, ingestion of contaminated water, or exposure of respiratory or conjunctiva membranes to contaminated poultry dust (Okoyo and Uzoukwu, 2005; Solomon and Abebe, 2007).

2.3.3 Immunosuppression effect of the virus

Immunosuppression caused by IBDV has a significant economic impact due to the widespread nature of the disease in commercial chickens. The virus infection at early age compromises the immune responses of chickens. All generated the earliest observation regarding the immunological disorder potential of IBDV. The extent of the immunosuppressive effect is related to the age at infection. The most pronounced damage results if the infection happens in the first 2-3 weeks of the hatch (Jackwood and Sommer-Wagner, 2005). The birds less than 3 weeks ages do not exhibit clinical signs however are immunological disorder.

After the virus was ingested by birds, the virus infected lymphoid cells and macrophage of intestine then the virus carry to bursa of Fabricius (Muller *et al*., 2003). Clinical signs and lesions of IBDV seem shortly after. The infected chicken with Infectious bursal disease is more prone to secondary infections especially Newcastle disease (ND). The infected chicken had a decreased humoral response to vaccines as well. Immunosuppression resulted in lower flock performance, a lot of secondary infections, poor feed conversion, less protecting response to vaccines and a higher rate of carcass condemnation at the process level (Van den Berg *et al*., 2000).

2.3.4 Morbidity and mortality

The infectious bursal disease is extremely contagious and its severity depends on the age and breed of the affected birds, the degree of passive immunity and the virulence of the strain of the virus, and secondary infections associated with the immunosuppressive effects of the disease.

The time wherever chickens are most prone to IBD is between 3 and 6 weeks once the bursa of Fabricius is at its maximum rate of development and the bursa follicles are filled up with immature lymphocytes. This is because the IBD virus replicates in and cytolytically affects the actively dividing B lymphocytes in the bursa of Fabricius (Van den Berg *et al*., 2000). Infections occurring before the age of 3 weeks are typically subclinical and immunological disorder. Clinical cases may be observed up to the age of fifteen to twenty weeks (Jackwood, 2014). Light strains of laying stock are more susceptible to disease than the heavy broiler strains.

Until 1987, the field strains isolated was of low virulence and caused just one to twenty of specific mortality. However, since 1987 a rise in specific mortality has been described in several part of the world. In the USA, new strains to blame for up to five percent of specific mortality were described. At a similar time, in Europe and later in Japan, high mortality rates of fifty to sixty percent in laying hens and 25% to 30% in broilers were observed. These hyper virulent field strains caused up to 100% mortality in specific pathogen-free (SPF) chickens (Van den Berg *et al*., 2000).

In Ethiopia the mortality rate of the disease in several poultry house ranges from 45-50% in Debre zeit. Broiler mortality was about fifty six percent whereas 25.08% for layer chickens (Zeleke *et al*., 2005). In totally prone flocks, mortality related to infection because of classic strain might vary from 1-60% with high morbidity of up to 100%. A variant IBDV strains do not produce overt clinical signs, however cause immunological disorder and could cause mortality because of secondary opportunist infections in immune compromised birds. In contrast, vvIBDV strains cause mortality of 50-60% in laying hens, 25-30% in broilers and 90-100% in prone leghorns. Susceptible chickens younger than three weeks of age may not exhibit clinical signs but develop subclinical infections. This results in a decreased humoral antibody response due to B lymphocyte depletion in the cloacal bursa and severe and prolonged immunosuppression. The most significant economic losses result from subclinical infections this form of IBD infection greatly enhances the chicken's susceptibility to sequels such as gangrenous dermatitis chicken anemia virus, inclusion body hepatitis, respiratory diseases and bacterial infections (Van den Berg *et al*., 2000).

2.3.5 Molecular epidemiology and phylogenetic analysis of IBDV

Immunization is the mainly significant quantify toward control IBD; but, widespread practice of live vaccines have resulted in the development of novel strain. in spite of the usual immunization program,there are still information of infectious bursal disease outbreak all over the world. Through intimately investigating the progression of infectious bursal disease virus, it is likely to trace the reason of the outbreak. The main capsid proteinVP2 has a critical function during antigenic difference through which the virus be able to break out immune system actions. The majority of the amino acid (aa) change between the antigenically diverse IBDVs are clustered in the hypervariable part of VP2 (hVP2). As a result, hVP2 is the understandable mark intended for IBDV finding, progression and virulence. The hVP2 region has two main hydrophilic domains, that is major hydrophilic peak A (aa 212–224) and peak B (aa 314–325) that form hairpin loops PBC (aa 219–224) and PHI (aa316–324), respectively. The minor hydrophilic peak 1 (aa248–254) and peak 2 (aa 279–290) of hVP2 form loops PDE (aa 249–254) and PFG (aa 279–284), respectively (Coulibaly *et al*., 2010).

Moreover lone or joint mutations in hVP2 region influence the virulence pattern of the virus. Current IBDV field outbreaks from different geographic location revealed amino acid exchanges at minor hydrophilic peak domain of hVP2 (Jackwood and Sommer-Wagner, 2011). Mutations at the variable domain linking amino acids position 206 and 350, where the neutralizing antibody binds, results in immune evasion of the virus. The substitution of the amino acids at positions 253 (Q253H), 279 (D279N) and 284 (A284T) on theVP2 domain of vvIBDV resulted in loss of virulence of thevirus. But a single point mutation in the 253 (H253Q/N) or 249(R249Q), however, drastically increased the virulence of anattenuated IBDV strain. The mutation at the amino acid position254 with serine in place of glycine (loop PDE) (Negash*et al*., 2013)results invaccination failure. Although sequencing of the whole IBDVgenome is useful for phylogenetic characterization, it isimpractical to replicate in large number of isolates. Recently,the virus has been classified into seven genogroups.Genogroup 1 generally comprises the classical IBDV and ispresent worldwide, genogroup 2 primarily comprises the antigenicvariants predominant in the USA and genogroup 3comprises predominantly of the vvIBDV pathotype or feware vvIBDVreassortants, distributed worldwide. An antigenicdrift at aa position 222, (A222T) the genetic hallmark, hasbeen observed in the population of genogroup 3 due to selectiveantigenic

pressure with vaccination. Moreover, the genogroup3 reassortants have a different segment B and do nothave the typical amino acids found in the vvIBDV. Most of thevirulent viruses of Indian origin also belong to genogroup 3 (Figure 2).

Nevertheless, the viral isolates that did not visibly fit into any of the three major genogroups were classified separately. The genogroup classification method classified these viruses into four new genogroups 4–7. A genogroup 4 virus is characterized by 222S, 272T, 289P, 290I and 296F and is distributed worldwide but was most commonly isolated from Latin America. Genogroup 5 viral strains included Mexican recombinant classical and variant viruses with amino acid changes in both PDE and PHI. These viruses have variant- type amino acid sequences in the PBC loop, whereas the PFG loop is more similar to the classical viruses. The PDE loop differed from reference variant and classical strains with the presence of 251N and 254N, and the PHI loop of genogroup 5 revealed two unique substitutions: S317K and A321P (Michel and Jackwood, 2017). Genogroup 6 consisted of samples from Saudi Arabia with 92.26–93.64% identity to the Italian genotype that is characterized by 220H, 222Q, 253E, 254S and 321Vand has 94.02–95.40% identity to Russian isolate. Genogroup 7 is composed of viruses from Australia and has two distinct groups of IBDV; the classical strains that are similar to V877 and 002-73 and antigenic variant strains that are similar to 05-5 and 08/95 viruses (Ignjatovic and Sapats, 2002).

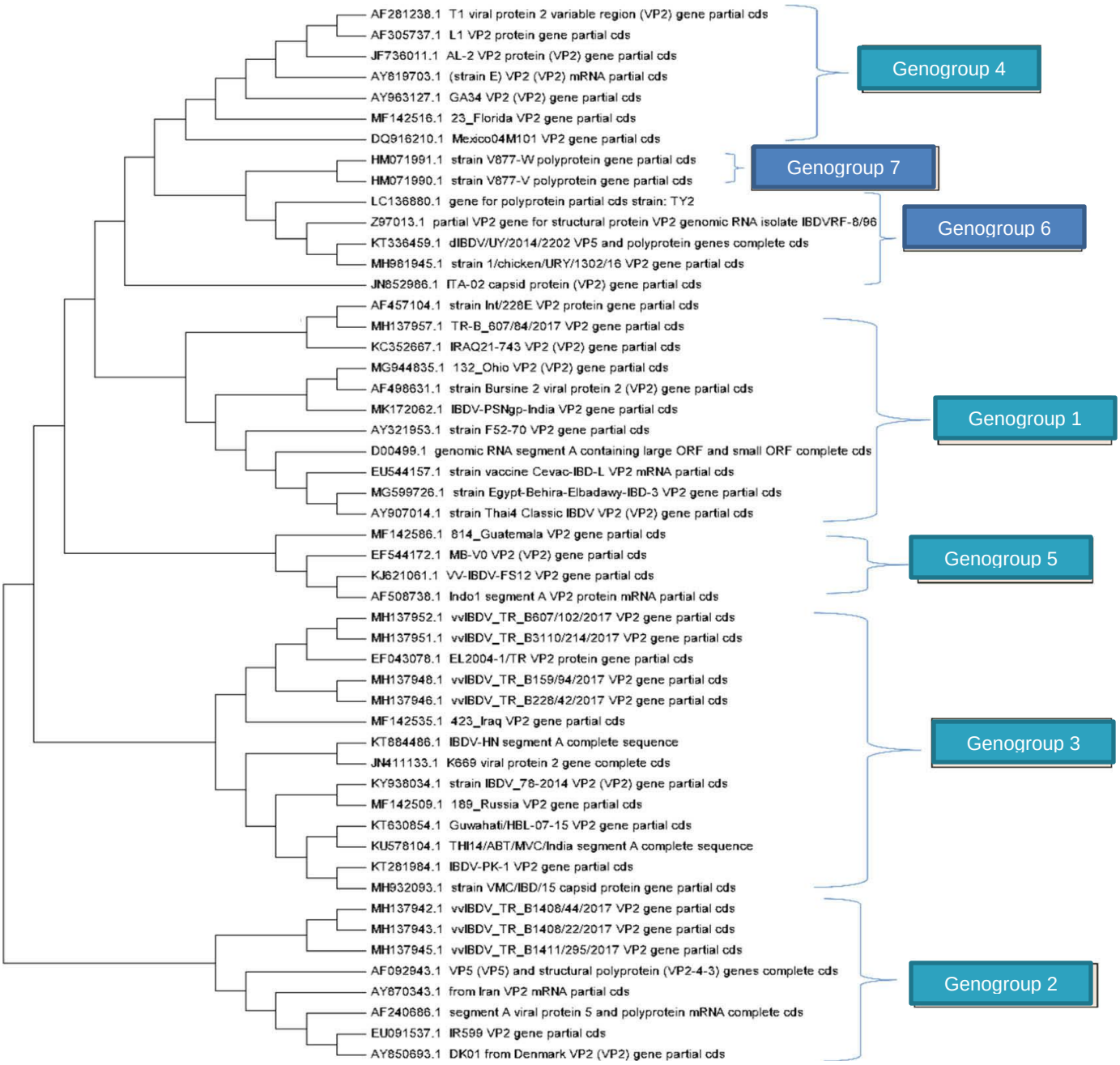


Figure 2: Phylogenetic analysis of the nucleotide sequences of hVP2 infectious bursal disease virus (IBDV). The evolutionary history was inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. (Jackwood *et al*., 2018).

2.3.6 Antigenic variation of IBDV strains

A high genetic mutation rate is the significant feature of RNA viruses (Vidalain and Tangy, 2010). The hyper-variable region (HVR) of IBDV is located in the vp2 gene (206 aa to 350 aa), which is responsible for the antigenic variation since VP2 induces neutralizing antibodies (Vakharia *et al*., 1994). IBDV takes advantage of the genetic flexibility to achieve antigenic variations, which help it to escape immune clearance and quickly adapts to the change of environment. The emergence of very virulent IBDV (vvIBDV) strains was reported in the late 1980s (Chettle *et al*., 1989). The putative amino acids responsible for high virulence were at Gln253, Asp279 and Ala284 in VP2 (Brandt *et al*., 2001). The residues 249 and 256 of VP2 were also found involved in the replication efficiency and virulence of IBDV (Qi *et al*., 2013). In addition to the mutations in HVR of VP2, genetic reassortment events and homologous recombination within segments also contribute to the variation of IBDV (Islam *et al*., 2001; Wei *et al*., 2006; He *et al*., 2009; Jackwood, 2012).

A molecular epidemiology study of IBDV isolates from seven provinces in southern China during 2000–2012 showed that the majority of the isolates (85.71%, 78/91) were identified to be naturally reassorted strains (He *et al*., 2014). The recombination leading to the variation of IBDV virulence can be evidenced in the different isolates having segment A region consistent with vvIBDV (Pitesky *et al*., 2013; Gallardo *et al*., 2014). With the intensive uses of live vaccines, the number of vvIBDV strains and their reassortants have continuously increased and the strains have become epidemic and posed a great threat to the poultry industry with the current anti-IBDV vaccination strategy (Kurukulsuriya *et al*., 2016; Hiraga *et al*., 1994), making the prevention and control of IBD more challenging.

