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MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS FIELD ISOLATES FROM SELECTED AREAS OF ETHIOPIA AND SEQUENCE BASED COMPARISON WITH CURRENTLY USED VACCINE STRAINS

MVSc THESIS

BY

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DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH MVSc PROGRAM IN VETERINARY MICROBIOLOGY

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BISHOFTU, ETHIOPIA

MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS FIELD ISOLATES FROM SELECTED AREAS OF ETHIOPIA AND SEQUENCE BASED COMPARISON WITH CURRENTLY USED VACCINE STRAINS

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiology

By

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Bishoftu, Ethiopia

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Title: Molecular characterization of infectious bursal disease virus field isolates from selected areas of Ethiopia and sequence based comparison with currently used vaccine strains

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First, I declare that this thesis is my *bona fide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/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 the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AC-ELISA	antigen capture Enzyme Linked Immunosorbent Assay
AGID	Agar gel immuno-diffusion
avIBDV	antigenic variant Infectious Bursal Disease Virus
BF	Bursa of fabricius
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CPE	cytopathic effect
cvIBDV	classical virulent Infectious Bursal Disease Virus
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization of the United Nations
FRET	fluorescence resonance energy transfer
IBA	infectious bronchitis virus
IBD	infectious bursal disease
IBDV	infectious bursal disease virus
IBV	infectious bursal agent
IgY	immunoglobulin Y
MAb	monoclonal antibody
OD	optical density
OIE	organization des epizootics
PBS	phosphate buffered saline
PCR	polymerase chain reaction

RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
rt-PCR	real time polymerase chain reaction
SAN	specific antibody negative
SPF	specific pathogen free
$TCID_{50}$	tissue culture infective dose 50
UV	ultra violet
VN	virus neutralization
vvIBDV	very virulent infectious bursal disease virus
LMP	livestock master plan
VP2	viral protein 2
CSA	central statistical agency
NMSA	national meteorological services agency
ARARI	amhara regional agricultural research institute
SDAO	Sululta District Agricultural Office
DF	Douglas Foster
DMEM	Dulbecco's minimum essential medium
aa	amino acid
MDA	maternally derived antibody

ABSTRACT

Matching of field isolates of infectious bursal disease viruses with vaccine strains is an important pre-requisite for production of effective vaccine. In this study isolation and characterization of infectious bursal disease virus was done from outbreaks reported from commercial poultry farms with the objective of investigation of the degree of genetic similarity between virulent viruses isolated from outbreaks and commercially available vaccines. Bursa samples were collected in pools from chickens affected by outbreaks for virus isolation. Isolation of the virus was done using chicken embryo fibroblast cell lines designated DF-1. Identification of the strains of the infectious bursal disease virus was carried out using reverse transcriptase polymerase chain reaction targeting VP2 gene of infectious bursal disease virus. The genetic similarity among the field strains and vaccine strains was performed by comparing the deduced amino acid differences at positions 222, 242, 256, 294 and 299. Nine outbreaks affecting 30250 chickens were investigated. All of the pooled samples collected from outbreaks and cultured yielded positive results. Molecular analysis confirmed that the viruses isolated were infectious bursal disease virus. Further sequence analysis revealed that the infectious bursal disease viruses isolated were all very virulent strains. The deduced amino acid sequence analysis showed that the virulent isolates reported in this study are more similar to European strains such as UK661 and DV86 than the vaccine strains used in Ethiopia. This study has showed that very virulent IBDV strains are circulating in the country affecting commercial poultry farms and distribution centers. Therefore, the veterinary and livestock authorities should take this into account.

Keywords: *Commercial poultry farms, Ethiopia, Filed strains, Infectious bursal disease, sequencing, vaccine, VP2.*

1. INTRODUCTION

Chicken are important livestock resources that can break the vicious cycle of poverty and malnutrition in developing countries. Family poultry production serve as a source of cash used to buy clothes, school supplies, medical costs, etc for children and women. Commercial production hires local people; also it contracts day old chicken to local people to raise it on payment basis. Taking this into account, the Ethiopian Ministry of Agriculture has identified poultry production as key sector to deal with food security issues (LMP, 2015). The target is to raise the amount of meat and eggs produced per year through increasing the number of poultry farms and introduction of improved breeds. Improved breeds are, however, susceptible to diseases. Therefore, it will be critically important to develop methods to expand poultry farms while lowering the prevalence of infectious diseases that inflict significant losses each year. Infectious diseases are among the several factors responsible for considerable losses. One of the infectious diseases that are of growing concern in poultry is infectious bursal disease (IBD). In Ethiopia IBD is prevalent in various areas (Zeleke *et al*., 2005) causing high mortality ranging from 49.89% to 72% in chicken (Woldemariam and Wossene, 2007; Tesfaheywet and Getnet, 2012). It became a serious threat and a challenge to the juvenile poultry industry in Ethiopia (Mazengia, 2012).

Infectious bursal disease also known as Gumboro disease is acute, highly contagious, and immunosuppressive (Rauf, 2011; Yao and Shijun, 2017) viral disease affecting mostly young chicks (Hair-Bejo *et al.,* 2004). It constrains poultry industries worldwide (Toro *et al*., 2009). The disease causes considerable economic losses resulting from mortality (Jackwood *et al.,* 2009; Mahgoub *et al*, 2012; Muller *et al*., 2012) and immunosuppression that lead to vaccine failures against other infectious diseases (Jackwood and Sommer-Wagner, 2010).). Besides, the immunosuppression increases susceptibility of chickens to other infectious diseases (Yao and Shijun, 2017).

Trivial causes apart, failure of vaccination against infectious bursal disease is associated mainly with early vaccination in flocks of unknown immune status and with the evolution

of viruses circulating in the field, leading to antigenic drift and a sharp rise in pathogenicity (Boudaoud *et al.*, 2016). Infectious bursal disease takes advantage of the genetic flexibility to achieve antigenic variations, which help it to escape immune clearance and quickly adapts to the change of enviroments. With the intensive use of live vaccines, the number of vvIBDV strains and their reassortants has continuously increased. These strains have become epidemic and posed a great threat to the poultry industry as they escape current anti- IBDV vaccines, making the prevention and control of IBD more challenging (Yao and Shijun, 2017). The emergence of antigenic variant as well as very virulent strains in vaccinated flocks considerably stimulated research efforts on both, IBD and IBDV (Aregitu, 2015). Detection and strain identification of IBDV is important because antigenic subtypes found within serotype 1 make it necessary to tailor vaccination programs to the antigenic type found in the bird's environment (Jackwood, 2004).

So far vaccines used against IBD in Ethiopia are being imported from abroad or locally produced from imported master seed. This has its own drawbacks. There may be divergence between the vaccine strain and the field strains. Besides, it could re-assort with the field strains and end up with emergence of new strain of the virus. The frequent reports of outbreaks of IBD from several parts of the country despite vaccination might be due to theses reasons. The use of molecular techniques to detect and identify IBDV strains has increased in recent years. Reverse transcriptase polymerase chain reaction (rt-PCR) has been used to amplify sections of the VP2 gene from IBDV genome because VP2 gene encodes for the major protective epitopes, contains determinants for pathogenicity, and is highly variable among strains. Matching of vaccine strains with filed strains of IBDV using sequence alignment is an important pre-requisite for production of effective vaccine. This study attempts to isolate IBDV from various outbreaks reported from different parts of Ethiopia and explore the relatedness of the field viruses with vaccine strains.

Therefore, the objectives of this study are:

- ✓ To isolate and characterize field IBDV samples
- ✓ To investigate the degree of genetic similarity between IBDV isolated from outbreaks and commercial vaccine strains

2. AN OVERVIEW OF INFECTIOUS BURSAL DISEASE

2.1. History

Infectious bursal disease (IBD) was first recognized as a distinct clinical entity in 1957 (Cosgrove, 1962) when it was described "avian nephrosis" on account of the tubular degenerative lesions found in the kidneys of infected broiler chickens. The syndrome adopted the name "Gumboro disease" since the first outbreaks occurred in and around the area of Gumboro, Delaware, USA. Predominant signs of illness included trembling, ruffled feathers, watery diarrhoea, anorexia, depression, severe prostration, and death. In addition, hemorrhages in the thigh and leg muscles, increased mucus in the intestine, liver lobe infarction, renal damage, and enlargement of the bursa of Fabricius were lesions commonly observed at necropsy (Cosgrove, 1962). Early studies suggested that the causative agent of IBD was a nephro-pathogenic strain of infectious bronchitis virus (IBV) on the basis of similar gross lesions observed in the kidney (Winterfield and Hitchner, 1962).This misconception was further evident from concurrent occurrence of IBDV and other infections, which was difficult to isolate with the available methods (Lasher and Davis 1997). Following successful isolation of IBA in embryonated chicken eggs (Hitchner, 1970), proposed that the disease be termed "infectious bursal disease" due to its pathognomonic bursa lesions. Subsequent studies (Pejkovski*, et al.,* 1979) however, revealed that IBV immunized birds could still be infected with the "infectious bursal agent" (IBA) and develop lesions in their cloacal bursas specific for the disease.

The virus is very resilient in the environment and is highly contagious. Classical strains of the virus have circulated since the 1960s, but the disease was adequately controlled by vaccination until the emergence of variant then very virulent strains in the 1980s made the task of controlling IBD more challenging. Despite a growing arsenal of vaccines, the infection remains enzootic worldwide, and still ranks among the top five infectious problems facing the poultry industry in almost all countries surveyed (CEVA, 2015). The disease was controlled by vaccination, however in 1985 variant (v) strains were first identified in the USA that were antigenically distinct from the classical strains (Oppling

et al., 1991; Van der Marel *et al*., 1990) and, in 1986, very virulent (vv) strains emerged in Europe and subsequently spread to Africa, Asia, South America, and eventually the USA (Chettle *et al*., 1989).

2.2. Etiology

Infectious bursal disease (IBD) is caused by *Avibirnavirus* of the family *Birnaviridae*. Two serotypes of IBDV designated serotypes 1 and 2 are recognized, which can be differentiated by cross-neutralization assays (OIE, 2008). Clinical disease has been associated with only serotype 1 and all commercial vaccines are prepared against this serotype. That is serotype 1 IBD viruses are important pathogens of chickens (Muller *et al*., 2003; Van den berg *et al*., 2004).

They replicate in the bursa of Fabricius and some of them cause clinical disease in chickens. Antibodies or virus are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 viruses are immunologically distinct from serotype 1 viruses since vaccination with serotype 2 viruses did not confer protection against serotype 1. They have been detected from the respiratory tract of turkeys, cloacal swabs of ducks or in the bursae of Fabricius of chickens. Antibody has been detected but no clinical disease has been reported in chickens or turkeys as a result of infection with IBD virus serotype 2 (Eterradossi & Saif, 2013). Antibodies against serotype 2 viruses are very widespread in turkeys and are sometimes found in chickens and ducks. Serotype 1 of IBD viruses are further classified as attenuated (vaccine strains), classical (standard), antigenic variant, and very virulent based on the virulence. Very virulent strains of classical serotype 1 are now common and are causing serious disease in many countries (OIE manual, 2008). IBD has not been reported to have any zoonotic potential (Eterradossi & Saif, 2013; OIE, 2016).