2.3.7 IBDV reassortment, recombination and reversion to virulence

Mainly, reassortment enhances to the first emergence of vvIBDV in the late 1980s in Europe (Chettle *et al*., 1989; Hon *et al*., 2006). Prediction of the most recent common ancestor of vvIBDV describes an approximate most recent common ancestor of vvVP2 around 1960 and of vvVP1 from an unidentified avian reservoir around 1980 indicating that these two proteins of vvIBDV have evolved at different time points (Hon *et al*., 2006; Le Nouën *et al*., 2005). Recent genetic analysis showed that most reassortant IBDVs reported from around the world have

segment A from vvIBDV that maintain key virulence marker aa in their VP2 (222A, 256I, 294I, and 299S, (Yamaguchi *et al*., 1997; Xia *et al*., 2008) attenuated strains have 253H and 284T, and 253Q and 284A are often found in variant strains33) and segment B from attenuated vaccine strains (Le Nouën *et al*., 2005; Lu *et al*., 2015). Reassortants with segments from serotype 1 and serotype 2 IBDV or segment A from an attenuated and segment B from vvIBDVs have also been reported (Pitesky *et al*., 2013). Different genotypes of IBDV are sometimes isolated from the same bursa implying coinfection being common in the field (He *et al*., 2012).

The frequent isolation of reassortant IBDV field isolates indicates that phylogenetic markers need to include VP1 in the molecular epidemiology of IBDV, especially if considering the contribution of VP1 to IBDV virulence (Alfonso-Morales *et al*., 2015; Liu and Vakharia, 2004; Boot *et al*., 2005). A field reassortant IBDV comprising segment A of vvIBDV and segment B of an attenuated strain caused reduced mortality in specific pathogen free (SPF) chickens compared with a typical vvIBDV isolate (Le *et al*., 2006). When regions of VP1 of a vvIBDV strain were exchanged with VP1 counterparts of an attenuated IBDV, the resulting recombinant virus showed reduced virulence and bursal lesions in chickens (Le *et al*., 2012). Recent studies have identified putative virulence markers in VP1 of IBDV field isolates (Yu *et al*., 2010). A TDN (threonine/aspartic acid/asparagine) motif was identified at aa residues 145, 146, and 147 of all vvIBDVs tested, which is absent in most non-vvIBDV isolates (Jackwood *et al*., 2012). Replacement of the TDN motif of a vvIBDV strain with TEG (threonine/glutamic acid/glycine) or NEG (asparagine/glutamic acid/glycine) resulted in loss of virulence, and the change of NEG to TDN increased the virulence for an attenuated strain, indicating the contribution of these three aa to the polymerase activity (Gao *et al*., 2014). Yu et al. (2013) further evaluated the pathogenicity of a vvIBDV strain by exchanging a single aa at VP1 position 4 (V4I) which resulted in attenuation of the respective mutated virus.

2.3.8 Immune response towards IBDV infection

IBDV causes immunosuppression in chickens, and BF is the target organ for viral replication. The stage of B cell differentiation in the BF plays an important role in viral replication as the stem cells and peripheral B cells do not support viral replication. The acute phase of the disease lasts for 7–10 days, during which the B cells are depleted in the bursal cortex and medulla,

peripheral blood and thymic medulla, and the BF becomes atrophic. The viral antigen can also be detected in the peripheral lymphoid organs like caecal tonsils, spleen and thymus besides BF. The disease when not fatal leads to immunosuppression with reduced antibody response. The chickens fail to produce antibodies against other viral diseases and lead to subsequent outbreaks with the surviving birds tend to show high anti-IBDV antibody titers. Protection against the disease does not depend solely on the humoral immunity; cell-mediated immunity through T cell involvement is also important. IgM+ B cells serve as targets for IBDV (Sharma *et al*., 2000) and CD4+ and CD8+ T cells, along with the macrophages accumulate in the BF even as early as 1 dpi (Withers *et al*., 2005) with up-regulation of IFN-γ, IL-8 and IL-6 transcription and apoptotic mediators like nitric oxide or TNF-α. The role of cell-mediated immunity following IBDV infection is well established (Dey *et al*., 2017; Maity *et al*., 2015) with the localization of bursal mRNA transcription of the pro-inflammatory cytokines IL-1β, IL-6, CXCLi2 and IFN-γ together with down-regulation of transcription growth factor-β4. In vivo challenge with vvIBDV UK661 strain upregulated the transcription levels of types I, II and III IFNs as well as IL-18, IL-4 and IL-13 cytokines (Eldaghayes *et al*., 2006).

2.4. Pathogenesis of IBDV

The disease attacks young chickens at 3-6 weeks of age, while sub-clinical form of infection is established in older birds. Layer type chickens are more susceptible to vvIBDV than broiler type (Tippenhauer *et al*., 2013) and higher mortality rates are observed in light than heavy breeds. As mentioned earlier, IBDV has two serotypes, serotype 1 and serotype 2 however serotype 1 develops IBD in chicks. Apparent pathological lesions are observed with experimental infection on pigeon and guinea fowl (Kasanga *et al*., 2008). The primary organ of predilection is bursa of Fabricius (BF) where majority of the B cells are in actively dividing stage in young chicks. Faeco-oral route and inhalation are the major routes of entry of the virus that replicates in gut-associate macrophages and lymphoid cells and results in primary viremia through portal circulation. Following primary viremia, the virus reaches BF by 11-hr post inoculation and after active replication in bursal follicles and B cells, the virus enters the bloodstream to cause secondary viremia. This leads to spread of the virus in other organs like kidneys and muscle tissue that leads to pathognomonic clinical signs and death. Following infection of the BF,

degeneration and necrosed B-cell follicles especially IgM+ cells are detected immediately, with associated infiltration of inflammatory cells such as heterophils. As the inflammation reduces, there is necrosis and phagocytosis of heterophils and plasma cells, fibroplasia in the interfollicular connective tissue, and ultimately bursal atrophy (Eterradossi and Saif, 2008).

With concurrent histological changes, molecular variations like upregulation of antiviral genes that are involved in the type I interferon (IFN) response, pro-apoptotic genes, and proinflammatory cytokines and chemokines, presumably from the infected B cell population appears (Ruby *et al*., 2006). VP2 and VP5 proteins of the virus induce apoptosis in B cells and other lymphoid cells thereby causing cell depletion (Qin and Zheng, 2017). Other organs show pathological signs like splenomegaly, petechial hemorrhages on the mucosa at the juncture of the proventriculus and diffused hemorrhages in the thigh and breast muscles. Lesions in the caecal tonsils, thymus, spleen and bone marrow confirm infection with vvIBDV, with the harderian gland being severely affected following infection in the day-old chicks (Abdul *et al*., 2013). Repopulation of B cells in the BF happen in the recovered birds. The cells arrange in two different types of follicles either the long follicles which are the repopulated endogenous bursal stem cells in the survived birds, and can mount their own immunity or the small, poorly developed follicles lacking a distinct medulla and cortex due to the damage following infection (Withers *et al*., 2005).

2.5. Clinical signs of IBD

Depending on the virulence of the strain, age and breed of the chicken, and the level of MDA, the severity of the clinical signs varies among flocks, localities, and countries. The disease occurs in two forms, acute and subclinical (Van der Berg, 2008). The acute form usually starts suddenly following a short incubation period of 2-3 days and lasts about 7 days. The clinical manifestations include anorexia, depression, prostration, ruffled feathers, white watery diarrhea, and sudden death (Figure 3). Morbidity may reach up to 100%, whereas mortality ranges from 20% to 30%. Higher mortalities approaching 90–100% are recorded in certain areas of the world like Europe, when associated with very virulent strains (Chettle *et al*., 1989; Ivanyi and Morris 1976). Birds of age less than 3 weeks or infected with low virulent and variant stains usually show subclinical form of the disease. Subclinical disease is a mild form, characterized by

immunosuppression and has been mainly reported from the United States. It is usually not associated with any apparent signs except for retardation of growth, secondary infection with opportunistic microbes, and ineffective vaccination programs (Jackwood *et al*., 2006; Kegne and Chanie 2014; Snyder 1990).

Figure 3: Clinical signs (severe depression and high mortality) of Gumboro disease

Source: (Musa *et al*., 2012)

2.6. Gross pathology

As mentioned earlier, BF is the primary target organ for the IBDV and shows typical gross pathological features. On day 3 PI, BF shows edematous changes and hemorrhage (Figure 4), resulting in increasing size and weight. This enlargement continues until it reaches double size and weight in comparison to birds with similar age. The bursa returns to its normal size on day 5 PI, then begins to atrophy gradually until it reaches approximately one third of its original size (Eterradossi and Saif, 2013). Gelatinous yellowish material may be seen covering the external surface of the bursa on the second and third day of infection. The bursal mucosa reveals multifocal areas of necrosis and hemorrhages (Mwenda *et al*. 2018).

Extra-bursal lesions include petechial and ecchymotic hemorrhages in the pectoral and thigh muscles (Figure 4) and hepatosplenomegaly associated with grayish necrotic foci on their surfaces. Moreover, dehydrated birds have swollen kidneys with accumulation of urates, owing

to obstruction of the ureters by the enlarged bursa. The intestinal lumen may contain large amount of mucous. Infrequently, the mucosa at the junction between gizzard and proventriculus show hemorrhages (Kegne and Chanie, 2014).

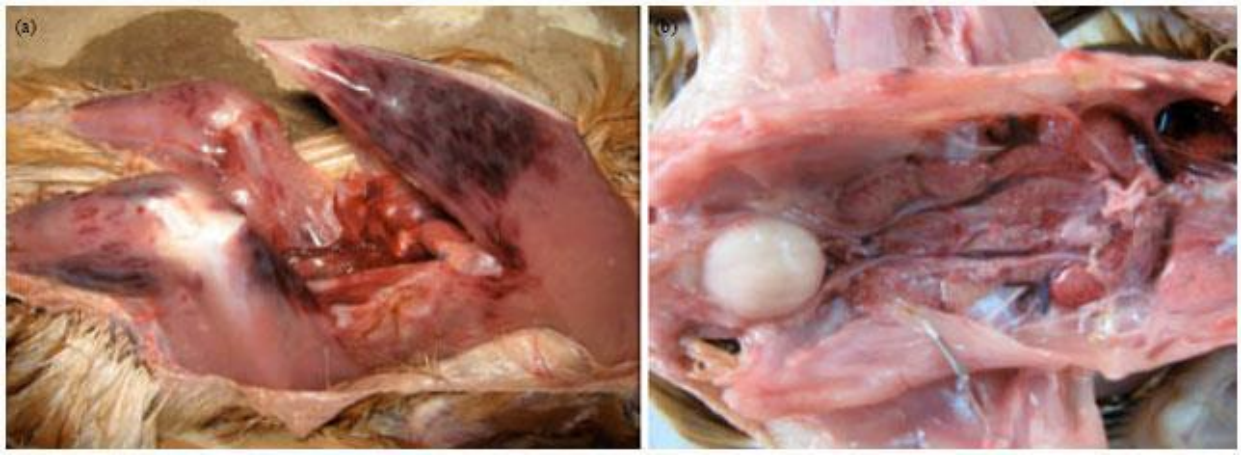


Figure 4: Gross pathology of (hemorrhagic muscles, inflamed bursa of fabricius), in the Gumboro infected bird.

Source: (Musa *et al*., 2012)

2.7. Diagnosis of IBD

Infectious Bursal Disease is diagnosed by considering the flock's history, clinical signs and post mortem lesions. Obviously, chickens less than 3 weeks of age present no clinical signs of disease but chickens greater than 3 weeks of age present clinical signs (Kegne and Chanie, 2014). Clinical IBD can be diagnosed by a combination of characteristic signs and post-mortem lesions. Subclinical IBD can be confirmed in the laboratory by demonstrating a humoral immune response in unvaccinated chickens, or by detecting viral antigens or viral genome in tissues. In the absence of such tests, histological examination of bursae may be helpful (OIE, 2016).

Clinical manifestations and postmortem findings of affected birds may aid to diagnose a disease but laboratory diagnosis is necessary for confirmation of the diseases (Banda, 2002). Isolation and identification of the agent provide the most certain diagnosis of IBD but are not usually attempted for routine diagnostic purposes because the virus is difficult to isolate. In practice, laboratory diagnosis of IBD depends on detection of specific antibodies to the virus, or on

detection of the virus antigen and nucleic acid in tissues, using immunological or molecular methods (OIE, 2016). Confirmatory diagnosis of IBDV is most commonly performed by serology using Enzyme linked immunosorbent assay (ELISA), Agar gel precipitin test (AGPT) and Virus neutralization test (VNT) of bursal sections (Jordan and Pattison, 1996).

2.7.1 Clinical diagnosis

Infectious bursal disease virus has a short incubation period of 2-3 days and the infection generally last 5-7 days. One of the earliest sign of IBDV infection is the tendency for the bird to engage in vent picking (Minalu *et al*., 2015). In a typical outbreak in 2- to 15-week-old chickens, some 10-20% of the flock may show sudden signs. Observing any signs is difficult during the very early stages. One of the earliest signs is whitish or watery diarrhoea, with vent feathers soiled by urinary material. This is followed by anorexia, depression, trembling, severe prostration, and death (Cosgrove, 1962). The disease is manifested by debilitation, dehydration and development of depression with the swollen and bloodstained vent (Islam and Samad, 2014).

2.7.2 Differential diagnosis

The lesions and symptoms of coccidiosis are very similar to IBD. Similar to IBD, in coccidiosis there are sudden onset, ruffled feathers bloody droppings but no bursal lesion (Kaufer and Weiss, 1980). However, muscular haemorrhages and edema of bursa differentiate IBD from coccodiosis. Other diseases that resemble IBD are infectious bronchitis virus, haemorrhagic syndrome, Marek's disease (Lukert, 1986). Quinn reported that if there are enlarged muscular haemorrhages and enlarged edematous or haemorrhagic cloacal bursas, it would suggest IBD, however, the involvement of cloacal bursa usually will distinguish IBD from other nephrosis causing condition. Marek's (causes bursal atrophy, but nerve lesion is very distinct and also marek's forms tumors). Haemorrhages syndrome (cause bursal muscular mucosal haemorrhage, but with no bursal lesion) is the usual manifestation of the diseases (Kaufer and Weiss, 1980). Non-infectious diseases like mycotoxins are causes of bursal atrophy (Swayne, 1998). In aflatoxins there is a degeneration of both thymus and the bursa and trichothecenes generally depress lymphocytopoesis. High doses of zearalenon also cause decreased bursal weight. As in other species, steroidal anti-inflammatory drug like, corticosteroids cause apoptosis and decrease

the production of lymphocytes and smaller bursas can be occurred in animals that are in poor nutritional states or stressed from other reasons (Farner *et al*., 1983; Ridell, 1996).

2.7.3 Gross lesions

Bursal of Fabricius is turgid, oedematous, heamorrhages and atrophic seven to ten days in chicken that died by acute stage of vvIBD through post mortem examination. This atrophy may be more quick, even 3 to 4 days after immunization. Likewise, dehydration and nephrosis with enlarged kidneys are normal, and heamorrhages in the muscle and mucosa of proventriculus are known in the greater part of affected chicken. Extreme depletion of lymphoid cell is perceived both in bursa of Fabricius and non-bursal lymphoid tissues. Pathogenicity of IBDV has been related with virus distribution in non-bursal lymphopoietic and hematopoitic organs. To be sure, utilizing various immune staining ways, a higher frequency of antigen-positive cells can 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 specific, atrophy of the thymus has been related with the acute phase of the disease and could be characteristic of the virulence of the isolate, though it is not related to extensive viral replication in thymic cells.. An increasing number of macrophages are found in various organs (Tanimura and Sharma, 1997). Thrombocytes also represent a target for IBDV, and acute disease is characterized by disseminated hemorrhages presumably related with impairment of the clotting mechanism (OIE, 2008).

2.7.4 Histopathological lesions

As per OIE, (2016), tissue of bursa of fabricius is taken out aseptically from affected chickens in the beginning phases of the disease. Bursae is chopped by utilizing two surgical blades, include a modest quantity of peptone stock containing penicillin and streptomycin (1000 μg/ml each), and homogenize in a tissue blender. The homogenate is centrifuged at 3000 g for 10 minutes. And then harvest the supernatant liquid for use in the examinations. The bursal tissue are fixed by immersion in 10% buffered formalin and processed for histologic examination by standard methods of paraffin embedding, sectioning and haematoxylin and eosin staining (Tanimura *et al*., 1995). The tissue sections are examined by light microscope and the severity of lesions is graded on the basis of the extent of lymphocyte necrosis, follicular depletion and atrophy

(Sreedevi *et al*., 2007). Histological finding depends on the recognition of changes happening in the bursa. The capacity to cause histological sores in the non-bursal lymphoid organs, for example, the thymus, (Hussain *et al*., 2004) the spleen or bone marrow (Inoue *et al*., 1999) has been reported as a potential characteristic of hypervirulent IBDV strains. Necrosis and infiltration of heterophils and plasma cells occur within the follicle, as well as, the interfollicular connective tissue. In addition, a fibroplasia, the inter-follicular connective tissue, may appear and the surface epithelium of the bursa becomes involutes and abnormal (Eterradossi et al., 1992). Histological lesions in the kidney are nonspecific and probably occur because of severe dehydration of affected chickens. Lesions observed consisted of large casts of homogeneous material infiltrated with heterophils, and also glomerular hypercellularity (Mahgoub, 2012). Proliferation of the bursal epithelial layer generates a glandular structure of columnar epithelial cells that contains globules of mucin. During this stage of the infection, scattered foci of repopulating lymphocytes were observed; however, these did not develop into healthy follicles (Campbell and Coles, 1986).

2.7.5 Virological diagnosis

2.7.5.1 Virus Isolation in cell culture

The cloacal bursa and spleen are used for the isolation of the virus (Lukert and Saif, 2003; Muller *et al.,* 2003).The virus can be found in other organs such as the thymus, liver and bone marrow but in significantly low quantities than in the bursa (Eterradossi and Saif, 2008; Elankumaran *et al.,* 2002; Kabell *et al.,* 2005). The inoculums 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). A filtered homogenate of the bursa of Fabricius is inoculated in nine- to eleven-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).

2.7.5.2 Virus isolation in embryos

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 (OIE, 2012). It is important especially for wild-type IBDV, usually not replicating in conventional cell culture, can also be regenerated by the reverse genetics approach, but can grow in embryonated chicken eggs (Brandt *et al.,* 2001; Islam *et al.,* 2001c). Some strains grow well in embryos 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).

2.7.6 Serological diagnosis

The commonly used tests for serological diagnosis are AGID, (Hirai *et al*., 1974) virus neutralization test (VNT), (Weisman and Hitchner, 1978) or ELISA (Dey *et al*., 2009).

2.7.6.1 Agar Gel Immunodiffusion (AGID)

Agar gel immunodiffusion (AGID) test distinguishes the antigen in the bursa by setting the minced bursa from defenseless chicks in the wells of the AGID plate against known positive serum. Freeze-thaw patterns of the minced tissue discharge the IBDV antigens from the tissue and the freeze-thaw exudate is utilized to fill the wells (OIE, 2016). Antigen-capture ELISA is described for the detection of serotype 1 IBDV in which the ELISA plates were covered with mouse against IBDV monoclonal antibodies (Mabs) or chicken anti-IBDV polyclonal sera (Snyder *et al*., 1983). A panel of seven Mabs, namely 1, 3, 4, 6, 7, 8 and 9 are utilized in which Mabs 2 and 3 bound with classical strain however not vvIBDV strain (Eterradossi *et al*., 1998).

2.7.6.2 Enzyme Linked Immunosorbent Assay (ELISA)

Marquardt et al. (1980) first described the indirect ELISA test for IBDV antibodies, but it failed to differentiate between antiserum to IBDV serotypes I and II, (Ismail and Saif,1990; Ismail *et al*., 1988). Moreover, many commercial ELISA kits detect antibodies to both serotypes and vary

considerably in their sensitivity and specificity (De Wit *et al*., 2001; Ashraf *et al*., 2006; Wu *et al*., 2007; Eterradossi and Saif, 2008). The antigen capture ELISA (AC-ELISA) system using monoclonal antibodies (MAbs) was developed for characterizing the antigenic properties and relatedness of IBDV strains (Van der Marel *et al*., 1990; Eterradossi *et al*., 1997).

Commonly used IBDV to measure serum antibody titers, this sensitive and rapid method allows for differentiation of classic, variant, and vvIBDV subtypes (Van den Berg *et al*.,1996; Sapats and Ignjatoivic, 2000; Islam *et al*., 2001). These titers are useful for evaluating the flock immunity level during an outbreak, as well as, the efficacy of vaccination program,(Eterradossi and Saif, 2008). Polyclonal antibodies with AC-ELISA assays are more likely to be used for general screening of tissue samples for IBDV (Lukert and Saif, 1997; Rosenerger *et al*., 2008). The disadvantages to use such ELISA tests are the extensive preparation of the MAbs, the cultivation of IBDV in CEF or chicken bursae, and the fact that results do not always correlate with VN test findings (Wu *et al*., 2007).

2.7.6.3 Agar Gel Precipitation Test (AGPT)

The AGP test can be used for the rapid detection of IBDV group-specific soluble antigens or IBDV antibodies in convalescent birds. While simple to perform, low sensitivity, the inability to detect serotypic differences, and non-quantitative results are serious limitations (Wu *et al*., 2007; Eterradossi and Saif, 2008; Rosenerger *et al*., 2008).

2.7.6.4 Virus Neutralization Test (VNT)

The VN test is a more sensitive antibody detection tool and can be used for both antibody quantitation and differentiation of IBDV serotypes and subtypes (Ismail and Saif, 1990; Wu *et al*., 2007). The ability of serum to neutralize a reference virus is determined and a neutralization endpoint titer (VN titer) is indicated as the mutual of the biggest dilution of serum that prevents cytopathic effects (Wit, 2006; Wu *et al*., 2007; Eterradossi and Saif, 2008). VN tests are often employed for IBDV identification in embryos and tissue culture after its adaptation to these host systems and for assessment of antigenic and immunogenic changes (Wu *et al*., 2007; Rodriguez-Chaez *et al*., 2002; Sapats and Ignjatovic, 2000).

VN tests serve to compare virus strains by evaluating the ability of antiserum raised against one IBDV strain to neutralize a heterologous strain (Wu *et al*., 2007). However, only IBDV strains that efficiently replicate in embryos or cell culture can be used in the VN assay. Attempts to adapt the virus to a host may result in antigenic and pathological changes to the virus that may render VN results as suspect (Rodriguez-Chaez *et al*., 2002; Wu *et al*., 2007).

2.7.7 Molecular diagnosis of IBD

Another method that is used to detect IBDV is molecular technique. Reverse-Transcription Polymerase Chain Reaction (RTPCR) is one of the most important frequently used molecular methods that is used to detect the genome of IBDV (Lin *et al*., 1993). Reverse transcription-polymerase chain reaction (RT-PCR) enable us to detect viral RNA in homogenates of infected organs or embryos, as well as in cell cultures, without considering the viability of the virus present (Van den Berg *et al*., 2000). It is also used to detect the genome of viruses that don't replicate in cell culture because it doesn't require the growth of the virus before amplification. There are three steps in which RT-PCR is performed .These are; extraction of nucleic acids from studied sample, change of IBDV RNA into cDNA by Reverse Transcription (RT) and amplification of cDNA by PCR. The IBDV double stranded RNA stranded RNA (dsRNA) can't be degraded by RNAases, unlike single stranded RNA (OIE, 2016).

2.7.7.1 Extraction of IBDV RNA

Infectious bursal disease RNA can be extracted from infected tissues by using some kits which is available from commercial suppliers of molecular biology reagent. In another way IBDV RNA can be extracted by adding 1% sodium dodecyl sulphate and 1 gm/ml proteinase K to 700 µl of bursal homogenate and Incubated for 60 minutes at 37 °C. Nucleic acids are harvested from the final aqueous phase by ethanol precipitation and are re-suspended in RNasefree distilled water or a suitable buffer. Water-diluted RNA should be kept frozen at a temperature below -20 °C until use (OIE, 2016).

2.7.7.2 First Strand cDNA Synthesis

The extracted RNA is used for the synthesis of cDNA. The following reagents are mixed in PCR tubes to a final volume of 25 µL. These are; Template RNA 1 µg, OligodT primer 1 µL and Nuclease free water to 12 µL. Then the above mixture are kept at 65 °C for 5 min in a thermal cycler, followed by the addition of the following components in the indicated order: 5 X reaction buffer 4 µL, Rnase inhibitor 1 µL, 10 mM dNTP 2 µL and Reverse transcriptase 1µL. The above mixture was kept at 42 °C for one hour and 5 minutes at 70 °C in a thermal cycler (Adamu *et al*., 2013). Synthesized cDNA is used as template for polymerase chain reaction (PCR) (Rai Shafqat *et al*., 2017).

2.7.7.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR allows IBDV differentiation based upon time and number of samples that can be tested simultaneously. The RT-PCR SYBR green technology is robust and may serve as a useful tool with high capacity for diagnostics as well as in viral pathogenesis studies. Different types of infectious IBDV strains including, virulent strain DK01, classic strain F52/70 and vaccine strain D78 were quantified and detected in infected BF and cloacal swabs by real-time RT-PCR with SYBR green dye (Li *et al*., 2007).

2.8. Treatment, prevention and control

2.8.1 Treatment

No practical therapeutic or supportive treatment has been found to change the course of IBDV infection (Parkhurst, 1964). Experimental immunotherapy where passively transferred antibody is injected intraperitoneally after challenge greatly reduced birds showing clinical signs, but this approach has not been tested in the field (Malik *et al*., 2006). There are no reports in the literature concerning the use of some of the newer antiviral compounds and interferon inducers for the treatment of IBD. Ketotifen was reported experimentally to prevent the development of bursal damage, and reduce clinical signs and mortality induced by vvIBDV challenge when administered one hour before IBDV inoculation, but it is not licensed for food animal veterinary use (Wang *et al*., 2009).