2.3. Morphology and genome of the virus

IBDV is a double strand RNA virus (dsRNA) and a nonenveloped, icosahedral capsid with bi-segmented genome (Wu *et al.*, 2007; Zhu *et al.*, 2008). The capsid shell exhibits icosahedral symmetry composed of 32 capsomeres and a diameter ranging from 55 to 65 nm. Its structure is based on a T = 13 lattices composed of trimeric subunits. Cryoelectron microscopy and image processing analysis showed that the outer surface of the viral capsid is made up of 260 trimeric VP2 clusters, while the inner surface is composed of 200 Y-shaped trimeric VP3 structures (Caston *et al.*, 2001). The detailed structure of the IBDV is depicted in figure1.

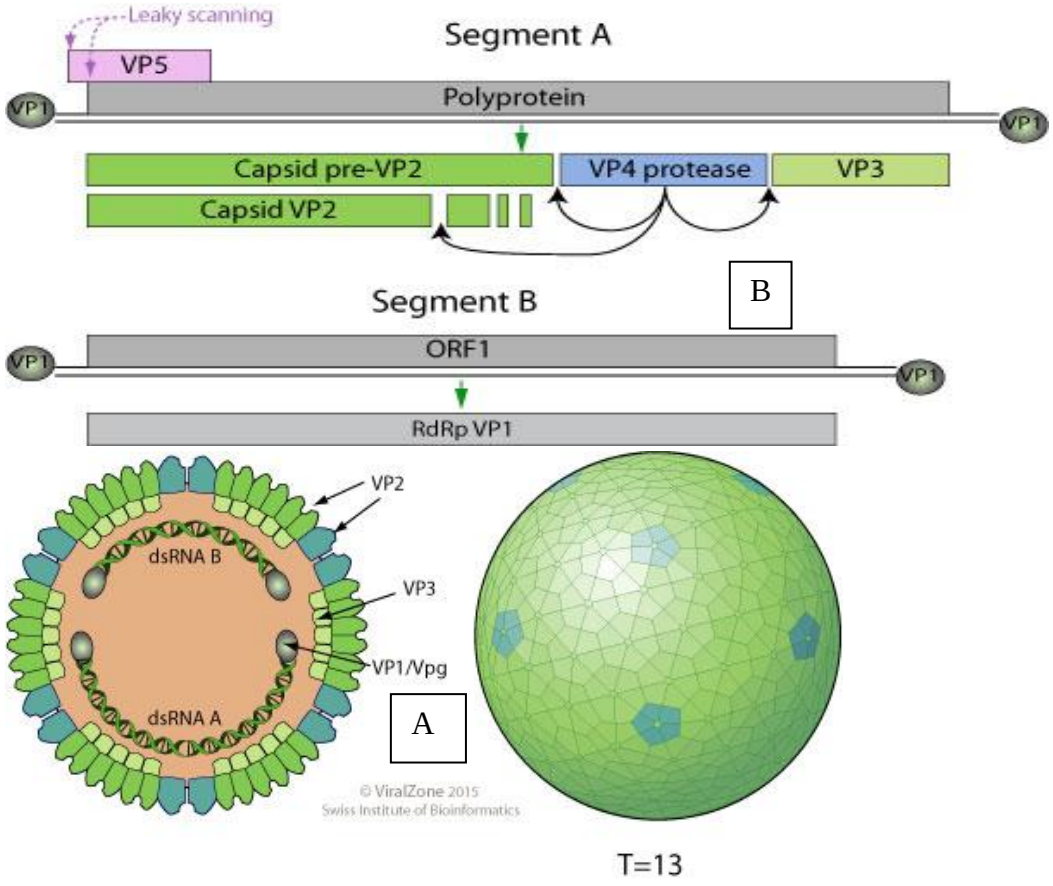


Figure 1: Morphology and genome structure of IBD (A). Morphology of IBDV (B) Genome structure of IBDV (2 segments (A and B) encode for 5-6 proteins. VP1 is found in a free form and covalently attached at the 5' genomic RNA end (VPg). Segments size is about 2.3-3 kb. Genome total size is about 6 kb. (*Source*: Viral zone, 2015).

2.4. Physico-chemical characteristics

The virus is non-enveloped and quite resistant to physical and chemical agents, resistant to: pH conditions of 2–11, but it is inactivated at pH 12 (Lukert and Saif, 2003). Due to this ability of stability and hardiness, it persists in poultry premises even after thorough cleaning and disinfection (Lukert and Saif, 2003), for up to 4 weeks in the bone marrow of infected chickens (Elankumaran *et al.,* 2002). The virus has been shown to remain infectious for 122 days in a chicken house, and for 52 days in feed, water and faeces (Benton *et al.,* 1967; Shamila, 2005).

A marked reduction in infectivity of the virus was observed after treatment with 0.5% Formalin for 6 hours. The virus remained unaffected by ether, chloroform, phenol, thiomaesal, Staphene and Hyamine 2389 treatments. The virus survived treatments with various concentrations of three disinfectants (an iodine complex, a phenolic derivative and a quaternary ammonium compound) for a period of 2 minutes at 23^{o}C, only the iodine complex had any deleterious effects. Cho and Edgar (1969) reported that the virus was inactivated by exposure for 1 hour to 1% formalin, 1% cresol and 1% phenol. It remained stable at 60°C for 90 minutes and was still infectious at room temperature for approximately for 21 days (Shamila, 2005).

2.5. Epidemiology

2.5.1. Geographical distribution

Classical IBDV have traditionally affected poultry worldwide ever since the first outbreak of disease was reported from Delaware, Maryland and Virginia (Delmarva) region. By 1970, the disease had been reported from Canada, Mexico, Europe, Africa, the Middle East and Asia as presented in figure 2. The virus is resistant to chemical agents and hard to eradicate from poultry houses, infections are thus potentially carried over from one cycle to the next. IBDV is not vertically transmitted (no transmission from parent to day old chick through the egg). Horizontal transmission through infected faeces, contaminated equipment (especially footwear) or other organic material is the most likely

route of spread. It has been demonstrated that the lesser mealworm (*Alphitobius diaperinus)* could act as a vector carrying IBDV from one cycle to the next. The infection is endemic in nature and birds are constantly exposed to the virus. Breeder flocks are vaccinated against the virus to provide maternal immunity to the off-springs so all chicken flocks are sero-positive for the virus (Shamila, 2005). Classic (cvIBDV), antigenic variant (avIBDV), and virulent (vvIBDV) isolates have been identified in Africa, Asia, Europe, South America, and Oceania. In North America and the Caribbean, only cvIBDV and avIBDV isolates were identified. This high frequency of cvIBDV and avIBDV isolates in North America was also observed by Jackwood and Sommer-Wagner (2005).

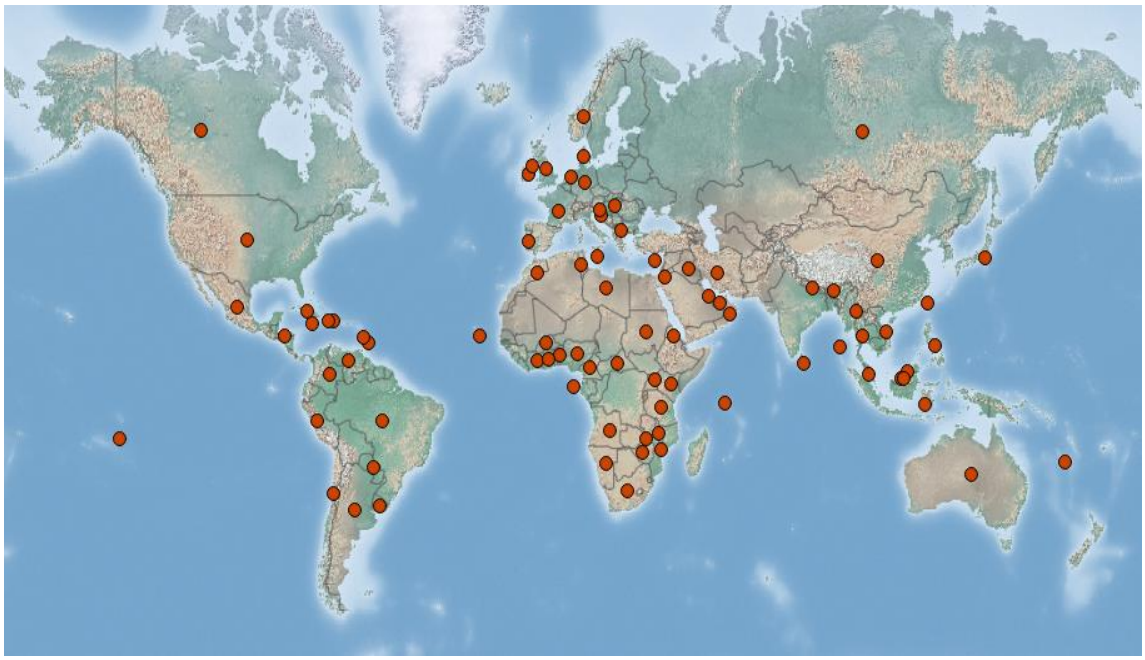


Figure 2: Geographic distribution of IBD (source: CABI.org, 2017)

2.5.2. Host range

Although turkeys, ducks, guinea fowl, pheasants and ostriches may be infected, clinical disease occurs solely in chickens. Only chickens younger than 10 weeks are usually clinically affected. Older chickens usually show no clinical signs. Severe acute disease of 3- to 6-week-old birds is associated with high mortality, and signs including prostration,

diarrhoea, and sudden death. A less acute or subclinical disease is common in 0- to 3-week-old birds (OIE, 2016).

Serotype 2 viruses have been detected from the respiratory tract of turkeys, cloacal swabs of ducks or in the bursae of Fabricius of chickens. Antibodies against serotype 2 viruses are very widespread in turkeys and are sometimes found in chickens and ducks. There is no report of clinical disease caused by infection with serotype 2 virus. IBD has not been reported to have any zoonotic potential (Eterradossi & Saif, 2013).

2.5.3. Transmission

Infected birds excrete virus in their droppings at least for 14 days (Baxendale, 2002). It is excreted in the faeces and then contaminates water, feed and litter, where it persists and from where it commonly spreads. The most common mode of infection is through the oral route, Conjuctival and respiratory routes may also be involved (Sharma *et al.,* 2000) but the virus is highly contagious so that the disease is transmitted by direct contact with excreting subjects, or by indirect contact with any inanimate or animate (farm staff, animals) contaminated vectors between infected and susceptible flocks (OIE, 2008). The high tenacity of the virus and its resistance to several disinfections and virucidal procedures may contribute to the rapid distribution of the virus (Garriga *et al*., 2006). Infectious bursal disease virus may spread through contaminated equipment (Flensburg *et al*. 2002; Jackwood and Sommer-Wagner, 2010).