2.8.2 Control and prevention

The IBDV is contagious and contact with infected birds and contaminated fomites could result in the spread of infection (AHA, 2009). The virus is environmentally stable and resistant to many chemical and physical agents (Eterradossi and Saif, 2008). Its spread between flocks can be restricted through implementation of strict biosecurity measures (Lukert and Saif 2003; Saif 2008). However, with the integrated nature of commercial poultry operations, litter reuse and the possibility of interaction with free-living wild birds, the control of IBD faces difficulties (AHA 2009). The use of therapeutic treatment has been reported to have no effect on the course of infection (Cosgrove 1962; Lukert and Saif, 2003).

Control of IBD has been more difficult by the popularity of variant strains of the IBD virus. Variant viruses induce damage within the BF in chickens, even once high and uniform protein titers are presence. Variant strains do not cause obvious clinical disease, but immune-suppression. In Chicken affected by classical IBDV the bursa of Fabricius undergo rapid atrophy (lymphocyte depletion) without inflammatory changes observed early in the infection. These variants are not from a different serotype, but are antigenically different enough to cause immunosuppression problems (Jackwood and Sommer-Wagner, 2005). An additional important feature of IBD is its immunosuppressive action that may interfere with the efficiency of vaccination programs.

2.8.2.1 Vaccination and management strategies

Strict hygiene measures, vaccination with conventional live attenuated and inactivated viral vaccines have been used to prevent IBD. However, eliminating the sturdy and the persistent IBDV particles from the farm is a difficult task as the virus remains infectious for 122 days in a chicken house and for 52 days in feed and water (Müller *et al*., 2012). Therefore, routine sanitary measures must rigorously be followed to control IBDV. Disinfection may reduce the virus load and thus reduce the risk of transmission. The mechanical vectors such as mosquitoes, mealworms, and smaller rodents must be eradicated. On the farms where IBDV outbreaks have occurred, the virus may be considered endemic. Improper cleaning of the hoods will expose the young birds to the virus at an early age. Early subclinical infection is the main cause of economic loss as the disease can cause severe, long-lasting suppression of the immune system and the

immunocompromised birds do not respond well to vaccination and are more susceptible to other infections. Culling the infected chickens and controlling other flocks from being infected is costly. Thus, vaccination remains the method of choice to control IBD. However, vaccine failures do occur due to the evolution of the virus even with strict vaccination practices. An early method of prevention is exposing the young chicks to IBDV (Lasher and Box, 1993) and this technique although reduces mortality but often results in immunosuppression and further dissemination of the field virus. Live-attenuated vaccines based on mild field isolates after passaging in specific-pathogen-free eggs were developed. They are still widely used today in parent stock as a primary vaccine for controlling the very virulent IBD in many countries. Until the 1980s, mortality caused by IBD was effectively controlled by vaccination. With the emergence of Delaware variants in the USA in the mid-1980s and the emergence of very virulent forms of the virus in Europe and Asia in 1989 resulted in vaccination failures (Berg, 2000; Eterradossi and Saif, 2008; Lasher and Box, 1993).

In addition to hygienic farm management and biosecurity, the current IBD control methods involve passive and active immunization (Fussell, 1998). It has been shown that the timing of IBD vaccine administration in chicken progeny is pivotal. The optimal vaccination time depends upon the maternal-derived antibody (MDA) level of the chicks, the vaccine strain used, vaccine break through titer, and the IBDV field pressure (de Wit, 2001). Vaccination in the presence of IBDVantibody levels above the breakthrough titer of the vaccine will lead to a significant delay in induction of immunity, and also IBD vaccine virus may even be completely neutralized by maternally derived antibodies (Moraes *et al*., 2005). In order to have chickens protected against IBDV field challenge, it is crucial to determine the optimal timing for IBD vaccine delivery. The optimal timing is often predicted based on serological data following detection of IBDV MDA by an ELISA system during the first few weeks post-hatch (Kouwenhoven and van den Bos, 1994).

It is essential to prevent the infection at an early age, so that the immunosuppressive effect of IBDV could be controlled. This can be achieved by immunization of the parent stock. Inactivated vaccines along with oil-adjuvants boost the immune response and the maternal immunity may be extended to 3–5 weeks. When young chickens are to be vaccinated with attenuated vaccines, timing of vaccination is important as too early vaccination may lead to neutralization of the

vaccine by MDA, and on the contrary, the birds may remain unprotective if vaccinating too late due to the low level of MDA. Monitoring the antibody level in a breeder flock or its progeny can aid in determining the right time to vaccination (Eterradossi and Saif, 2008).

The MDA level can be determined by serological monitoring and the right time of vaccination could also be determined. Vaccines may be administered by intramuscular injection, by spray or by mixing in drinking water. Chickens vaccinated with IBDV in early life before 7 days of age and revaccinating with an inactivated, oil-adjuvant IBD vaccine at 18 weeks of age can produce and maintain high levels of virus-neutralizing antibody through 10 months of lay (Naqi et al., 1983). Moreover, due to early vaccination, the vaccine virus will spread in the poultry farm and indirectly could provide immune response to the other susceptible chicks (van Den Berg *et al*., 2000).

Live-attenuated vaccines are referred to as mild, intermediate, or "intermediate plus" (hot) vaccines based on the ability to cause varying degree of histopathological lesions and are suitable for mass vaccination preferably through drinking water to induce robust cellular and humoral immunity. The mild vaccines do not cause bursal damage in chicks but have poor efficacy in the presence of MDA or vvIBDV infection. Vaccines of higher pathogenicity (intermediate or "intermediate plus") may break through the high levels of maternal immunity but may produce bursal lesions, with subsequent immunosuppression leading to a secondary infection. In addition, they may not protect against infection with vvIBDV (Rautenschlein *et al*., 2005) or antigenic variants. Inactivated vaccines, mostly formulated as water-in-oil emulsions, are usually administered in the breeder hens for vertical transmission of high, uniform, and persistent antibody titers to the progeny (Wyeth and Cullen, 1978).

Intermediate and "hot" vaccines are mostly used to overcome the MDA in young broilers. The possibility of reversion to virulence, generation of reassortment strain and vaccine reactions resulting in disease or production loss may be the few undesirable side effects. Due to these limitations, the new generation vaccines such as subunit vaccines (Maity *et al*., 2015), *Virus-like particle (VLP) based vaccine* (Martínez-Torrecuadrada *et al*., 2000), DNA vaccines (Fodor *et al*., 1999), and Immune complex vaccines (Ignjatovic *et al*., 2006) have been developed to control IBDV.

2.8.2.2 Vaccination failures and potential causes

In general, vaccine efficacy profoundly relies upon the dose and strains of the vaccine and challenge viruses, as well as the route of administration, the appropriate vaccination time, and the degrees of maternal antibodies (OIE, 2012). The potential causes that influence the result of an IBDV vaccine are generally founded on the gap on relationship between strains of the vaccine with pathogenicity and antigenicity kind of the circulated virus, the proper immunization time, the age and the breed of the bird, and the presence or nonappearance 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 vaccine In addition to this immunization isn't common practice in smallholder poultry and control is additionally complicated by the customary development of new strains that may not be secured by existing immunization. On head of this, most control procedures planned in the nation don't think about the local chickens, and this may lead into the failure of most techniques (Tadelle and Ogle, 2001; Hailemariam *et al*., 2006).

The reasons for failures of live-virus immunizations are various. Interference from MAB is one of the most frequent causes of failure (AL-Natour et al., 2004). 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). Hence it requires ceaseless checking of the neutralizer level in a in a breeder flock or its progeny to help in deciding the perfect opportunity to immunize (Eterradossi and Saif, 2008). Classical live attenuated vaccines may actuate expansive, long lasting protection, however they additionally convey residual pathogenicity and the potential to revert to virulence (Van sanctum Berg et al., 2000). Yet inactivated immunizations failures is uncommon, yet may happen, either because of 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; consequently, they will in general be best in chicks that have been "primed" with a live virus vaccine or naturally infected through field introduction to IBDV (Eterradossi and Saif. 2008).

2.9. Economic importance of IBD

Immunosuppressive viral diseases are a great concern for the poultry industry for several years. Indeed the reemergence of IBDV in variant or highly virulent forms have been the cause of significant economic losses (Rautenschlein *et al.,* 2001). IBDV has an economic impact not only due to the direct losses it provokes, but also to the indirect losses as a consequence to immunosuppression or due to interaction it might have with other factors. The direct losses are due to specific mortality, depending on the virulence and the dose of the inoculums, the age and the breed of the bird, and the presence or absence of passive immunity. Moreover, IBDV is also responsible for indirect losses due to acquired immunodeficiency, impaired growth and condemnation of carcasses (Shane *et al*., 1994).

Furthermore the increase use of antibiotics and chemicals to fight against opportunistic (secondary) infections is a major concern of human health, if we consider the risks linked to the presence or residues in meat products, the release of residues into environment and increased antibiotic resistance (Marian, 2001). It can be stated that there is a significant variation in body weight in Gumboro affected broilers due to the existing and imposed vaccination program under farm condition and thus the imposed vaccination program should be recommended for use in farm condition to attain better body weight (Paul *et al*., 2004). The economic importance of this disease is fundamentally based on these two aspects: on one hand, the high mortality rate caused by some IBDV strains in 3-week old chickens, and even older, and on the other hand, the second clinical manifestation of the disease consisting of a prolonged immunosuppression of the birds infected at early ages (Naqi *et al*., 2001). The main sequelae associated to said immunosuppression are: dermatitis gangrenosa, anemia-hepatitis syndrome with inclusion bodies, *E. coli* infections and failures in the efficacy of other vaccinations, such as the vaccinations against the Newcastle disease and infectious bronchitis (Naqi *et al*., 2001).

2.10. The status of IBD in Ethiopia

Devastating outbreaks of the disease have been reported in many parts of the world and recently in Ethiopia ((Zeleke *et al*., 2005). The importance of the disease is represented by the high mortality, reduced productivity amongst infected chicks and accrued prone to other infections (accordingly, chickens also develop a poor immune reaction to vaccination against alternative pathogens (Zeleke *et al*., 2005; Sharma *et al*., 2000). The IBDV infection has as spread to all commercial farms and multiplication centers occur at an average outbreak rate of 3-4 farms per year. The disease was encountered usually in backyard poultry production systems similarly. Gumboro disease investigation was conducted by the NAHDIC in different Regions and with the result of overall prevalence rates to be about 77.48 % from the 706 samples collected and analyzed (Animal Health Yearbook, 2011).

There were also studies carried out by different authors where the disease is prevalent in different parts of our country (Table 2). Seroprevalence of 45.05% (173/384) of Infectious bursal disease (IBD) in chicken reared under backyard poultry production systems in Mekele town was reported (Zegeye *et al*., 2015). Out of 552 serum samples tested 458 (83%) in backyard chickens at selected districts of Eastern Ethiopia by Tadesse and Jenbere (2014) of 82.2% (227/276) reported on backyard chickens in both peasant associations and kebelles of Debre Zeit revealed the presence of IBDV specific antibody in the absence of vaccination, which indicate the presence of field exposure of household chickens to the virus by Tesfaheywet and Getnet (2012) and 27.8% of with a case fatality rates of 98.56% and 77.73% the incidence of IBD in chickens owned by 775 households in Amhara region of Bahir Dar and Farta district respectively by Hailu et al. (2009). Agar gel Immuno-diffusion test revealed the presence of antibodies against IBD in the serum of most recovered birds from IBD. Thus, it's of very importance design cost-efficient management ways against IBD so as to enhance the productivity and welfare of village chickens and additionally to conserve the indigenous chicken genetic resource (Hailu *et al*., 2009).

The study conducted in eight districts of Ethiopia showed that among the total of 2,597 chicken serum samples, 83.1% (2158/2597) positive for IBD examined using ELISA. Among the predisposing factors location, age, breed are influenced the occurrence IBD. The highest seroprevalence was recorded in Mekele (90.3%) while the lowest was recorded at Gondar

(69.8%). Moreover, higher seroprevalence was reported in crossbreed of chicken (91.4%) while the lowest was recorded in indigenous breed of chicken (81.4%). The production system can as well influence the occurrence of IBD (Shiferaw *et al*., 2012). Jenberie et al. (2013) stated that phylogenetically, Ethiopian infectious bursal disease virus characterizes two genetic lineages: very virulent infectious bursal disease virus or variants of the classical attenuated vaccine strain (D78). The nucleotide identity between Ethiopian vvIBDVs ranged between zero and 2.6%. Ethiopian vvIBDVs are clustered phylogenetically with the African IBDV genetic lineage, differently from the Asian/European lineage. This report demonstrates the circulation of vvIBDV in business and breeding poultry farms in Ethiopia. Besides, among all IBDV strains represented within the study for phylogenetic comparison of VP2 nucleotide sequences, Ethiopian strains type cluster inside the vvIBDV lineage. There was also shown that Ethiopian IBDV strains have mutations in the VP1 region. Similarly, Mekuriaw et al. (2017) has also reported sequence result which showed that the IBDV strain circulating in Ethiopia is the very virulent IBD virus (vvIBDV) and also isolates from the same area clustered together. On the other hand, Shegu et al. (2020) has reported vvIBDV which are more similar to European strains such as UK661 and DV86 than the vaccine strains used in Ethiopia. These reports could help to select the most appropriate vaccination program for the genomic sequences of field strains through diagnostic testing (Tamiru *et al*., 2012).