There is no evidence to suggest that IBDV is spread via transovarial transmission (Eterradossi and Saif, 2008). No specific vectors or reservoirs of IBDV have been established, but the virus has been isolated from mosquitos (*Aedes vexans*), rats, and lesser mealworms (*Alphitobiusdiaperinus*) (Eterradossi and Saif, 2008). Viable vvIBD virus was recovered after 2 days fromthe faeces of a dog that had been fed tissues from experimentally infected chickens, indicating that dogs may act as mechanical vectors for the virus (Pages-Mante *et al*., 2004).

2.5.4. Risk factors

Previous studies revealed that low biosecurity standards contributed to the spread of IBDV in poultry farms. Poultry farms that receive visitors on the farm premises have higher risk of infection with IBDV, compared with farms that do not. It is notable that most of the visitors are related with poultry business (the person himself buy or sell the products or act as a middle man for other person), they have higher chance of visiting several farms per day. In this way, they are more likely to transmit infection from infected farm to healthy farm (Rashid *et al*., 2013). The odds of IBDV infection in farms that purchase live poultry from the open market was higher than farms that do not purchase. Sales of live poultry are related with quick dissemination of infections through slaughtering the birds, evisceration, haphazard disposal of visceral organs and feathers, and contaminated equipment used by the farmers (Henzler *et al*., 2003).

Another risk factor of IBDV is 'workers living outside the farm premises'. An investigation in Nigeria showed that these workers tend to follow no biosecurity rules because the workers have poultry in their own house, they offer service to other farms, vaccinate other farms chickens, sometimes exchange equipments from other farms and rarely change their clothes and shoes before entering into a farm (Adene and Oguntade, 2007). Access of vendor vehicles on the farm premises was a strong risk factor. If the farm owner has no transport facilities, vehicles belonging to vendors enter farm premises to pick up products. Thus, these vehicles visit many farms per day. In the course of conducting trade, the vendor may inadvertently transmit infection from an infected site to a healthy one (Rashid *et al*., 2013).

2.5.5. Morbidity and Mortality

Infectious bursal disease is the major health and production constraint of young chicken. IBD is acute, highly contagious globally occurring viral poultry disease. A flock will show very high morbidity with severe depression in most birds lasting for 5–7 days. Mortality rises sharply for 2 days then declines rapidly over the next 2–3 days. Usually

between 5% and 10% of birds die, but mortality can reach 30–40% or more with very virulent IBDV (vvIBDV) (OIE, 2016).

However, molecular classification of isolates has no direct correlation with the mortality in the field. The IBDV strain might cause a high death rate if outbreak occurred within an unvaccinated flock infected with bacteria, other viruses and coccidia than in the vaccinated flock with no complication of disease. A high mortality rate can be observed because of a reovirus infection and staphylococcosis, in spite of the flocks' vaccination. These results mean that concurrent bacterial and viral infections are one of the factors that affect mortality (Kim et al., 2010).

Table 1: Morbidity and Mortality due to IBD in chicken from different countries of the world

Country	Morbidity	Mortality	source
Egypt	38%	10-13%	Nevin *et al*., 2015
Pakistan	1.847%	7.037%	Raj *et al*., 2009
Bangladesh	100%	7-38%	M.N.Islam *et al*., 2008
Nigeria	80-100%	43.3-95.8	H.B.Aliyu *et al*., 2016
India	NA	8.66-19.2%	Morla *et al*., 2016
USA	NA	5-30%	Maurice, 2012
Ethiopia	NA	60%	Aregitu *et al*., 2017
South Korea	NA	0.2-9.4%	Kim *et al*., 2010
Jordan	NA	25%-80%	Roussn *et al*, 2012

NA = data not available

2.6. Diagnosis

Vaccination of hens, with the subsequent maternal immunity imparted to chicks, is the primary means of controlling infectious bursal disease virus (IBDV). Effective vaccination depends on rapid and accurate diagnosis of the subtype present in a flock because vaccines based on the classic subtype of IBDV can fail to protect against challenge with a variant subtype (Wu *et al*., 2006). Isolation and identification of the

agent provide the most certain diagnosis of IBD, but are not usually attempted for routine diagnostic purposes as the virus may prove difficult to isolate. In practice, laboratory diagnosis of IBD depends on detection of specific antibodies to the virus, or on detection of the virus in tissues, using immunological or molecular methods. Several methods are available for diagnosis depending on the objectives (OIE, 2016).

2.6.1. Clinical signs and post mortem lesions

Diagnosis involves consideration of flock history, clinical sign and post mortem lesions. Pathological change observed at the bursa of fabricius is characteristic and histo-pathological investigations combined with the demonstration of viral antigen by immunohistochemistry confirm an IBDV infection. Infectious bursal disease virus has 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 bird to engage in vent picking. Clinical sing are described as acute onset of depression, trembling, white and watery diarrhea, anorexia, prostration, ruffled feather, and vent feather solids with urates. In severe cases, bird became dehydrated and in terminal stages subnormal temperature and death (Zeleke *et al*, 2005).The lesions observed in birds that are common to IBDV infection include dehydration, hemorrhage in breast and leg musculature, darkened discoloration of pectoral muscles, occasional hemorrhage in thigh muscle and pectoral muscle, increasing mucus in the intestine and renal changes. In bird that die or are in advanced stage of the disease, kidneys frequently show swelling and pallor with accumulation of urates in the predominant lymphoid organ affected by IBDV (Weissi and Kaufer, 2010).

2.6.2. Histopathology

The tissues is processed and the 4 μ thick tissue sections are cut out of the paraffin embedded tissue blocks and stained with haematoxylin and eosin staining as per the protocol of Bancroft and Gamble (2002) for routine histopathology. Microscopic examination of tissues shows moderate haemorrhages in the muscles and kidneys (Figures 3 (A) and (B)) and the spleen shows moderate lymphoid depletion in the lymphoid nodules (Figure 4 (A)). There is marked interfollicular oedema and depletion of

lymphocytes from the lymphoid nodules in the BFs (Figure 4(B)). Other lymphoid nodules of the BF show degeneration and necrosis of lymphocytes and cystic cavitations with heterophil infiltrates (Figure 4(B)).

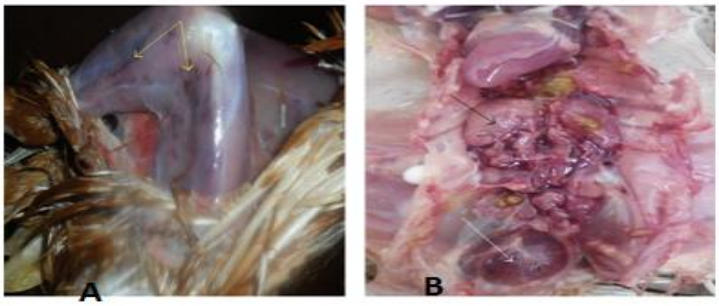


Figure 3: Gross pathological lesions due to IBD: A= hemorrhage in the thigh and leg muscles (yellow arrow); B = inflammation and degeneration of kidneys and bursa (arrows)

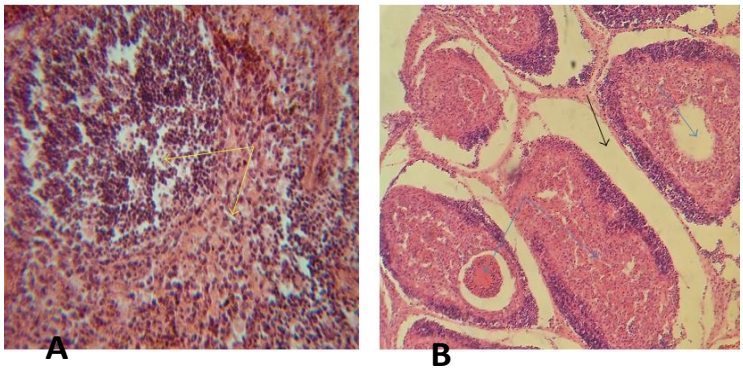


Figure 4: Histopathological appearance of spleen and bursa in IBD infected chicken: A= lymphocyte depletion in the lymphoid nodules of the spleen; B= marked interfollicular oedema (black arrow), and cystic cavitation and necrosis in the medullar of bursal follicles (blue arrows)

2.6.3. Sample preparation

Remove the bursae of Fabricius aseptically from approximately five affected chickens in the early stages of the disease. Chop the bursae using two scalpels, add a small amount of peptone broth containing penicillin and streptomycin (1000 μg/ml each), and homogenize in a tissue blender. Centrifuge the homogenate at 3000gfor 10 minutes. Harvest the supernatant fluid for use in the investigations. Filtration through a 0.22 μ filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titre (OIE, 2016).

Sera samples can be collected for detection of antibodies against IBDV. Sterile 3 ml disposable syringes with needle size 21 gauge and 1½² are used to collect blood samples from brachial (wing) vein of chickens. The whole blood collected from local chickens must be labeled and allowed to clot under normal atmospheric temperature in the slant position within the syringe for 24 hours. Then, the clear serum is harvested in to labeled cryo-vials and stored at - 20°C until the ELISA test was carried out (Zeryehun and Fekadu, 2012).

2.6.4. Virus Isolation

2.6.4.1. Virus isolation on cell culture

Inoculate 0.5 ml of sample into each of four freshly confluent chicken embryo fibroblast (CEF) cultures (from a specific pathogen free (SPF) source) or DF-1cell lines in 25 cm^2 flasks. Adsorb at 37°C for 30–60 minutes, wash twice with Earle's balanced salt solution and add maintenance medium to each flask. Incubate the cultures at 37°C, observing daily for evidence of cytopathic effect (CPE). This is characterized by small round refractive cells. If no CPE is observed after 6 days, discard the medium, then freeze–thaw the cultures and inoculate the resulting lysate into fresh cultures. This procedure may need to be repeated at least three times. If CPE is observed, the virus should be tested against monospecific IBDV antiserum in tissue culture virus neutralization (VN) test. The

more pathogenic IBDV strains usually cannot be adapted to grow in CEF unless the virus has first been submitted to extensive serial passage in embryos (OIE, 2016).

2.6.4.2. Virus isolation in chicken embryo

Inoculate 0.2 ml of sample into the yolk sac of five 6- to 8-day-old specific antibody negative (SAN) chicken embryos and on to the chorio-allantoic membrane (American Association of Avian Pathologists, 2008) of five 9- to 11-day-old SAN chicken embryos. SAN embryos are derived from flocks shown to be serologically negative to IBDV. Candle daily and discard dead embryos up to 48 hours post-inoculation. Embryos that die after this time are examined for lesions. Serotype 1 IBD produces dwarfing of the embryo, subcutaneous oedema, congestion and subcutaneous or intracranial haemorrhages. The liver is usually swollen, with patchy congestion producing a mottled effect. In later deaths, the liver may be swollen and greenish, with areas of necrosis. The spleen is enlarged and the kidneys are swollen and congested, with a mottled effect. If lesions are observed, the virus should then be tested against a monospecific anti-IBDV serum in an embryo-revealed virus neutralization assay.

Serotype 1 IBDV usually causes death in at least some of the embryos on primary isolation. Serotype 2 IBDV does not induce subcutaneous oedema or haemorrhages in the infected embryos, but embryos are of a smaller size with a pale yellowish discolouration. For the preparation of embryo-propagated stock virus or for subsequent passaging, embryos with lesions or embryos suspected to be infected, respectively, are harvested aseptically. Their head and limbs are discarded and the main body is minced for the preparation a virus suspension (OIE, 2016).

2.6.4.3. Virus isolation in chickens

This method has been used in the past but is no longer recommended due to animal welfare concerns. Five susceptible and five IBD-immune chickens (3–7 weeks of age) are inoculated by the eye-drop route with 0.05 ml of sample. Humanely euthanise the chickens 72–80 hours after inoculation, and examine their bursae of Fabricius. The bursae of chickens infected with virulent serotype 1 IBDV appear yellowish (sometimes haemorrhagic) and turgid, with prominent striations. Peribursal oedema is sometimes present, and plugs of caseous material are occasionally found. The plicae are petechiated.

The presence of lesions in the bursae of susceptible chickens along with the absence of lesions in immune chickens is diagnostic of IBD. The bursae from both groups may be used as antigen in an AGID test against known positive IBD antiserum. The extent of bursal damage may vary considerably with the pathogenicity of the studied IBDV strain. However, as the samples submitted for virus isolation may vary in virus content, the extent of bursal damage observed in susceptible chickens at the isolation stage gives only a limited indication on strain pathogenicity. The bursae of chickens infected with serotype 2 IBDV do not exhibit any gross lesions (OIE, 2016).

2.6.5. Identification by Agar Gel Immuno-diffusion Test (AGID)

For detection of antigen in the bursa of Fabricius by AGID, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in scissor movement, and then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue, and the freeze–thaw exudate may be used to fill the wells (OIE, 2016).The antigen and the antibody meet in a 1% agar gel and their diffusion is according to size, structure and charge. At the equivalence point of the antigen and antibody a precipitation arc is formed. In case of mixed antigen and antibody, the specifically reacting antibodies and antigens

reach the equivalence point at different distances, so several arcs may be formed. Evaluation of the reaction is usually after 24 hours.

2.6.6. Identification by Immunofluorescence

Sections of bursa are prepared using a microtome cryostat, dried at room temperature and then fixed in cold acetone. Fluorescent-labeled IBDV-specific antisera are applied to the sections, which are then incubated at 37°C for 1 hour in a humid atmosphere. At the end of the incubation period, they are washed for 30 minutes using phosphate-buffered saline (PBS), pH 7.2, and then rinsed in distilled water. The sections are mounted using buffered glycerol, pH 7.6, and examined by UV microscopy for IBDV-specific fluorescence (OIE, 2016).

2.6.7. Identification by ELISA

2.6.7.1. Identification by antigen- capture enzyme-linked immunosorbent assay (AC-ELISA)

Since the first protocol was described by Snyder *et al.* (1988) for the detection of serotype 1 IBDV using an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA), many other assays have been developed (Eterradossi & Saif, 2013). Briefly, ELISA plates are coated with IBDV-specific antibodies. Depending on the chosen AC-ELISA protocol, the capture antibody may be a mouse anti-IBDV monoclonal antibody (MAb), or a mix of such MAbs, or a chicken post-infectious anti-IBDV polyclonal serum. It has been suggested that AC-ELISAs using polyclonal antibodies may have a higher sensitivity.

Samples of bursal homogenates diluted 1/10 to 1/25 (w/v) in a suitable dilution buffer are incubated in the coated wells. Unbound antigens are discarded at the end of the incubation period by washing with a suitable washing buffer (e.g. PBS, pH 7.2 + 0.2% Tween 20). The captured antigens are then revealed, as in an indirect ELISA, with a

detection antibody (which must have been developed from a different animal species than the capture antibody), followed by an enzyme conjugate that binds to the detection antibody only (in some protocols the detection antibody may be directly conjugated to the enzyme), followed by the enzyme substrate. Finally, optical densities, which parallel the amount of captured IBDV antigens, are read with an ELISA reader.

AC-ELISA is based on the use of samples possibly containing live virus and should be performed only in suitable containment facilities such as a class II safety cabinet. All liquid (washing buffers) and solid wastes should be considered to be contaminated by IBDV and decontaminated accordingly before disposal. Critical steps in the implementation or assessment of AC-ELISA are i) the need to perform extensive washings between each step of the reaction to keep background reactions low, ii) the requirement for known positive and negative samples to be included in each assay as controls, and iii) the need for both the capture and detection antibodies to positively react with all serotype 1 IBDV strains (i.e. neither capture nor detection should critically depend on IBDV antigenic variation that occurs among serotype 1 strains) (OIE Manual, 2016).

2.6.7.2. Antibody detection by indirect ELISA

The flat bottomed microtitre plates are coated with 100µl per well of working volume of antigen diluted 1:5000 in coating buffer, then incubated for 3 hours at 37^{o}C and subsequently overnight at 4^{o}C. After incubating overnight at 4^{o}C,the plates are thereafter washed 3 times with PBS-Tween 20, PH 7.2 and blocked with PSB-Tween 20 (Phosphate buffered saline containing Tween 20 and 0.1% gelatin) and incubated at 37^{o}C for 30 minutes. The washing of plates is done three times after every 5 minutes with PBS-Tween-20 PH.7.2.

Working serum dilution is 1:200. Sera are prediluted to 1:20 in a pre-dilution microtitre plate and 10µl per well transferred to the test plate containing 90µl/well of the diluting buffer, to give a 1/200 dilution. 10µl of prediluted sera for the pre-dilution plate are

transferred to the respective wells on the test plate, then covered and incubated at 37^0C with the shaker set at 100rpm, for 30 minutes.

Working dilution is 1:1000 in diluting buffer. Washing and blotting of the plates on absorbent tissue before adding the conjugate is done.2^{nd} antibody conjugate (Goat anti-chicken IgY) diluted as above was dispensed to all wells (100µl/well) and incubated at 370C for 30 minutes. Washing and blotting of the plates on absorbent tissue was done before adding the conjugate. 100µl/well of the substrate TMB was added to all wells and the plates incubated at room temperature for 5 minutes. The reaction was stopped using 0.5M H_3PO_4 at the end of incubation period mentioned above and the results read first by looking at the colour development on the plates, the colour reactions were viewed or quantified by measuring the optical density (OD) of each well using a micro-ELISA reader at 450nm, recorded and then analyzed (E. Baraza *et al*, 2011).

2.6.8. Virus Neutralization test

VN tests are carried out in cell culture. The test is more laborious and expensive than the AGID test, but is more sensitive for detecting antibody. This sensitivity is not required for routine diagnostic purposes, but may be useful for evaluating vaccine responses or for differentiating between IBDV 1 and 2 serotypes. The test uses either SPF chicken embryo fibroblast cells, or a suitable continuous cell line (such as QT-35, BGM-70, MA-104, Vero or DF1), in conjunction with an adapted strain of IBDV.

First, 0.05 ml of virus diluted in tissue culture medium to contain 100 $TCID_{50}$ (50% tissue culture infective doses) per 0.05 ml is placed in each well of a tissue-culture grade microtitre plate. The test sera are heat-inactivated at 56°C for 30 minutes. Serial doubling dilutions of the sera are made in the diluted virus. After 30 minutes at room temperature, 0.2 ml of cell suspension, with a cell density allowing confluent layers to be obtained after 24 hours of incubation, is dispensed into each well. Plates are sealed and incubated at 37°C for 4–5 days, after which the monolayers are observed microscopically for typical CPE. The end-point (serum titre) is expressed as the reciprocal of the highest

serum dilution that did not show CPE. To reduce test-to-test and operator-to-operator variation, a standard reference antiserum may be included with each batch of tests and the titre of the virus suspension must be reassessed in each new experiment using a sufficient number of repeats (wells) per virus dilution (OIE, 2016).

2.6.9. Identification by Molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation. This method can detect the genome of viruses that do not replicate in cell culture, because it is not necessary to grow the virus before amplification.

2.6.9.1. Reverse-transcriptase polymerase chain reaction (RT-PCR)

The reverse transcriptase–polymerase chain reaction (RT-PCR) assay has been used by several laboratories to identify IBDV. Most researchers have focused on a variable sequence region of the VP2 gene that is known to encode one or more neutralizing epitopes of the virus (Jackwood, 2004). RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription (RT) of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotidic primers that are short sequences complementary to the virus-specific nucleotidic sequence. Different areas of the genome will be amplified depending on the location from which the primers have been selected (OIE, 2016).

2.6.9.2. RT-PCR with Restriction Fragment Length Polymorphism (RFLP)

This assay has been useful in placing vaccine strains of the virus into molecular groups. Within a molecular group, IBDV strains are related by ancestry (Jackwood *et al*., 2001). Furthermore, viruses within a molecular group have nucleotide and amino acid sequences that are relatively more alike compared with viruses in different molecular groups. The

results indicate that RFLP profiles can be used to predict the relative similarities and differences among unknown IBDV strains. The RT-PCR–RFLP procedures used to generate molecular groups of IBDV are designed to assess the nucleotide similarity or diversity among viruses (Jackwood, 2004).

2.6.9.3. Real time RT-PCR

Moody *et al*. (2000) used the TaqMan real-time RT-PCR assay to quantify viral load in the blood of IBDV infected chickens. Although very rapid and sensitive, this assay was not used to differentiate different strains of IBDV. Real-time RT-PCR with hybridization probe system is able to differentiate among IBDV strains. This real-time RT-PCR probe system employs fluorescence resonance energy transfer (FRET) to identify the RT-PCR products. There are two probes, one labeled with fluorescein isothiocyanate and the other with a Red 640 fluorophore. These probes are not destroyed during amplification, as they are in the TaqMan system. Thus, they can be used after RT-PCR amplification to generate a melting temperature (Tm) for each IBDV strain. The Tm is the temperature at which one of the probes (usually identified as the mutation probe) will dissociate from the RT-PCR product (Jackwood, 2004).