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

Study area	Prevalence	Authors
Gondar and west Gojjam	73.50%	Kassa and Molla, 2014
Southwest showa of Ethiopia	76.64%	Hailu et al., *2009*
Mekelle town	45.05%	Zegeye et al., 2015
Central Ethiopia	82.20%	Tesfaheywet and Getnet, 2012
Andassa poultry farm	98.90%	Solomon and Abebe, 2007
Eastern Ethiopia	83%	Tadesse and Jenbere, 2014
Debre Brehan	94.70%	AHY, 2011
Selected sites of Ethiopia	83.1%	Jenberie *et al*., 2012
Bahir Dar	29.4%	Mazengia *et al*., 2010
Farta	21.7%	Mazengia *et al*., 2010
Debre Zeit	93.3%	Zeleke *et al.*, 2005

Source: (Tulu, 2019; Teshager, 2015)

3. MATERIALS AND METHODS

3.1. Study areas

An outbreak based cross sectional study was conducted in Bishoftu and Modjo towns which are known to be potential sources of different poultry production systems with frequent IBD outbreaks. The area is located at 9°N latitude and 40°E longitudes at an altitude of 1850 meters in central high land of Ethiopia. Bishoftu is located at 47 km from capital city of the country South-East of Finfinne main asphalt road and 52 km from capital city of East Shewa zone Adama and surrounded by different peasant associations (PAs) (Figure 5). The highest wind speed is registered in March (2.24m/s) and the most common wind direction seen in the city is easterlies. In Bishoftu town there are nine (9) kebeles, peri-urban areas and many surrounding PAs. Bishoftu is a town where several commercial farms of different scale, hatcheries and breeding farms are found and from which pullets of various ages are distributed to different parts of the country. (http://www.mwud.gov.et/web/bishoftu/home). Modjo is located in the East Shewa zone of the Oromia region, Ethiopia. It is located at 66 km Southeast of Addis Ababa and lies at 8°35'N and 39°7'E at an altitude 1790 meters above sea level (Figure 5). The area gain rainfall twice a year those known as long and short season rainy season. The main rainy season extends from June to September. The average annual rainfall, temperature, and mean relative humidity are: 776mm, 19.4 ^{0}C and 59.9% respectively. In Modjo town, there are different poultry production systems (CSA, 2005).

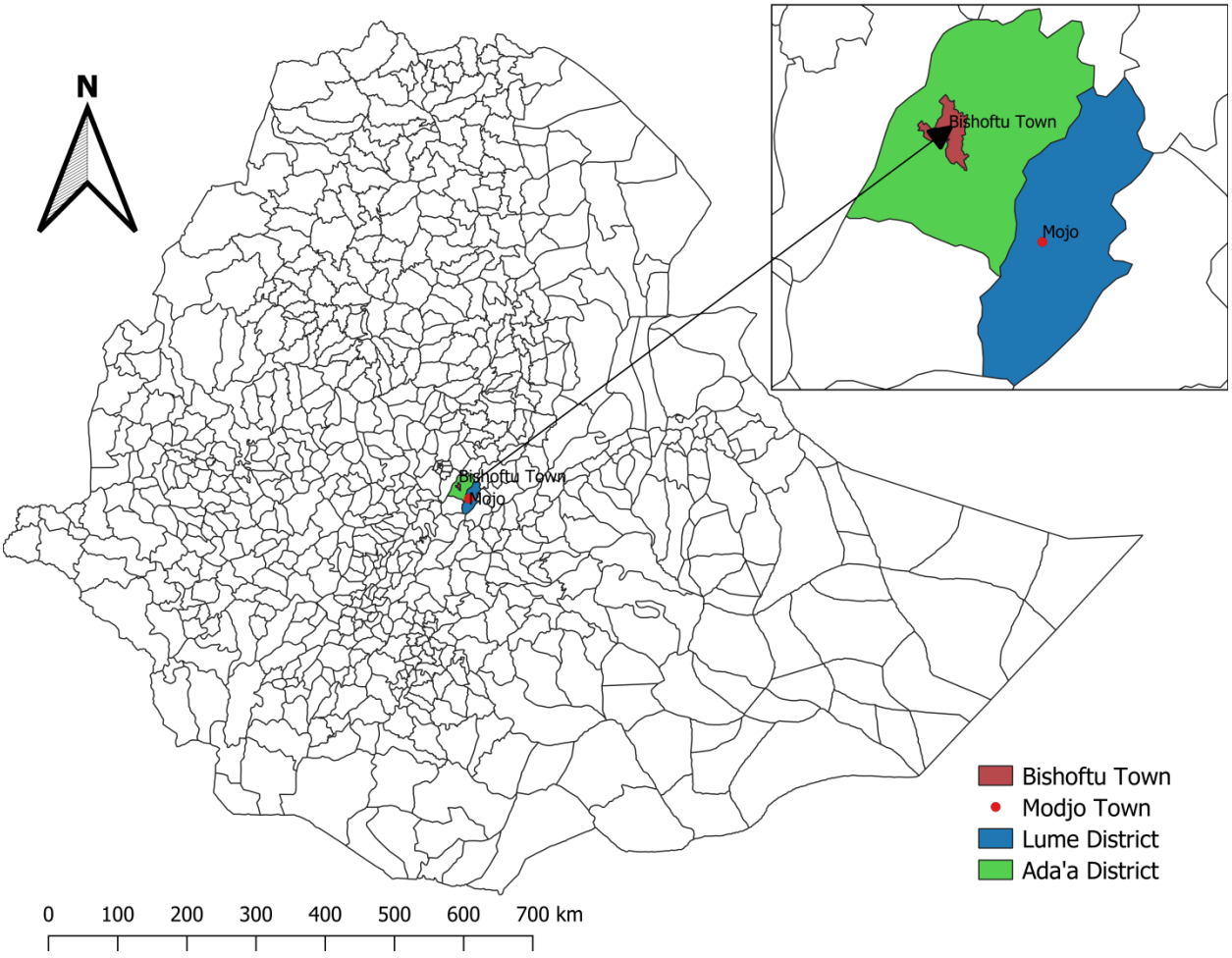


Figure 5: Map of the study areas where IBD outbreak samples were collected from clinically diseased chickens.

3.2. Study population

The study animals were lowman tradition, bovans brown, and cockreles breed type chickens; 28-70 days age and both sex groups kept under private ownership s smallholder production systems. Chickens which were diseased or dead recently were opened. Both broilers and layer production systems were included in the study.

3.3. Study design

An outbreak based cross-sectional study was carried out from October 2019 to May 2020 in Bishoftu and Modjo towns, Central Ethiopia, with the objective of outbreak investigation and molecular characterization of IBDV. The areas included in the study were visited according to the frequency of occurrence of outbreaks of IBD during the study period. In this study, outbreaks of IBD were investigated based on the information gathered from diagnostic laboratories of the National Veterinary Institute/NAHDIC, and private poultry practitioners. Outbreaks which occurred in private poultry producers mostly equivalent to small scale production systems were investigated. At least 4/5 chickens were taken per outbreak per farm (OIE, 2018) and the total number of samples in the study were determined based on the frequency of outbreaks reported.

3.4. Sample collection and laboratory investigation

3.4.1 Questionnaire survey

In order to collect useful information for each sample, questionnaire survey was implemented on each poultry farm owners and information includes farm category, location, contact with other farm (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 poultry farm and one questionnaire was administered per farm.

3.4.2 Clinical examinations (signs and necropsy findings)

Chickens were examined both individually and in a flock for the typical signs and pathlogical lesions of IBD (Gumboro disease). All clinical signs and necropsy findings which are indicators of IBD were recorded. A total of six farms were visited as per the reported outbreak and representative chickens which showed typical clinical signs of IBD were used for futher investigation of the disease.

3.4.3 Virus isolation

For virus detection, bursa samples (4/5 per outbreak per farm) were aseptically collected from suspected cases, placed into individual sterile universal bottles and transported under cold chain with viral transport medium to the virology laboratory of National Animal Health Diagnostic and Investigation Center (NAHDIC), Ethiopia. Then, samples were chopped into small pieces using a sterile scalpel blade, and minced using a mortar and pestle. A 10% suspension of each bursal samples were prepared in sterile phosphate buffer saline supplemented with penicillin and streptomycin (1000µg/ml each). The suspensions were transferred into sterile centrifuge tube and were centrifuged at 3000×g for 10min. The supernatants were harvested and filtered using 0.22µm milipore filters and and stored at -20oC before inoculation (OIE, 2018). Samples of the resulting suspensions were added to FTA card (Whatman) and sent to University of Padova (Italy) for molecular detection and sequencing) while the other suspensions were inoculated onto 80% confulent DF-1 cell for virus isolation in NAHDIC's virology laboratory.

An immortalized chicken embryo fibroblast cells (DF-1, passage 14) obtained from GD, Netherlands, were revived from liquid nitrogen and re-cultured in 25cm2 tissue culture flask. The confluent flask was then sub-cultured to multiple 25cm2 TC flasks and maintained in Dulbecco's modified Eagle"s medium (DMEM) supplemented with 10% fetal bovine serum at 37^{o}C in a humidified incubator at 5% CO2 (OIE 2016, ATCC 2018). Cultures were observed microscopically for up to seven days for the presence of cytopathic effect (CPE). After seven days, samples with no CPE were blindly passed further three times following two cycles of freeze-thawing. Samples showing no CPE after the third passage were considered negative.

3.4.4 Molecular characterization

3.4.4.1 RNA extraction and reverse transcription

The RNA extraction and reverse transcriptase PCR were done at Padova University , Department of Animal Medicine, Production and Health, Molecular biology Laboratory (Italy). Briefly, RNA was eluted from FTA card by placing a section of the filter card (approximately 0.5cm×0.5 cm) in 300µl of Elution buffer (Qiagen), vortexing and incubating on ice for approximately 15min. Subsequently, 140µl was further processed using the QIAGEN Viral RNA extraction kit

as outlined by the manufacturer. Complementary DNA was generated from RNA using the reverse transcriptase Revert Aid^{TM} (Fermentas).

Ribonucleic acid (RNA) was first incubated at 95 ^{0}C for 3min and placed on ice for at least 3min in the presence of the gene specific primer which binds nucleotides 1194–1213 of the positive strand of IBDV segment A (Bayliss *et al*., 1990), and 20% DMSO (Martin *et al*., 2007). RNA was reverse transcribed in a final volume of 20µl containing reaction buffer (Fermentas), 1mM of each dNTP (ThermoScientific), 20U RiboLock^{TM} RNase Inhibitor and 200 units RevertAid^{TM} Reverse transcriptase. Reverse transcription reactions were performed at 42 ^{o}C for 60min and the reverse transcriptase inactivated at 70 ^{o}C for 10min.

3.4.4.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplification of products intended for sequencing was carried out using a high fidelity DNA polymerase, Pfu DNA polymerase (Fermentas). A typical 25µl reaction contained Pfu Buffer with MgSO4 (Fermentas), 0.2m M dNTPs (ThermoScientific), 200nM of each primers: IBDV Forward-2: 5'-GCCCCAGAGTCTACACCAT-3', IBDV Reverse-5: 5'-CCCGGATTATGTCTTTGA-3' which binds nucleotide position 1194–1213 and 658–677 of IBDV segment A, respectively (Bayliss *et al*., 1990), 2.5units (U) Pfu DNA polymerase (Fermentas) and 2µl of cDNA template. PCR reactions were carried out for 1 cycle at 95 ◦C for 3min, 35 cycles at 95 ◦C for 30s, 60 ◦C for 30s, 72 ◦C for 1min and 1 cycle at 72 ◦C for 7min. The amplified 645 base pair product contained the VP2 hypervariable region coding sequence. Amplicons were separated from reaction components using the QIAGEN Gel extraction kit, with the concentration of DNA determined spectrophotometrically using a NanoDrop Spectrophometer 1000 (Thermo Scientific). Purified amplicons were sequenced using both primers by a commercial sequence provider (Macrogen) using the Big Dye terminator cycling (Applied Biosystems) condition and analysed by the automated sequencer ABI 3730XL (OIE, 2018).

3.4.4.3 Agarose gel electrophoresis of PCR products

The equipment and supplies necessary for conducting agarose gel electrophoresis were an electrophoresis chamber and power supply, gel casting trays, sample combs, electrophoresis buffer (Tris-EDTA 1% buffer), 6Xloading buffer, Gel red and transilluminator (an ultraviolet light box). An agarose powder was mixed with Tris-EDTA 1% buffer to make 1.5% concentration, and then heated in a microwave oven until completely melted. PCR product containing loading dye was mixed with gel red and molecular ladder was added in separate well. 4 µl gel red with loading dye was added into 20 µl PCR products and then 10 µl of each PCR products were loaded into separate well. 10 µl molecular marker (Ladder) was also loaded in the first lane. The lid and power leads were placed on the apparatus, and a current was applied.

The Electrophoresis was run for 1:20 hour at 120V. It was confirmed that whether the current was flowing by observing bubbles coming off the electrodes. RNA was migrated towards the positive electrode which was colored red. The amplified fragment (amplicon) was visualized on 1.5% agarose and compared with the band of the molecular marker after the gel was placed on an ultraviolet transilluminator. A 100 bp DNA ladder marker was used and the PCR result was around 645bp positive for IBDV. The PCR products band was visualized by gel documentation under UV-lamp camera and the size of the PCR products was estimated by comparing with the band size of the molecular marker 1Kb ladder (marker) that was loaded on a separate lane (OIE, 2012). The result of gel picture captured by the camera was saved and printed out for documentation (Figure 8).