2.6.9.4. Sequence analysis of VP2 gene

Gel containing RNA band of the expected size was excised and purified with the QIAquick Gel Extraction Kit (Qiagen, USA) according to the manufacturer instruction. The purified PCR products were sequenced directly using the ABI PRISM® BigDyeTM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM® 3130 genetic analyzer (Applied Biosystems) with 80 cm capillaries, assembly of the consensus sequences and alignment trimming was performed with the BLAST tool (Neven et al, 2015). Conventional RT-PCR has been useful in detecting IBDV serotypes and, to a lesser extent, differentiating IBDV subtypes. Conventional RT-PCR, amplifying the VP2 hypervariable region, in combination with DNA sequencing of the PCR product, can differentiate classic, variant, and vvIBDV strains because variant

and vvIBDV have characteristic nucleotide and amino acid substitutions. These methods potentially allow for more rapid, sensitive, and specific detection and differentiation of IBDV classic, very virulent, and variant subtypes. This approach is a valuable tool for molecular epidemiological studies on IBDV (Islam *et al.*, 2012). The comparative analysis will indicate that if these viruses are genetically close to the vvIBDVs or classical strains (Singh *et al.*, 2012).

Table 2: Summary diagnostic methods available for detection and identification of IBD

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection-surveillance	Post vaccination immune status
Pathology and virus detection						
Histopathology	+	-	-	+++	+	+
Virus isolation	+	-	-	+	+	-
Virus characterization	+	-	-	+++	+	+
Immunoassays	+	-	-	+++	+	-
Rt-PCR	+	-	+	+++	-	+
Detection of immune response						
AGID	++	++	++	-	-	+
ELISA	+++	+++	+++	-	-	+++
VNT	+	++	-	-	-	++

Key: +++ = recommended method, ++ = suitable method, + = may be used and - = not appropriate. Although not all of the tests listed as category +++ or ++ have undergone formal standardization and validation, their routine nature and the fact that they have been used widely without dubious results, make them acceptable (OIE, 2016).

Within serotype 1, two subtypes, classic and variant, can be differentiated by the virus neutralization assay. Antigen capture enzyme-linked immunosorbent assay (AC-ELISA) with MAbs has been successful at differentiating the very virulent IBDV phenotype (vvIBDV) from less pathogenic types. More rapid and sensitive molecular diagnostic methods based on reverse transcription–polymerase chain reaction (RT-PCR) for amplification of the IBDV VP2 gene have been a major focus of investigation in recent years. Conventional RT-PCR has been useful in detecting IBDV serotypes and, to a lesser extent, differentiating IBDV subtypes. One of the approaches has been the use of SspI and NgoM IV restriction enzymes, for restriction endonuclease (RE) analysis of RT-PCR products (RT-PCR-RE) and BstNI and MboI for restriction fragment length polymorphism (RFLP) analysis (RT-PCR-RFLP) to find unique banding patterns associated with antigenic variation within the variable region of the IBDV VP2 protein. However, these approaches were ultimately found to be unreliable because subtypes could not be consistently distinguished with restriction enzymes. These limitations led to studies in differentiating subtypes by detection of single nucleotide differences in sequence through real-time RT-PCR or DNA sequencing of RT-PCR products. Conventional RT-PCR, amplifying theVP2 hypervariable region, in combination with DNA sequencing of the PCR product, can differentiate classic, variant, and vvIBDV strains because variant and vvIBDV have characteristic nucleotide and amino acid substitutions. Real-time RT-PCR, targeting different regions of the IBDV genome, including VP1, VP2, and VP4 genes, in conjunction with melting-curve analysis is being investigated as a promising tool for molecular diagnosis of IBDV infection. These methods potentially allow for more rapid, sensitive, and specific detection and differentiation of IBDV classic, very virulent, and variant subtypes (Wu *et al*., 2007).

2.7. Status of IBDV in Ethiopia

An outbreak of infectious bursal disease, affecting 20-45 days old broiler and layer chickens, was investigated for the first time in Ethiopia in the months of March and April 2002. Death of chickens started at the 30th day of age and continues to the 55th day. The mortality due to the disease in different poultry farms ranges from 45 to 50 % with average mortality of 49.89%. In broiler farms the mortality was 56.09% while it was 25.08% in layer chickens (Zeleke et al, 2005).

The report of Zeleke and colleague (2005) and that of Woldemariam and Wossene (2007) showed high seroprevalence of IBD that was 93.3 and 100%, respectively. Mazengia (2008) reported an overall prevalence of 51.10% from Bahir Dar and Farta districts and Nigussie (2007) reported a high seroprevalence of 61% in backyard chickens of Addis Ababa and Adami Tulu areas. The study conducted on backyard chickens in both Peasant Associations and Kebelles of Bishoftu revealed the presence of IBDV specific antibody in the absence of vaccination, which show the presence of field exposure of household chickens to the virus. According to this study, the overall seroprevalence of IBD in Bishoftu was 82.20% (Zeryehun and Fekadu, 2012). The results of previous investigations are presented in Table 1. The previous studies have focused on estimation of seroprevalence of infection with IBDV. Information on the molecular epidemiology of the disease and its etiologic agent is extremely scarce.

Table 3: Reports of serosurvey and molecular diagnosis of IBD in chicken in some poultry rearing areas of Ethiopia

Site	IBD prevalence	Confirmation by rt-PCR	References
Adama	75.3%	Confirmed	Tadesse, and Jenbere, 2014
Modjo	76.6%	Confirmed	Tadesse, and Jenbere, 2014
wenji	92.1%	NA	Tadesse, and Jenbere, 2014
West Gojam	75%	NA	Samson, 2017
Gondar	72%	NA	Samson, 2017
Tigray	45.05%	NA	Yishak *et al*., 2015
Adiss Ababa	NA	Confirmed	Samson, 2017
Woliso	89.78%	NA	Degefu *et al*., 2010
Ambo	70.69	NA	Degefu *et al*., 2010
Holeta	40.81	NA	Degefu *et al*., 2010
Sebeta	38.3	NA	Tesfaye *et al*., 2016
Bishoftu	82.2	NA	Zeryehun and Fekadu, 2012
Andassa	100%	NA	Woldemariam and Wossene, 2007
Bahir dar	29.40%	NA	Mazengia *et al*., 2009, 2010
Farta	21.7%	NA	Mazengia *et al*., 2009, 2010

2.8. Vaccines and Vaccination against IBD

The right strategy for infectious bursal disease (IBD) control and its success rate under field conditions depends on hygiene management, IBD field pressure, level and variation in maternally derived IBD antibodies, and the IBD vaccine strains to be used. Usually, standard vaccination programmes are used, which are not always adapted to the specific conditions on the farm and to the immune status of chickens (Block, *et al*., 2007). Since the first report of IBD in Ethiopia by Zeleke *et al*. (2005), it has become a priority problem in commercial and backyard poultry production system despite regular

vaccination practices and improved biosecurity measures. Although classical attenuated IBDV vaccines protect chickens from vvIBDV, a hallmark of vvIBDVs is the ability to cause disease in the presence of higher levels of neutralizing antibodies in comparison to the classical virulent IBDVs. Therefore, highly attenuated vaccines that induce lower levels of neutralizing antibody may not provide adequate vaccination induced protection against vvIBDVs (Jenberie, *et al.*, 2014). 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. On top of this, most control strategies designed in the country do not take into consideration the local chickens, and this may lead into the failure of most strategies (Tadelle and Ogle, 2001; Hailemariam *et al.,* 2006).

3. MATERIAL AND METHODS

3.1. Study area

The study was conducted on tissue samples collected from five different areas of the country depicted in figure 5 (Addis Ababa, Bishoftu, Assela, Sululta and Kombolcha). 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. The town is situated at 9^{o}N latitude and 40^{o}E longitude. It is located at a distance of 47km south east of Addis Ababa and 52km from Adama to the northwest. The town is boredered with Genda Gorba Peasant Association in the east, with Kaliti Peasant Association in the west, with Kurkura in the south and with Wedo and Keta Jara Peasant Association (Oromia finance and economic bureau, 2011). Addis Ababa covers about 540km^2 of which 18.2km^2 is rural. Addis Ababa lies between 2200 and 2500 meters above sea level. The city lies at the foot of the 300 meters high Entoto mountains. Despite its proximity to the equator, Addis Ababa enjoys a mild, Afro-Alpine temperate and warm temperate climate. The lowest and highest annual average temperature are between 9.89 and 24.64^{o}C (Addis Ababa city administration, 2018). Kombolcha is located at 11^{o}07' N latitude, 39^{o}44'E longitude and an altitude of 1864m above the sea level. It has 1038 mm annual rainfall and 18^{o}C average temperature (ARAAI, 2008). Sululta district is one of the six districts of Oromia Special Zone Surrounding Finfinne of Oromia National Regional State. The districts' capital town, Chancho, is 40 kms away from Addis Ababa towards the North-west. It lies on the geographical coordinates of 9° 11' 0" N latitude, 38° 45' 0" E longitude. The area is characterized by shallow valley with an elevation of *2500 meters* above sea level. The average annual temperature in Sululta is 14.7 °C with an average rainfall of *1119 mm* (SDAO, 2012). Arsi Zone is found in the central part of the Oromiya National Regional State. The zone astronomically lies between 60 45' N to 8058'N and 38032 E to 40050' E. It is located at 175 km from Adis Abeba on Adis Abeba-Adama-Bale Robe main road. Also Asela is located at 75 km south of Adama town. Having the total area of 23881

Km^2, it accounts for about 7 percent of the total area of the Regional State of Oromia (OBFED, 2011).

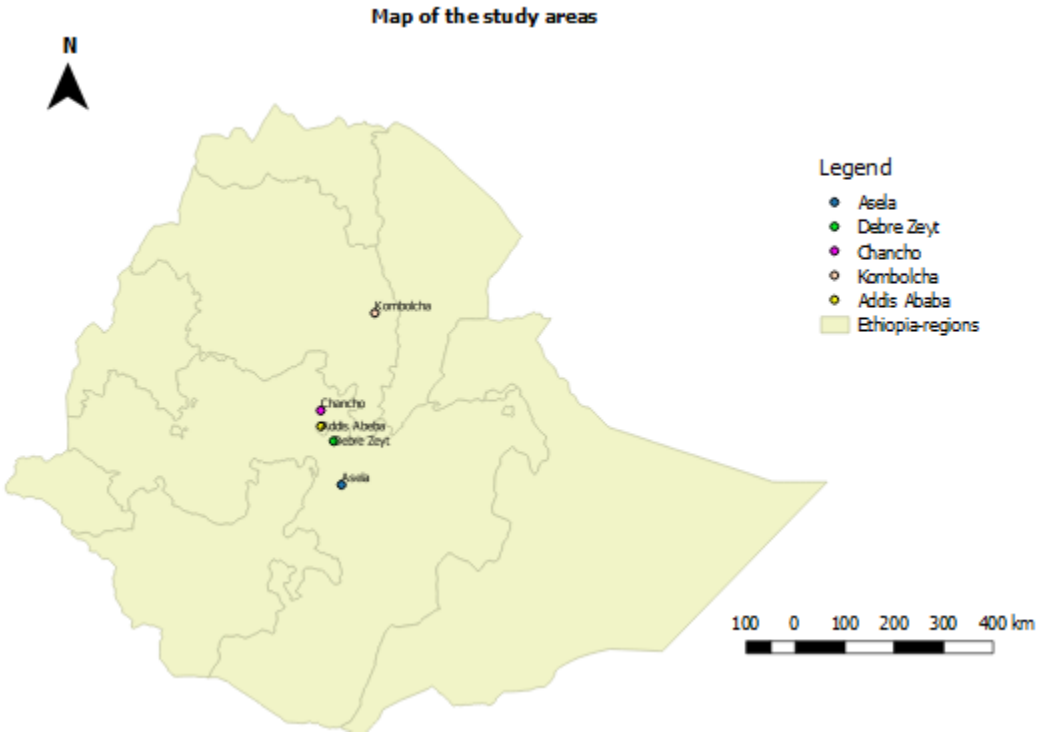


Figure 5: Map of Ethiopia showing the locations of outbreaks of IBD investigated

3.2. Study population

The study population consists of chickens raised by commercial poultry farms in the areas indicated above which were affected by outbreaks of IBD. Sick birds and Bursal tissues from intensive and semi-intensive commercial broiler farms suspected of having IBDV were submitted to the National Veterinary Institute (NVI) from June 2016 to February 2018 for isolation and identification IBD virus. The ages of the flocks ranged from 14 to 50 days.