3.4.4.4 Sequence and Phylogenetic analysis

After amplification, Sanger sequencing was performed on the positive samples using both primers, and then a longer contig sequence was obtained by overlapping the respective sequence reads in ChromasPro (after trimming and quality check, performed with FinchTV). After that, the reference strains required for the classification by Jackwood et al. (2018), along with several other vaccine strains for a better characterization inside lineage G1, and performed a MUSCLE alignment in MEGA X.

3.5. Ethical statement

Ethical clearance for this research was obtained from Addis Ababa University College of Veterinary Medicine and Agriculture Animal Research Ethical Review Committee, and all animal work was conducted according to animal research ethics (Annex II).

3.6. Limitations of the study

Outbreak follow-up (field investigation) was challenging as the cases were not reported on time (delayed report of the case, and sometimes ignorance), that is why only one outbreak sample was processed from Modjo town. Besides, the COVID-19 pandemic has also its own impact in following up the outbreaks for remaining of the study periods and collecting additional samples, plus on time reporting of the outcomes.

4. RESULTS

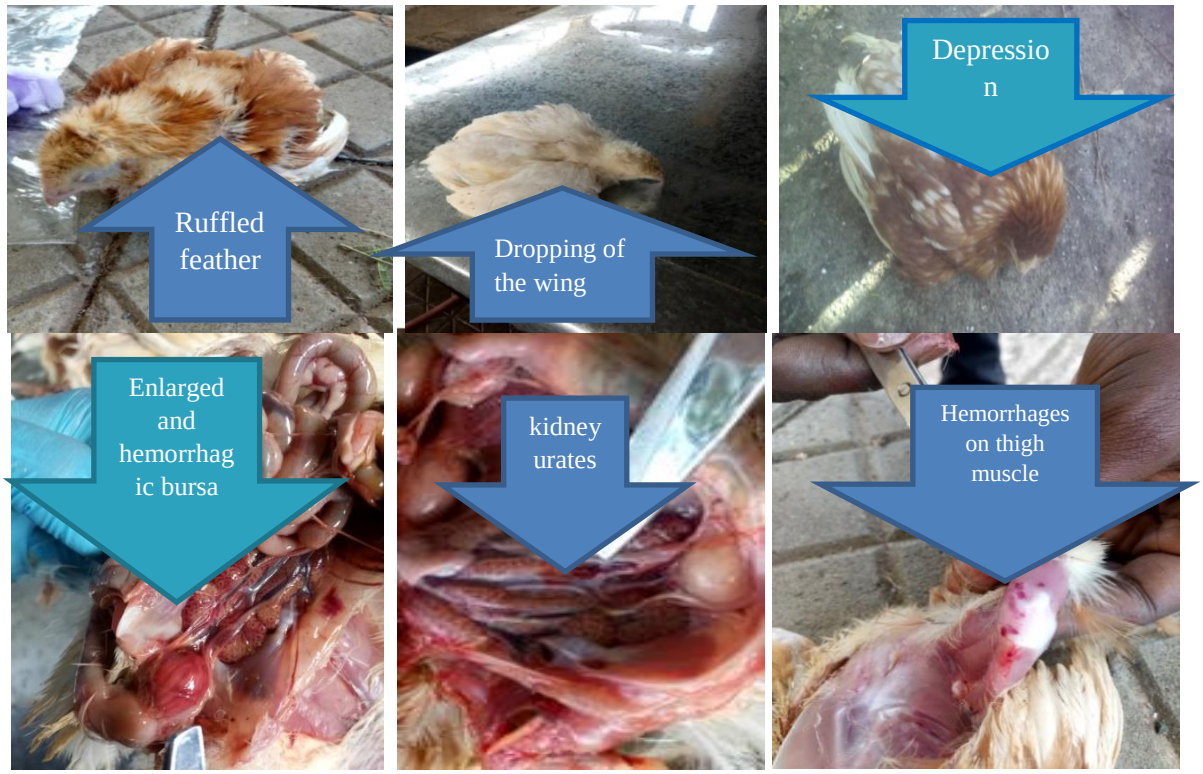
4.1. Questionnaire survey

The questionnaire surveys made in order to collect useful information for each sample per farm were useful in that they have differences among the visited farms. The outbreak has occurred both in layer and broiler farms, the latter constituting the larger percentage (over 60%). From a total of six farms visited with reported outbreaks, five confirmed IBD cases were seen. Except the source of the chicks, all visited farms use their own equipment and other usable materials. Major pathological lesions, morbidity and rate of mortality seen are mostly indicators of IBD. The main variation seen during the survey was the schedule of vaccination though most farms' follow the same program as their origin of chick is similar. For instance, one farm had vaccination history with the following schedule: Marek disease vaccination at day-one, ND at day-two, twelve, and thirty five, Gumboro at day-seven and seventeen, and FT and FP at day 42 whereas the other farm with different chick source had this one: HVT (IBD + MD) vaccination at day-one, ND Lasota at day ten, Gumboro at day-21 (Annex II). So, these differences in vaccination program might have their own contributions in the outbreak of IBD in the study areas as more mortality has been observed in the latter farm.

4.2. Results of clinical and necropsy examinations

A total of 15,000 chickens reared on six (6) farms were affected by the outbreak in the areas visited during the study period. Almost all farms have similar flock sizes as they are smallholder poultry rearing farms. Among this, 50% (7500) of the flocks were at risk of being infected with IBD. Of these, 3000 (20%) of them were affected with IBD. The clinical signs observed in affected chicken were typical of IBD such as emaciation, ruffled feather, dropping of the wing, depression (Figure 6A), anorexia, watery diarrhea and discharge and massive death within short period of time. Four to five chickens were opened per farm during the outbreak time and more than 90% of the birds have shown gross lesions indicative of IBD such as enlarged and hemorrhagic bursa, haemorrhage on thigh muscle, and kidney urates (Figure 6B).

(A)



(B)

Figure 6: Different age groups and breeds of chickens with ruffled feathers, dropping of the wing, and depression (A); Enlarged and hemorrhagic bursa, hemorrhages on thigh muscles, and kidney urates (B)

4.3. Virus isolation

Of 8 (eight) pooled bursal suspensions inoculated onto DF-1 cell lines for isolation of IBD virus, 6 (six) of them have shown characteristic cytopathic effect (CPE) starting on day two post infection after second passage. The viral growth was indicated by cell swelling, cell rounding, detachment and floating (Figure 7).

Figure 7: IBDV growth on DF-1 cells: Unaffected DF-1 cell line (A), cell swelling and rounding on dpi 2 (B), cell rounding, detachment and floating on dpi 4 (C) dpi 5 (D).

4.4. Molecular characterization of the isolates

The smeared samples on FTA cards (from bursal suspensions and from original bursa) were used for the molecular characterizations of the IBDV samples.

4.4.1 Polymerase chain reaction (PCR) results

From a total of 8 (eight) pooled viral suspensions isolated in cell culture and 6 (six) original bursal samples that were smeared on FTA cards were analyzed using Reverse-transcriptase polymerase chain reaction (RT-PCR), of which 11 (78.57%) yielded positive results showing the required amplicon for IBDV (Figure 8).

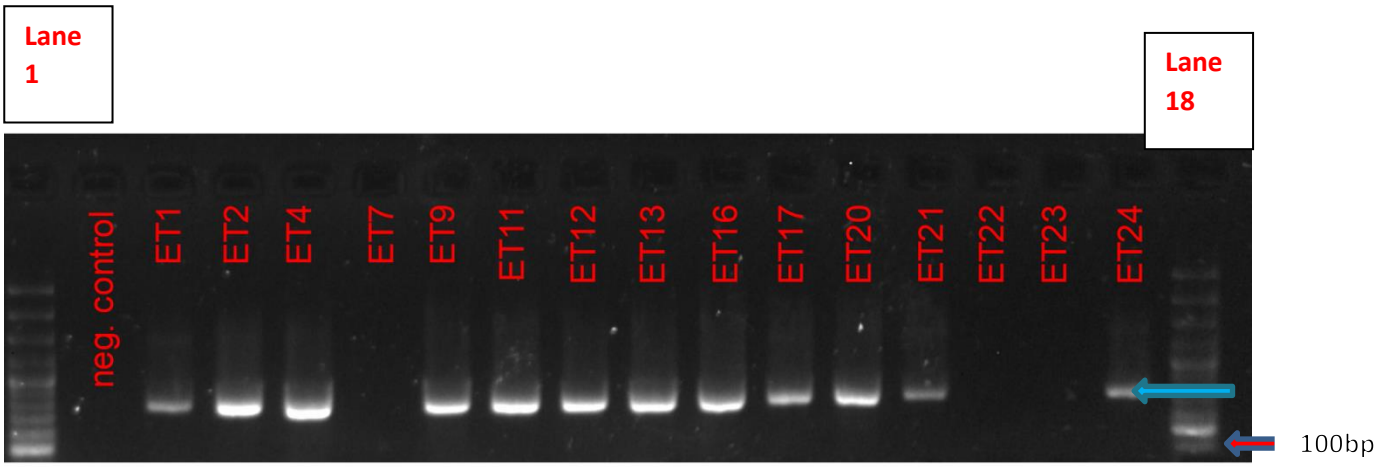


Figure 8: Gel electrophoresis of RT-PCR showing the amplification of the VP2 gene of IBDV, Where, Lane 1 and Lane 18: 1000bp DNA ladders, ET-1, 2, 4, 9, 11, 12, 13, 16, 17, 20, & 21 are positive isolates from field outbreaks, ET-7, 22, & 23 are samples which are found negative, and ET-24 is Positive control-NVI Vaccine seed D78 strain- Positive around 645bp (blue arrow).

4.4.2 Sequencing and phylogenetic analysis

Among the 11 isolates detected using the Sanger sequencing, 6 (six) of them were vaccinal strains belonging to the IBDV G1 while 5 (five) of them were wild (vvIBDV) strains belonging to the IBDV G3 (Table 4).

Phylogenetic tree was obtained with the Neighbor Joining method adopting pairwise deletion of missing sites to cope with the different sequence lengths (Figure 9). Both the vaccinal and field strains of IBDV isolates in the current study are indicated by black dot on the phylogenetic tree. The current isolates made three clusters as depicted in figure 9. One of the clusters was made of isolates identical to vaccinal strains ((Winterfield 2512-like) - CEVAC® IBD L)) and (Faragher 52/70 used in HVT+IBD vaccines). The second cluster was made of wild strains identical to very virulent strains deposited in the Genbank. The third cluster was made of isolates identical to vaccine strain manufactured and marketed by National Veterinary Institute, Ethiopia.

Table 3: Results of RT-PCR based sequence analysis of the IBDV isolates obtained from tissue samples collected from commercial farms in Bishoftu and Modjo towns

Sample codes	Tissue sampled	IBDV RT-PCR	Similarity with vaccine or wild strains
ET1	Bursa	IBDV Genogroup 1	(Winterfield 2512-like) - CEVAC® IBD L
ET2 and ET21	Bursa	IBDV Genogroup 3	(very virulent strain)
ET7	Bursa	Negative	-
ET9, ET12 and ET13	Bursa	IBDV Genogroup 1	(Winterfield 2512-like) - CEVAC® IBD L
ET11, ET16 and ET17	Bursa	IBDV Genogroup 3	(very virulent strain)
ET20	Bursa	IBDV Genogroup 1	Identical to IBD NVI vaccine
ET22	Bursa	Negative	-
ET23	Bursa	Negative	-
ET24	IBD NVI vaccine strain		