3.3. Sample preparation

Samples collected were pooled together (with one pool consisting of five bursal tissues from five different chicken showing clinical signs of IBD from one flock). Bursa samples were chopped in to small pieces using a sterile scalpel blade, and minced using a mortar and pestle. A 10% suspension of bursa samples were prepared in sterile phosphate buffer saline supplemented with 1% antibiotic-antimycotic solution. To facilitate the release of viruses, suspensions were freeze-thawed three times between room temperature and -20^{o}C. The suspensions were then centrifuged at 3000rpm for 10minute and the supernatant is harvested, filtered using 0.22µl Millipore filters and stored at -20^{o}C before inoculation (OIE, 2016).

3.4. Cell culture preparation

An immortalized chicken embryo fibroblast cells (DF-1, passage 14) obtained from GD, Netherlands, were revived from liquid nitrogen and re-cultured in 25cm^2 tissue culture flask. The confluent flask was then sub-cultured to multiple 25cm^2 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% CO_2 (OIE 2016, ATCC 2018).

3.5. Laboratory Investigation of Samples

3.5.1. Virus isolation

The bursal suspensions prepared and stored at -20^{o}C were thawed and 0.5ml of the samples inoculated in to each confluent DF-1 cultures in 25cm^2 flasks. After 60min adsorbtion at 37^{o}C, maintenance medium is added to each flask including one uninoculated flask as negative control and incubated at 39^{o}C in a humidified incubator at 5% CO_2. Cells were monitored every 24 h post-infection and inspected for cytopathic effects (CPEs) using an inverted microscope. On 6th day, the cultures were freeze-thawed and the resulting lysate was inoculated into fresh cultures until the third passage (OIE, 2016).

3.5.2. RNA extraction and reverse transcription

Viral RNA was extracted from the samples using the QIAGEN RNeasy Mini kit (QIAGEN, Austin, Texas, USA). The resulting RNA pellets were re-suspended in 70% ethanol, sedimented, dried and dissolved in 50ul RNase-free water according to manufacturer's instruction. Reverse transcription was performed using SCRIPTUM Reverse Transcriptase first strand cDNA synthesis kit (Bio and Sell Company).

3.5.3. Polymerase chain reaction

PCR amplification of the resulting cDNA was performed based on the partial sequence of VP2 gene of IBD virus by using Taq DNA polymerase and IBDV specific designed Forward primer IBDF3 (5'-TGT-AAA-ACG-ACG-GCC-AGT-GCA-TGC-GGT-ATG-TGA-CGC-TTG-GTC-AC-3') and Reverse primer IBDR3 (5'- CAG-GAA-ACA-GCT-ATG-ACC-GAA-TTC-GAT-CCT-GTT-GCC-ACT-CTT-TC-3') 645bp amplification capacity, with negative and positive control reactions (OIE, 2016).

Touchdown PCR was carried out in a final reaction volume of 50µl using 200 µl capacity thin wall PCR tube containing 20µl IQ super mix, 16µl RNase free water, 4µl of each primers and 6µl of cDNA template. PCR reactions were carried out for 1 cycle at 95 °C for 5minutes, for initial denaturation; and 95°C for 30seconds, 60°C for 30seconds, 72°C for 30seconds for 15 cycles and also at 95°C for 30seconds, 56°C for 30seconds, 72°C for 30seconds for 20 cycles and finally elongation at 72°C for 7minutes.

3.5.4. Agarose gel electrophoresis of PCR products

After amplification, PCR products were analysed by electrophoresis on Agarose gel. 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 ladders are 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 in to separate wells. 10 µl molecular marker (Ladder) was also loaded in the first and last lanes. 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. The amplified fragment (amplicon) was visualized and compared with bands of the molecular markers after the gel was placed on an ultraviolet trans-illuminator. A 100 bp DNA ladder was used and the PCR result around 645bp considered positive for IBDV. The gel pictures captured by the camera were saved and printed out for documentation.

3.5.5. Sequencing,sequence analysis and phylogenetic tree construction

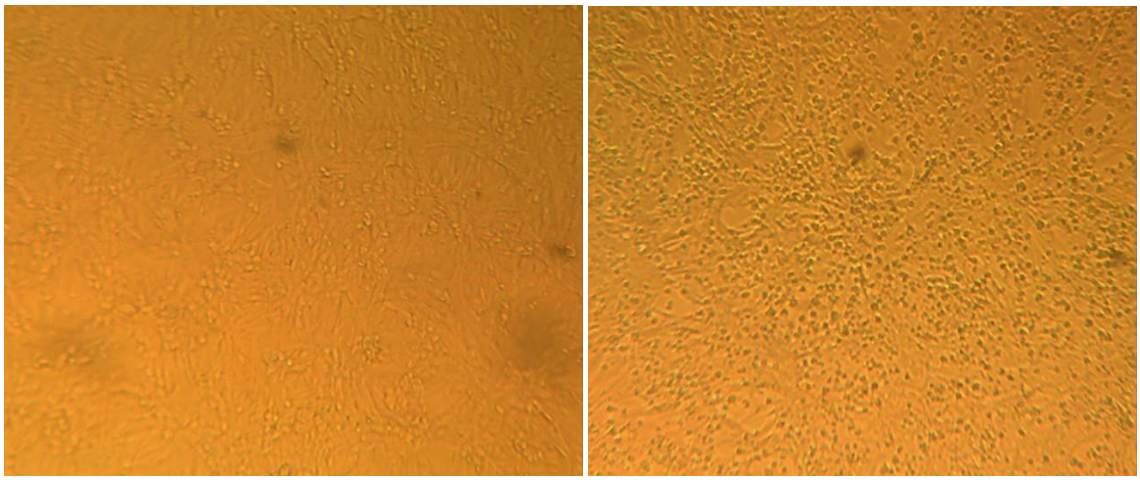
PCR positive product was purified individually using the Wizard SV Gel and PCR clean-up system kit (Promega, Germany) following the manufacturer's instruction. The concentration of the purified DNA was measured using a microvolume Spectrophometer (Nano Drop 2000c, USA). The concentration of the quantified Purified PCR product was adjusted following the requirements set by the sequencing company. The quantified DNA and IBDV VP2 gene specific forward and reverse sequencing primers were added in to the separated labeled eppendorf tubes. The eppendorf tubes containing DNA and primer were sent to the sequencing service company (LGC genomics, Germany). The raw sequence data was edited and phylogenetic tree was construed including the reference strain.

The raw sequence data were edited and fragments were assembled using Vector NTI Advance™ 11.5 software (Invitrogen, Carlsbad, CA, USA). Multiple sequence alignments were performed using the ClustalW algorithm implemented in BioEdit software package to compare the VP2 gene of the outbreak isolates and the vaccine strain. For construction of phylogenetic tree, multiple sequence alignments were performed to align the sequences as codons using the Muscle algorithm in MEGA6 (Tamura *et al*., 2013). The Neighbor-Joining algorithm was used with the maximum composite likelihood nucleotide substitution model with the pair wise deletion option was used. For construction of phylogenetic tree, 1000 bootstrap replicate was used.

4. RESULTS

4.1. Isolation of Infectious Bursal Disease Virus (IBDV)

During this study 9 outbreaks affecting 30250 chickens were investigated (Table 4). From these outbreaks 45 pools of bursa samples (a pool of 5 bursae) were collected and cultured for virus isolation. Viruses were isolated from all of the pooled samples. Isolation was evident by development of visible cytopathic effect of different degree on duck fibroblast cell lines designated DF-1. The growth of the virus was categorized as small, round and reflective cells that detached from the wall of cell culture flask and float in the medium (Figure 6).



A. B.

Figure 6: Uninfected mono layer of DF-1 cells (A) and DF-1 cells infected by IBDV (B) showing cytopathic effect

Table 4: Affected farms investigated for outbreaks

Site	No affected	Mortality (%)
Bishoftu	10700	66.35
Adiss Ababa	3200	41.93
Assela	7350	10.44
Kombolcha	5000	41.42
Sululta	4000	80
Total	30250	47.87

4.2. Results of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Nine of the culture positive samples that were supposed to represent various origins were analyzed by RT-PCR targeting VP2 gene of IBDV. The pair of primers used were IBDF3 (forward) and IBDR3 (reverse) that amplify a 645 bp fragment. All of the samples analyzed showed the presence of 645 bp PCR product (figure 7). The amplified fragment (amplicon) was visualized and compared with bands of 100bp molecular markers after the gel was placed on an ultraviolet trans-illuminator.