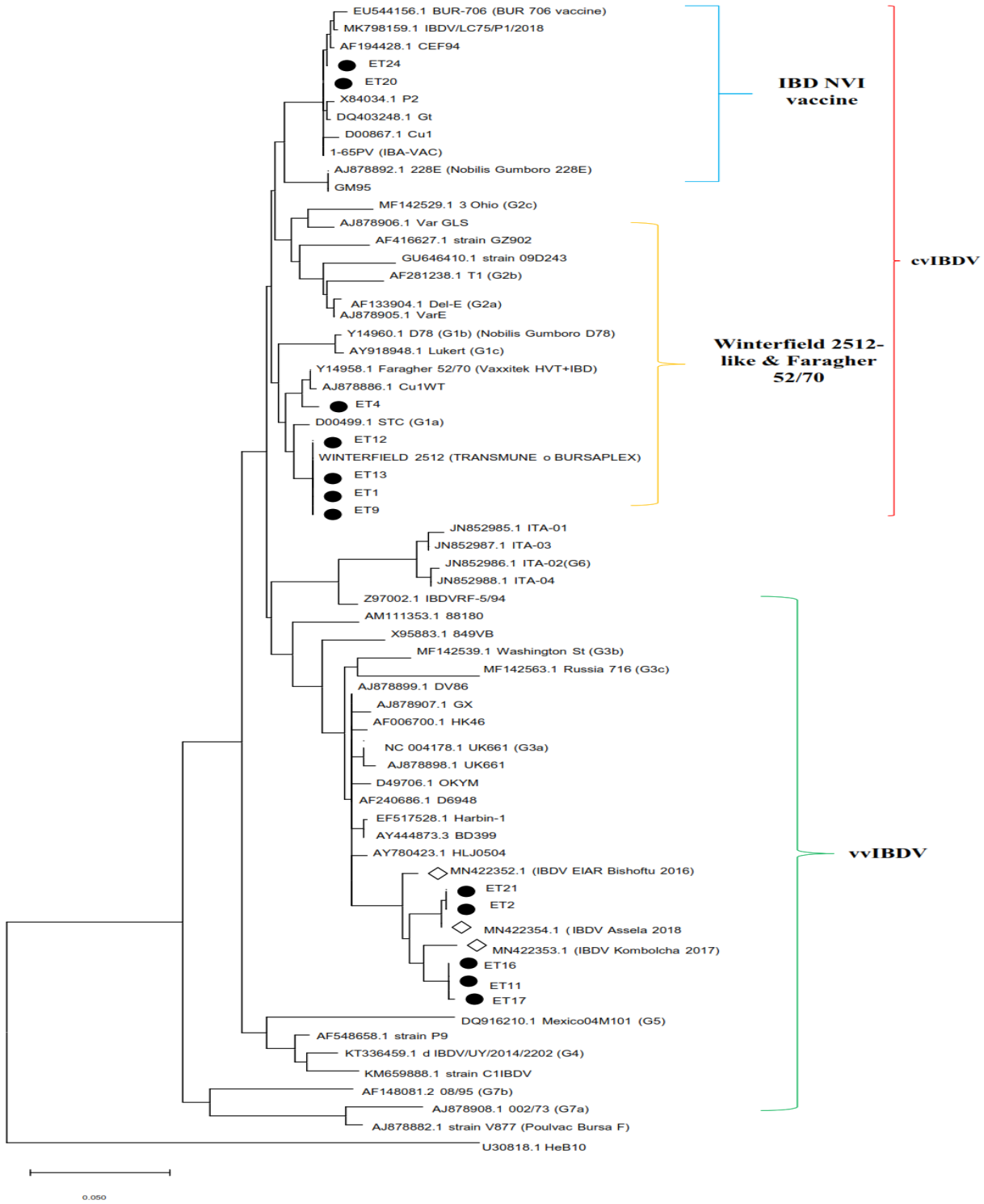


Figure 9: Phylogenetic tree analysis of the VP2 hypervariable coding sequence of IBDV field isolates, classical/attenuated vaccine strains and reference sequences of classical and very virulent strains retrieved from GenBank. The analysis involved 64 nucleotide sequences The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. This analysis involved 31 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 421 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

5. DISCUSSION

Poultry productions in Ethiopia are expanding rapidly in the recent years. Several reasons can be pointed out for this including its nature of easy manipulation/operation, quick response (benefit) gained, reasonable profit and others. In comparable with its benefit, the sector is vulnerable to diseases of different origins especially of viral origins. IBDV reassortment and recombination are responsible for the wild strains evolution, whereas vaccine types used and related practices are contributing for vaccine reversion. In this study, the main aim, which was characterizing wild strains of IBDV virus was successfully done from the outbreak cases during the study time.

It has been noted that, an interesting findings was the detection of both field and vaccinal strains of IBDV from outbreak cases up on sequencing of VP2 region. With regard to the production type, the disease is more common in broilers than layers implying the age vulnerability of the broilers. As the source of the chickens is different, this has contributed for the various vaccines type usage, administration and management practices which aided for the emergence of wild strains and vaccinal strains reversion. Apart from this, it was also noted that IBDV have shown their typical CPEs on DF-1 cell lines which is supportive of the virus presence. Moreover, both vaccinal and field strains of IBDV have been detected from outbreak samples. This indicates that different strains of the virus are circulating the poultry production systems in the study areas. Especially, the presence of vaccinal strains currently in use the country is a new discovery indicating the reversion of vaccines.

The findings of the current study are unique in their nature that they are combinations of both very virulent and classical virulent strains. This is because the reporting of vaccinal strains from outbreak is for the first time in Ethiopia, to our knowledge, although the circulation of vvIBDV has been reported (Shegu *et al*., 2020; Mekuriaw *et al*., 2017). Ethiopian poultry industry uses IBD vaccines from two sources: NVI (from Ethiopia) and CEVA santé animale (from France). Which type of vaccines is used intensively depends on the chicken origins from which the companies or private rears are using. For instance, producers which use Alemma's chicken use mostly CEVA based vaccines, whereas those using from Ethio-chicken implement mixed vaccine type and vaccination strategies (NVI and CEVA vaccines) according to the customers need.

The detection of vaccinal strains from outbreak cases is a clear indication that the currently used vaccines are being reverted to their virulence/pathogenicity. This finding is an indication for revision of our live virus importation policy as it is reverting. Apart from the vaccinal strains, vvIBDV is still the major factor for the occurrence of IBD outbreak in the poulty rearing areas of the country especially Central Ethiopia which alerts for continuous molecular characterizations of the wild viruses and their matching with the currently used vaccinal strains.

From the investigated outbreaks, farms which CEVA based vaccines have shown critical revert in four out of six vaccinal strains detected whereas those of mixed users are with less reversion percentage, only one. Another interesting finding is that, vectorized vaccine, marketed by Merial /Boehringer Ingelheim has also shown reversion. Referrring to their last date of vaccination and the occurrence of IBD, there is at least 14 days interval which implies that the administered vaccines were unable to generate ample immune response. From these indicators, it is necessary to re-consider the safety and efficacy (importation) of live-virus/vaccine for extensive usage of vaccinations in poultry productions in Ethiopia especially smallholder ones where the vaccination and management practices are not intensive. Besides, the cause of their reversion has to be investigated from different aspects so as to safeguard the industry.

The current finding, especially the detection of vaccinal strains in the outbreak cases, is in line with the studies conducted in different parts of the world: Jemberie et al. (2014) who found two genetic lineages: very virulent (vv) IBDVs or variants of the classical attenuated vaccine strain (D78) representing Ethiopian IBDVs, Sara et al. (2014) from Egypt has identified classical IBDV similar to vaccine strains, Yilmaz et al. (2019) with classical and vvIBDV strains from Turkey, Dobrosavljevi et al. (2014) who detected vaccinal virus after 14 dpv in Serbia, Camila et al. (2009) from Brazil showed clustering of newly IBDV isolates with classical IBDV isolates used in attenuated vaccines, and Ali et al. (2019) from Egypt has identified isolates belonging to geno-group 1 according to newly proposed model of classification of IBDV into geno-groups (Michel and Jackwood, 2017). From these reports, it is possible to understand that vaccine viruses are being reported worldwide along with the vvIBDV recalling for the rapid design of vaccines and vaccinations strategies for proper control and prevention of this contagious disease which is headache for poultry industry.

Apart from the vaccine strains, vvIBDV is also continuously circulating in the country as this current detection being the third in characterizing it from Ethiopian field isolates. This sustained circulation of vvIBDV in Ethiopian chicken population and responsible for the frequently occurring outbreaks of IBD. Hence, continuous molecular characterizations of the hypervariable region of VP2 of the virus, which is responsible for controlling antigenicity and host-cell attachment (Brandt *et al*., 2001; Letzel *et al*., 2007) is necessary. In addition, there should be uniformity in vaccines type used and the management techniques implemented. This is impotant to overcome the pressure and effect of the disease in optimizing the vaccines used and their mode of delivery as one factor in addition to the chick breeds and viral strain diversity.

As to know and provide updated information about this contagious disease of chickens, IBD, this current study has shown important reminders to the concerned stake holders on the status of the disease in the study areas whare outbreak occurred. Hence, collaborative effort is needed to do continuous surveliance and characterizations of field isolates of IBDV, and to monitor the efficacy of the different vaccine types in use and also to re-consider their modes of delivery.

6. CONCLUSION AND RECOMMENDATIONS

To come up with the circulating strains of infectious bursal disease virus in poultry productions, it is important to apply molecular techniques. The current research aimed to characterize the wild isolates of IBDV from field outbreaks and explore their genetic relationship with strains deposited in the Gene Bank. Based on using reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing of VP2 gene, it was noted that both vaccinal and vvIBDV strains were detected. The results indicate that, in addition to the continuous circulation of vvIBDV strains in the country, vaccinal strains are also reverting to their virulence being one of the causes for the investigated outbreaks. This is an indication that currently used vaccines are not on the right track of appropriate usage, hence corrective measures should be taken.

Therefore, based on the above conclusion the following recommendations are forwarded:

- The efficacy and virulence of vaccines currently on market should be assessed and the cause of the reversion needs to be investigated.
- Field isolate-based vaccine candidates should be designed so as to tackle the circulation of the non vaccinal IBDV strains.
- Further and sustained molecular characterization of the vaccinal and field IBDV strains is crucial as the virus is resistant and prone to change in its nucleotide sequences.

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Website sources:

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8. APPENDIX

Annex I: Questionnaire sample collection format

Name, address and contacts									
Sample ID-Code									
Matrix (rhinopharyngeal, tracheal, cloacal swabs, FTA card, bursa)									
Collection date									
Farm / Shed / Flock									
Location (Address, City, Country)									
Origin of the chicks									
N° of sheds / N° of birds									
Species									
Production type / Sex (Broilers, Breeders, Layers)									
Genetic type (i.g. ROSS, Cobb, local breed)									
Age at sampling									
Vaccination protocol	Age	Vaccination	Route	Age	Vaccination	Route	Age	Vaccination	Route
Contact with other farms (trucks, Feed mill trucks, sharing equipment and personnel, others…)									
Integrated company or Owner? Please indicate also the name									
Clinical signs									
Lesions									
Morbidity									
Mortality									
Zootechnical parameters (e.g. mean weight, f.c.r)									
Treatment applied (i.e. antibiotics)									

Annex II: Summary of the results of questionnaire survey

Sample codes	Location	Origin of the chicks	N° of birds in a flock	Prodn type	Genetic type (breed)	Age at sampling	Vaccination protocol
ET1and ET20	Bishoftu	Alema	3000	Layer	Lowman tradition	10 weeks	Age-D1 (day-1), Vaccination-MD (Marek disease), Route-SC (subcutaneous); Age-D2, Vaccination-ND (Newcastle disease) NB1 Route-ED (Eye drop); Age-D7, Vaccination-Gomboro, Route-DW; Age-D12, Vaccination-ND LaSota, Route-DW; Age-D17, Vaccination-Gumboro, Route-DW; Age-D35, Vaccination-ND, Route-DW; Age-D42, Vaccination-FT (Fowl thyphoid), Route-SC; Age-42, Vaccination-FP (Fowl pox), Route-Wing web
ET2 and ET21	Bishoftu	Ethio-chicken	3000	Broiler	Bovans brown	4 weeks	Age-D1, Vaccination-HVT (IBD + MD), Route- SC; Age-D10, Vaccination-Gumboro, Route-DW,
ET4 and ET22	Bishoftu	Ethio-chicken	4000	Layer	Bovans brown	4 weeks	Age-D1, Vaccination-HVT (IBD + MD), Route- SC; Age-D1, Vaccination-HB1 ND, Route- Spray; Age-D14, Vaccination-Lasota ND, Route-DW; Age-D17, Vaccination-Gumboro, Route- DW
ET7 and ET23	Mojo	Ethio-chicken	3000	Broiler	Bovans brown	7 weeks	Age-D1, Vaccination-HVT (IBD + MD), Route- SC; Age-D10, Vaccination-ND Lasota, Route-DW; Age-D21, Vaccination-Gumboro, Route-DW
ET9, ET12, and ET13	Bishoftu	Alemma	2000	Broiler	Bovans white-cock	4 weeks	Age-D1, Vaccination-VECTORMUNE ND, Route- SC; Age-D2, Vaccination-ND HB1, Route-ED; Age-D7, Vaccination-Gumboro (IBD Intermediate), Route-DW; Age-D14, Vaccination-ND LASOTA, Route-DW; Age-D18, Vaccination-IBD Intermediate plus, Route-DW
ET11, ET16, and ET17	Bishoftu	Alemma	3000	Broiler	Bovans white-cock	5 weeks	Age-D1, Vaccination-VECTORMUNE ND, Route- SC; Age-D2, Vaccination-ND HB1, Route-ED; Age-D7, Vaccination-Gumboro (IBD Intermediate), Route-DW; Age-D14, Vaccination-ND LASOTA, Route-DW; Age-D18, Vaccination-IBD Intermediate plus, Route-DW

Annex III: Ethical clearance sheet

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/01/01/12/2020

Name of Applicant: Debebe Ashenafi (DVM, MVSc fellow)

Address: College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: O*utbreak investigation and genetic characterization of infectious bursal disease virus in Central Ethiopia*

Date of application:	**16/10/2019**
Nature of the project:	**mildly invasive**
Target animal species:	**Poultry**
Number of animals involved:	**depends on number of outbreaks**
Study area:	**Central Ethiopia**

Minutes No. and date of review: **VM/ERC/01/12/020, 03/01/2020**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when this is deemed necessary

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

Dr Getachew Terefe
Chairman

Signature

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Please quote Our Ref. No. When replying

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Fax 251-11-4339933 Tel. +251 114338450 P.o.x. Box}34 Bishoftu/Debre Zeit, Ethiopia

Annex IV: Different pictures taken from field and at postmortem

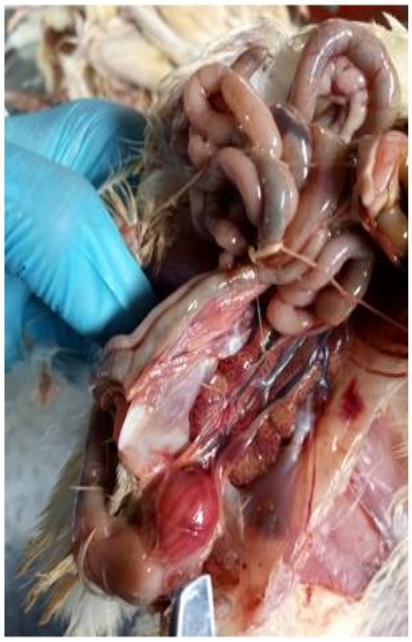
Housing style of one sampled farm…around Kajima

Typical clinical signs

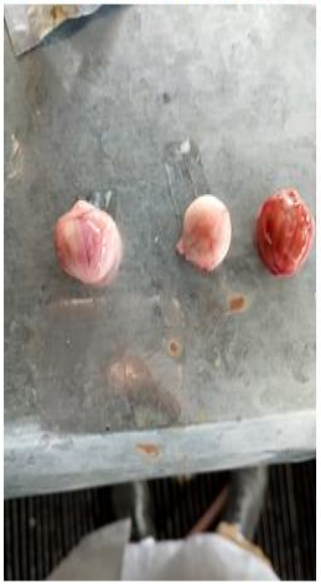
Pre-PM examination@PM room of CVMA

Enlarged Bursa insitu

Visible urated kidney

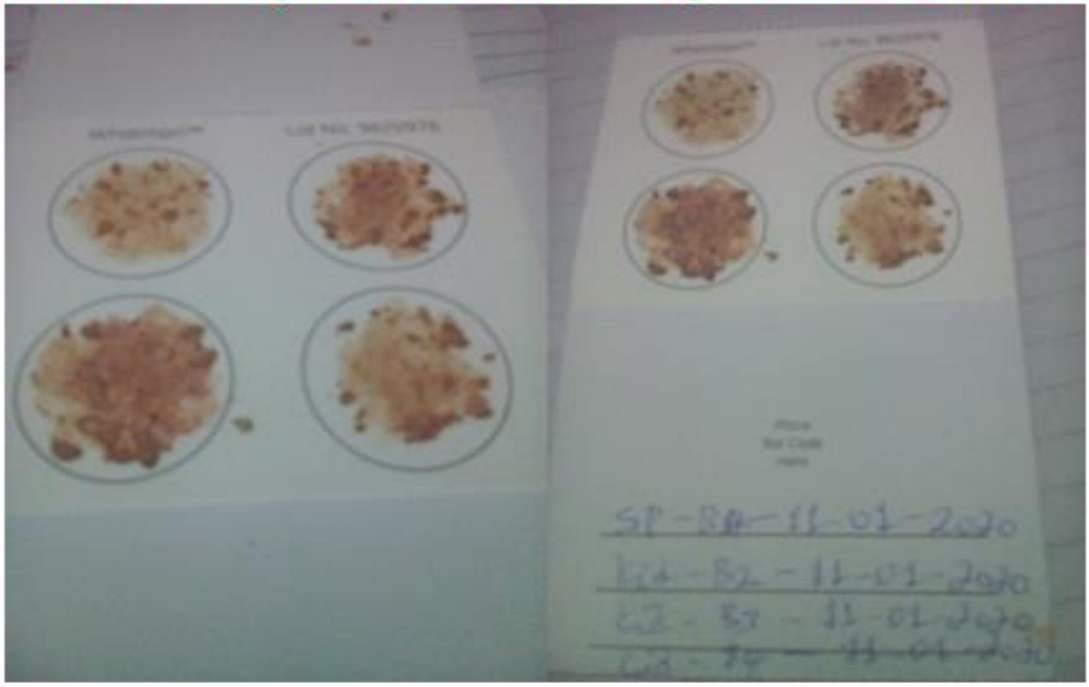


Bursa of different size and lesion types from different birds…observed during necropsy



Hemorrhagic Bursa and urated kidney

Smeared samples of different organs on FTA cards



Annex V: Formulation of growth and maintenance Cell Culture Medium

Base cell culture medium preparation

Ingredients	**Amounts**
GMEM (MEM) (powdered)	12.5 g
NaHCO3	2.75 g
H2O (bi distilled)	1000ml

Dilute and adjust pH to 7.35 to 7.4 by adding NaHCO3 solution.

Sterilized by filtration with a filter pad 0.22 µl pore size in to sterile bottles, place at +4oC

Preparation of maintenance cell culture medium

Base GMEM medium	800ml
Tryptose phosphate broth	100ml
Gamma irradiated Calf serum	20ml
Antibiotic	1ml/lit

Annex VI: Preparation of Balanced Salt Solution (PBSA)

Phosphate buffer saline (PBSA) without calcium and magnesium

Sodium Phosphate Dibasic (Na2HPO4)	1.60 gms
Potassium Phosphate (KH2PO4)	0.51 gms
Sodium Chloride (NaCl)	7.03 gms
Double distilled water	1 liter

Diluted completely and Sterilized by autoclave 121 oc for 15 minutes

Annex VII: Preparation Enzyme solutions for cell culture

Trypsin Solution (0.25%).

Ingredient	Amounts
NaCl	8.0 g
KCl	0.4 g
Glucose	1.0 ml

Phenol Red (0.5% solution)	1.0 ml
Trypsin (1:250)	2.5 g
NaHCO3	0.35 g
Purified H20	1 liter

Adjust pH to 7.4 with NaHCO3 solution. Sterilized by filtration

EDTA (sucrose, Phosphate, Glutamate and albumin (ethylenediamine tetra acetate acid) buffer preparation

Ingredients	Concentration	Amount
Sucrose	0.218M	7.462 gm
Mono potassium phosphate	0.0038M	0.052gm
Di potassium Phosphate	0.0072M	0.125gm
L-monosodium glutamate	0.0049M	0.083gm
Bovine Albumin powder	1.0%	1.0gm
EDTA	0.2%	0.2gm

Distilled water 1000ml_mixed thoroughly adjust PH at 6.5 and sterilized by filtration

Trypsin version working solution preparation (0.05%)

Sterile Phosphate buffer saline (PBSA)	970 ml
Trypsin 0.25%	20 ml
EDTA solution	10 ml

Note: Test its sterility and used for cell propagation and chicken fibroblast cell preparation.

Annex VIII: Reviving DF-1 cells from liquid nitrogen storage

o Collect all the materials required, prepare the medium and label the culture flask

o Retrieve the cryovial containing the frozen cells from liquid nitrogen storage and check the label that it is the correct one, then immediately place it into a 37°C water bath.

o Avoid getting water up to the cup as this will increase the chance of contamination.

o Then swab the cryovial thoroughly with 70% alcohol, and open it in a bio safety cabinet.

o Transfer the thawed cells drop wise into the centrifuge tube containing the desired amount of pre-warmed complete growth medium appropriate for your cell line

o Centrifuge the cell suspension at approximately 1500 rpm for 10 minutes. After the centrifugation, check the clarity of supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.

o Gently reconstitute the cells in complete growth medium, transfer them into labeled culture flask and incubate at 370C with 5% CO2.

ANNEX IX: Sub-culturing of DF-1 mono layers

o Prepare the hood, bring all the reagents and materials required to the hood

o Examine the cultures carefully for signs of deterioration or contamination

o Take the culture flasks to a sterile work area, remove and discard the medium

o Add PBS prewash (0.2ml/cm2) to the side of the flasks opposite the cells so as to avoid dislodging cells, rinse the prewash over the cells, and discard. This step is designed to remove traces of serum that would inhibit the action of trypsin.

o Add trypsin (0.1ml/cm2) to the side of the flasks opposite the cells. Turn the flasks over and lay them down. Ensure that the monolayer is completely covered. Leave the flasks stationary for 15-30seconds.

o Raise the flasks to remove the trypsin from the monolayer and quickly check that the monolayer is not detaching. Withdraw all but a few drops of the trypsin.

o Incubate, with the flasks lying flat, until the cells round up; when the bottle is tilted, the monolayer should slide down the surface.

o Do not leave the flasks longer than necessary, but on the other hand, do not force the cells to detach before they are ready to do so, or else clumping may result.

o Add medium (0.1-0.2ml/cm2), and dispense the cells by repeated pipetting over the surface bearing the monolayer. Finally, pipette the cell suspension up and down a few times, with the tip of the pipette resting on the bottom corner of bottle, taking care not to create foam. A single-cell suspension is desirable at subculture to ensure an accurate cell count and uniform growth on reseeding.

o Count the cells or follow the splitting ratio

o Dilute the cell suspensions to the appropriate seeding concentration and distribute among flasks.

o Incubate in 5% CO_2 at 37^0C.

NOTE: Maintenance media 2% calf serum.
Check plates daily for cytopathic effect (CPE) and condition of cells.
To harvest samples, freeze-thaw 2-3 times and collect.

ANNEX X: Sample preparation and inoculation on mono layer cells

o Tissues are removed from the buffered glycerin or VTM (virus transport media)

o Put in a mortar and wash several times with PBS containing antibiotics and antimycotic

o by using coarse sterile sand, triturate thoroughly and a 10 % suspension is made in PBS with antibiotics and antimycotic
o the suspension is free thawed three times to facilitate the release of viruses from the tissues

o centrifuge at 3000 rpm for 10 minutes and the supernatant is collected and filtered using 0.22µ pore size filter before inoculation

ANNEX XI: RNA extraction

o make all the necessary reagents and consumables ready in and around the work station

o add 350µl of cell suspension to labeled PCR tube and keep the remaining original sample in deep freezer

o add 350µl lysing buffer, vortex and centrifuge for 3min at 12500rpm

o add 350µl ethanol, mix gently with pipette and centrifuge for 1min at 12500rpm

o transfer the lysate to a labeled RNeasy mini spin column containing 2ml collection tube, centrifuge for 1min at 12500rpm (washed contaminant) , discard the tube containing the sediment and replace with a new 2ml collection tube

o add 700µl washing buffer AW1, centrifuge for 1min at 12500rpm and discard the flow through

o add 500µl washing buffer AW2, centrifuge for 1min at 12500rpm and discard the flow through

o add 500µl washing buffer AW2 again, centrifuge for 1min at 12500rpm discard the flow through and spin to dry for 2min; then remove the collection tube from the mini span and replace with the labeled eppendorf tubes

o add 50µl RNase-free water directly to the spin column membrane, centrifuge for 3min at 13400rpm, discard the mini span column and eluted RNA is finally harvested by Eppendorf tubes. Keep it at 40C until cDNA synthesis.

Annex XII. Test procedure for RT-PCR

cDNA synthesis for one reaction (1X)
RNAse free water 3 µl
10mM dNTPs mix 1 µl
IBD Specific primer 1 µl for each (IBD3 forward and IBD3 reverse

- Mix by votexing and dispense 5 µl mixtures in to PCR tube and add 5 µl of extracted RNA sample and mix by votexing and then incubate for 56^{o}C for s minute

Prepare cDNA synthesis mixture
10X RT buffer 2 µl
25 mM MgCl2 4 µl
0.1MDTT 2 µl
RNASe out 1 µl

RT enzyme (Taq polymerase) 1 µl

- Dispense 10 µl mixture to each tube incubate at 42^{o}C for 5 minute and add 1 µl RNase H in to each PCR tube incubate at 37^{o}C for 20 min chill on ice.
- Finally preserve cDNA product at -20^{o}C, or run PCR amplification as follows.

- Add 3µl template DNA (cDNA) to the master mix and run the PCR with the following program:

Process	**Temperature**	**Time**	**cycle**
Initial denaturation	95^{o}C	5min	1 cycle
1st denaturation	95 ^{o}C	30sec	15 cycles
Annealing	60 ^{o}C	30sec	
Elongation	72 ^{o}C	30sec	
2nd denaturation	95 ^{o}C	30sec	20 cycles
Annealing	56 ^{o}C	30sec	
Elongation	72 ^{o}C	30sec	
Final Elongation	72 ^{o}C	7min	1 cycle
Put at	4 ^{o}C	Till machine off	

Annex XIII. Agarose Gel Electrophoresis

- Prepare 60 ml of 1.5% agarose in 1X TBE buffer.
- Either heat in microwave for approximately 2min or boiling water until melt it.
- Allow to cool to about 45oc and add 2.5µl /red gel.
- Pour gel and insert well former (comb). Allow o set on a flat surface for about 15 min.
- Pour puffer 1X TBE in to tank and remove the comb from gel.
- Prepare samples in to tubes, multichannel pipette or on paraffin

Ingredints:

1 µl loading buffer

5 µl PCR product

Prepare molecular weight marker

0.5 µl ml molecular weight marker VI (Bioehringer)

1 µl loading buffer

4.5 µl H2O

- Load samples in to the wells formed in the gel. It is often use full to load the molecular weight markers in both the first and last lanes
- Electrophoresis at 120 volt for 50 minutes
- View the photograph the gel on an UV-trans illuminator. Use UV safety spectacle.