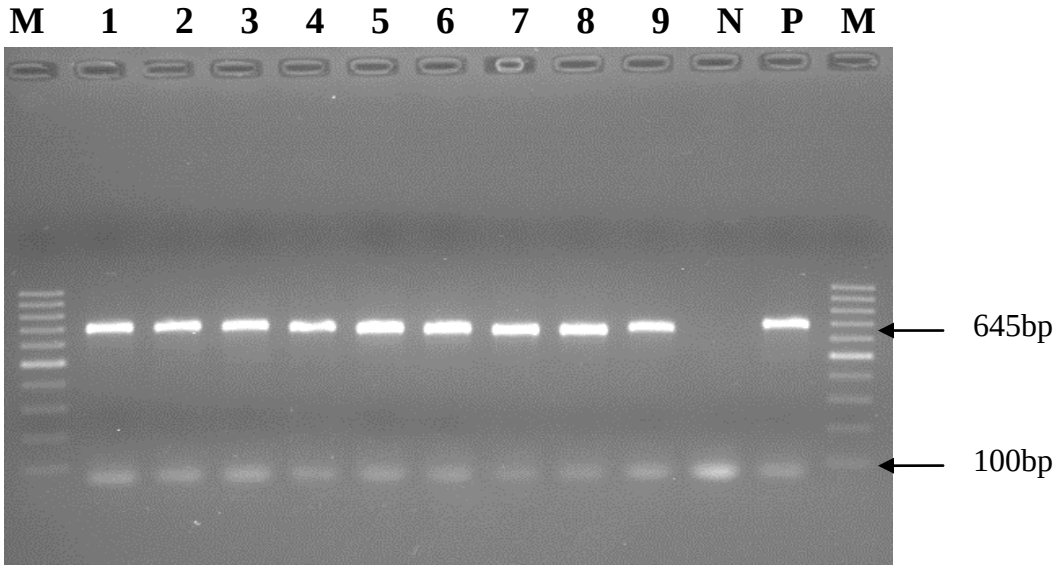


Figure 7: Gel electrophoresis result of the amplification of the VP2 gene of IBDV. M = DNA Ladder (100bp, Fermantas); Lane 1-9 = samples from field outbreaks; N = negative control; P = positive control

4.3. Results of sequencing

The results of sequencing after editing of the raw nucleotide sequence data and aligning the deduced amino acid sequences showed that the field isolates of IBDV isolated in this study all belong to very virulent strains (figure 9; Table 5). The infectious bursal disease viruses isolated from Bishoftu, Asella and Kombolcha all closely resemble the British strain designated UK661 and strain of IBD designated as DV86 than vaccine strains commonly used to vaccinate chicken in Ethiopia such as LC 75 and D 78.

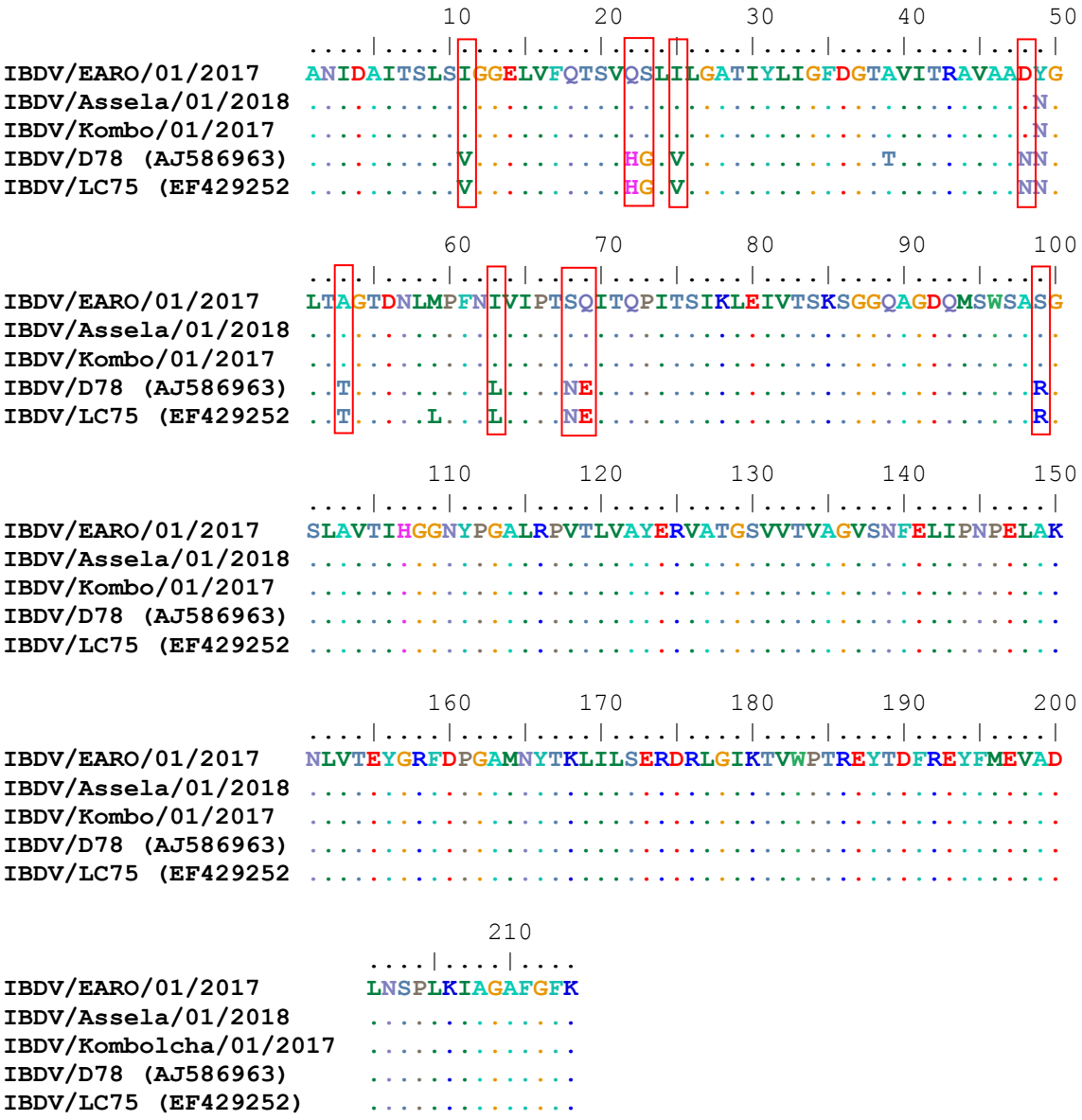


Figure 8: Comparison of aminoacid sequences of the partial VP2 variable domain among field isolates of IBDV and vaccinal strains (aa 232-446 of the full VP2 gene).

Table 5: Comparison of the selected position of amino acids showing differences among field isolates of IBD virus, vaccine strains and prototype strains for VV, CV and AV IBD viruses

Strain	Molecular type	Key amino acids positions				
		222	242	256	294	299
EIAR	VV	-	I	I	I	S
Assela	VV	-	I	I	I	S
Kombolcha	VV	-	I	I	I	S
CEVACIBDL	CV	P	V	V	I	N
D78	CV	P	V	V	L	N
LC 75	CV	P	V	V	L	N
UK661	VV	A	I	I	I	S
DV86	VV	A	I	I	I	S
Edgar	CV	P	I	V	L	N
F52/70	CV	P	I	V	L	N
Del E	AV	T	V	V	L	N
GLS	AV	T	V	V	L	N

VV- very virulent, **CV**- classical virulent and **AV**- antigenic variant

4.4. Phylogenetic analysis

Phylogenetic tree analysis of 22 IBD viruses based on nucleotide sequences of hyper variable coding (VP2) gene partial sequence was done. For this IBDV field isolates, classical/attenuated vaccine strains and reference sequences of classical and very virulent strains retrieved from the Genbank were included in the analysis. The current three field isolates are clustered with the very virulent IBDV isolates and marked with blue colored rectangles (figure 9).

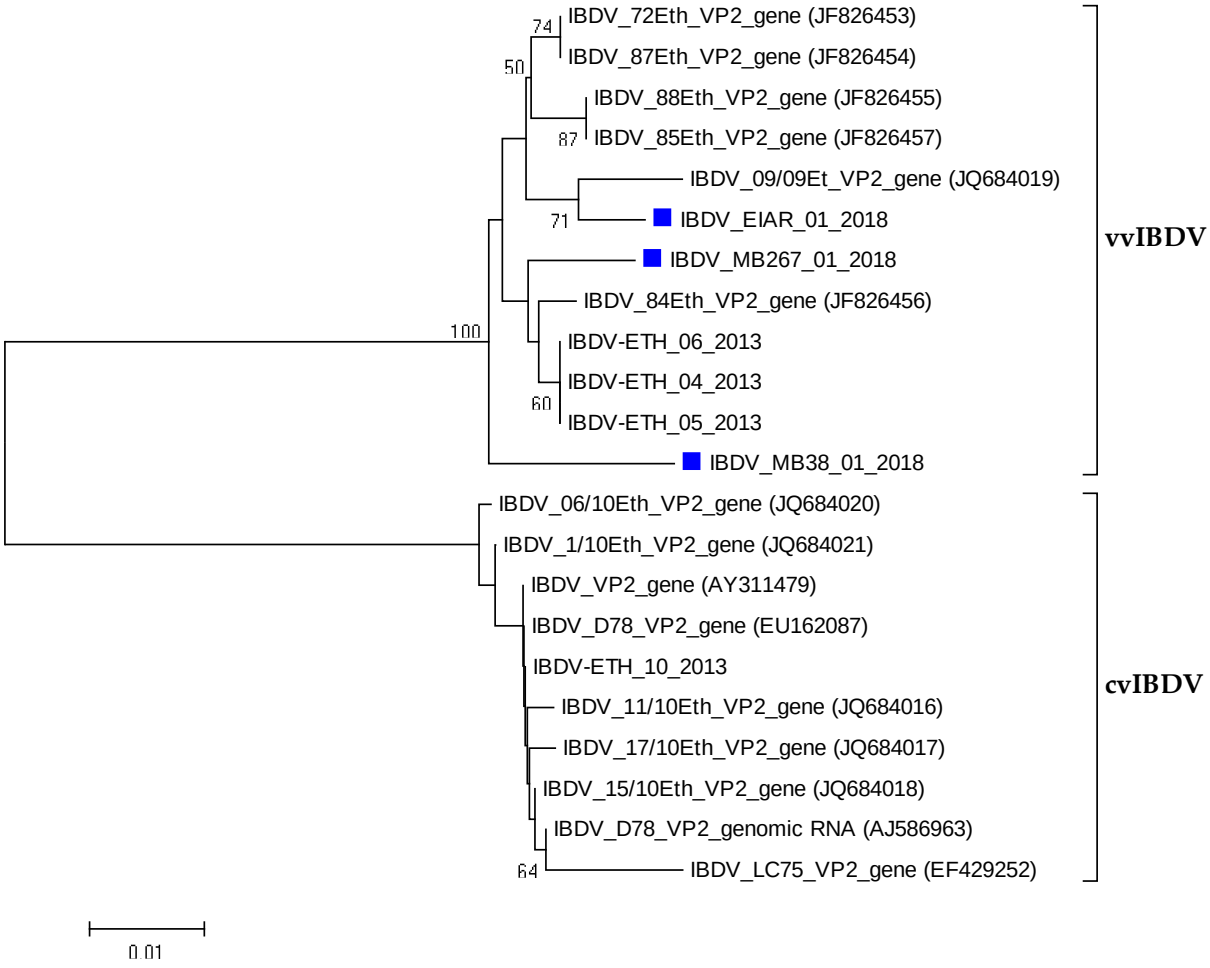


Figure 9: Phylogenetic tree analysis based on the 645bp sequences of the VP2 hypervariable coding sequence of IBDV field isolates, classical/attenuated vaccine strains and reference sequences of classical and very virulent strains retrieved from GenBank were included. The analysis involved 22 nucleotide sequences. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6. The present three IBDV isolates are marked by Blue square. Infectious bursal disease virus isolates grouped phylogenetically into classical virulent (cv) and very virulent (vv) strains

5. DISCUSSION

In Ethiopia, chicken production is very important economic activity that has multitude of functions to the community such as source of cash used to buy clothes, school supplies, medical costs, etc and provide nutritional and socio-cultural services (Hailemichael *et al.,* 2016).The Ethiopian government made chicken production a priority sector for food security. The goal is rise family chicken from 120,000 and number of commercial poultry farms from 290 in 2015 to 416,000 and 2400, respectively in 2020 (Shapiro *et al.,* 2015). To achieve this level of chicken production control of infactious diseases such as infectious bursal disease (IBD) is vital. Vaccination remains affordable option for control of IBD. Understanding of the epidemiology of diseases is useful for optimum control using vaccines, of necessity is the ability to catalogue the potential strains circulating in a given country. To this end our results provided accurate evidence on the types of IBD viruses inflicting mortality in chicken in various farms in central Ethiopia. All of the present characterized isolates are very virulent IBD viruses. Infectious bursal disease virus was isolated from nine outbreaks affecting more than 30,250 chickens inflicting up to 80% mortality. This suggests that the disease is responsible for considerable losses, which should be taken as an alarm for the livestock authorities. The veterinary and livestock authorities should consider the use of effective vaccines for control of IBD among priority intervention activities. As shown in the phylogenetic tree the Ethiopian isolates including the isolates reported in this study, are diversified suggesting the need for investigation of the extent of cross-protection among the field strains and the vaccine strains.

Most commercially available conventional live IBDV vaccines produced and used in Ethiopia are based on classical virulent strains. Those classified as ‘‘mild’’ vaccines exhibit only poor efficacy in the presence of certain levels of maternally derived antibodies and against vvIBDV. ‘‘Intermediate’’ and ‘‘intermediate plus’’ or ‘‘hot’’ vaccines have a much better efficacy and may break through higher levels of maternally derived antibodies, but they can induce moderate to severe bursal lesions and, thus, cause corresponding levels of immunosuppression (Rautenschlein, *et al*., 2005).They may not

fully protect chickens against infection by the vvIBDV strains or by antigenic variants. Safety and efficacy of this type of vaccine still remain a major concern. In addition, it should be kept in mind that the practical on-farm administration of the conventional live vaccines to a large number of chickens is also a technically demanding process, with difficulties inherent to farm-to-farm variability (variable chicks, variable farming conditions, variable skills in vaccination crews, etc.) that should not be underestimated when assessing the results of vaccination programmes (Muller *et al*., 2012).

In Ethiopia vaccines against IBD are either imported or produced from imported master seeds. Effective protection against IBD by vaccination can be obtained when the vaccine is prepared from viruses that are similar antigenically to the field strains causing outbreaks. In this study the sequence analysis of VP2 region of IBD virus showed divergence of the local isolates from the strains used to prepare vaccines suggesting possible spillover of the virus in vaccinated flock. This is in agreement with observation of Aregitu (2015). To this end it has been shown that vaccines prepared from cvIBD viruses offer partial protection against vvIBD viruses, which ultimately cause infection of vaccinated chickens that excrete the virulent viruses. This is considered one reason for appearance of vaccine mutant strains, which are responsible for outbreaks in vaccinated flock (Alkie and Rautenschlein, 2016). In consent to our observation previously an attempt was made to explore molecular epidemiology of IBD situation in Ethiopia in which local IBDV strains were characterized to antigenic level using molecular tools. Characterization of the IBD viruses isolated between 2013 and 2015 revealed some of the features of local IBDV strains previously not reported (Aregitu, 2015). That is, complete understanding of the epidemiology of IBD and its control methods require reliable molecular information. This study provided useful and reliable information on the genetic and antigenic relationship among the field strains causing outbreaks and those deposited in the Genbank.

In addition to genetic divergence, several factors can jeopardize the optimal immunization of vaccinated poultry. These negative factors can be summarized and classified into three main categories: those linked to the vaccine itself, those regarding

vaccine delivery, and those endogenous to the bird. Management conditions are also relevant and should be considered as the fourth factor. As a consequence of inadequate cleansing and disinfection of poultry premises over successive production cycles, the challenge dose could either be high enough to overcome the level of protection induced by maternally derived antibody and/or vaccination or infection might occur before vaccination is performed. This series of events can also occur in large multi-flock layer complexes in which the simultaneous presence of multi-age layer flocks has reduced the possibility of applying an effective all-in, all-out system (Margon and Busani, 2006). Given the physico-chemical characteristics of the virus, thorough cleaning, disinfection with appropriate disinfectant and a strict biosecurity measure must be practiced in order to break transmission cycle of IBDV. This calls into action further investigation of the likely cause vaccine failures.

The epidemiology of variant and vvIBDV in different regions of the world is important for understanding the trend in the evolution, spread and field status of IBDV for effective control of IBD in chickens. Earlier reports indicated Europe as the region of origin for vvIBDV, from where the strains spread rapidly to other regions (van den Berg, 2000). The situation in Ethiopia is not different from this. However, results of the present study suggest that the occurrence of vvIBDV which are more similar to European strains (UK661 and DV86) might have been introduced from Europe. This might have contributed to the adaptation of escape mutant vaccine strains to the new region alongside indigenous field strains (Adamu *et al*., 2013). There have been earlier reports of field isolates having sequences similar to IBDV vaccine strains (Jackwood *et al*., 2008). Ethiopia is one of the importers of these vaccines. Currently IBDL, GUMBOL and HVT-IBD recombinant vaccines are utilized in commercial farms and distribution centers in the country. However, the isolates reported in this study are divergent from IBDL and HVT-IBD.

Infectious bursal disease continues to be a problem in chicken flocks. Because IBD is primarily controlled using vaccination, the failure of vaccines to generate adequate maternal immunity in young chicks must be considered. Several factors can cause

breeder vaccination programs to fail, including the lack of apposite immunity due to newly emerging viruses that are antigenically divergent from vaccine strains. Because IBDV continues to evolve through genetic mutation, a continual surveillance for antigenically unique viruses is needed. Field viruses must be rapidly screened for unique sequence mutations that are known to cause antigenic drift resulting in the ability of IBDV to circumvent immunity generated with current classic vaccines.

6. CONCLUSIONS AND RECOMMENDATIONS

This study has showed that very virulent IBDV strains are circulating in the country affecting commercial poultry farms and distribution centers. Based on the criteria that can define very virulent IBDV- the mortality, nucleotide and amino acid sequences at key positions and their place in the phylogenetic tree – the field strains isolated are phenotypically very virulent which can break high and normally protective levels of MDA. In this study the sequence analysis of VP2 region of IBD virus RNA showed divergence of the local strains from the strains used to prepare vaccines in the country and also from imported vaccines. Based on these conclusions, the following recommendations put forwarded:

- Further study is recommended to develop vaccines from local strains
- Immunogenicity of imported vaccines against local very virulent IBDV challenge should be studied
- All IBD suspected outbreaks must be investigated and the virus need to be characterized genetically and antigenically

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7. ANNEXES

ANNEX 1: Reviving DF-1 cells from liquid nitrogen storage

- Collect all the materials required, prepare the medium and label the culture flask
- 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.
- Avoid getting water up to the cup as this will increase the chance of contamination.
- Then swab the cryovial thoroughly with 70% alcohol, and open it in a bio safety cabinet.
- 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
- 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.
- Gently reconstitute the cells in complete growth medium, transfer them into labeled culture flask and incubate at 37^0C with 5% CO_2.

ANNEX 2: Sub-culturing of DF-1 mono layers

- Prepare the hood, bring all the reagents and materials required to the hood
- Examine the cultures carefully for signs of deterioration or contamination
- Take the culture flasks to a sterile work area, remove and discard the medium
- Add PBS prewash (0.2ml/cm^2) 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.

- Add trypsin (0.1ml/cm^2) 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.
- 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.
- Incubate, with the flasks lying flat, until the cells round up; when the bottle is tilted, the monolayer should slide down the surface.
- 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.
- Add medium (0.1-0.2ml/cm^2), 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.
- Count the cells or follow the splitting ratio
- Dilute the cell suspensions to the appropriate seeding concentration and distribute among flasks.
- Incubate in 5% CO_2 at 37^0C.
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ANNEX 3: Sample preparation and inoculation on mono layer cells

- Tissues are removed from the buffered glycerin or VTM (virus transport media)
- Put in a mortar and wash several times with PBS containing antibiotics and antimycotic
- by using coarse sterile sand, triturate thoroughly and a 10 % suspension is made in PBS with antibiotics and antimycotic

- the suspension is free thawed three times to facilitate the release of viruses from the tissues
- centrifuge at 3000 rpm for 10 minutes and the supernatant is collected and filtered using 0.22µ pore size filter before inoculation

ANNEX 4: RNA extraction

- make all the necessary reagents and consumables ready in and around the work station
- add 350µl of cell suspension to labeled PCR tube and keep the remaining original sample in deep freezer
- add 350µl lysing buffer, vortex and centrifuge for 3min at 12500rpm
- add 350µl ethanol, mix gently with pipette and centrifuge for 1min at 12500rpm
- 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
- add 700µl washing buffer AW1, centrifuge for 1min at 12500rpm and discard the flow through
- add 500µl washing buffer AW2, centrifuge for 1min at 12500rpm and discard the flow through
- 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
- 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 4^0C until cDNA synthesis.

ANNEX 5: cDNA synthesis and rt-PCR

- make all the reagents and consumables ready in and around the work station
- Add 1µl random hexamer (oligodt) and 8µl RNase free water in to a single PCR tube
- Add 5µl sample in to a tube (14µl volume)
- Incubate at 70^{o}C for 10min
- Prepare a cDNA synthesis mixture of reverse transcriptase, DTT stock, Buffer 5X, RNease inhibitor, dNTP and RNease free water
- Add 12µl of the above mix in to a tube (26 µl volume) and incubate the tube with the following program- 55^{o}C/60min, 70^{o}C/10min, 25^{o}C/10min and keep at 4^{o}C.
- Prepare PCR master mix:
  - RNase free water (3µl)
  - Forward primer (2µl)
  - Reverse primer (2µl)
  - IQ super mix (10µl)

  } For a sample
- 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 6: Agarose gel electrophoresis

- Prepare 1.5% Agarose gel and heat in microwave oven until it melts completely
- Pour it on the gel tray with a comb and allow it to solidify on a level surface for about 15min
- Add 4µl gel red with loading dye in to 20µl PCR product
- put the gel on electrophoresis tank full of buffer, Remove the comb, and load 10µl PCR product in each well
- Add the same amount of molecular marker in wells of both flank
- Run electrophoresis for 1:20hr at 120V
- Finally read the result using UV trans-illuminator