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OUTBREAK INVESTIGATION AND MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS AND VACCINE IMMUNOGENESITY TRIAL IN ETHIOPIA

MSc Thesis

By

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LIST OF ABBEVATIONS

AA	Amino acid
Ab	Antibody
AC-ELISA	Antigen-capture enzyme-linked Immunosorbent assay
AGID	Agar gel immunodiffusion test
Bp	Base pair
BF	Bursa of Fabricius
BALT	Bronchial-associated lymphoid tissues
CALT	Conjunctiva-associated lymphoid tissues
CAM	Chorioallantoic membrane
CEB	Chicken embryo bursa
CEF	Chicken embryo fibroblasts
CEKC	Chicken embryo kidney cell
CMI	Cell mediated immunity
CPE	Cytopathic effect
CSA	Central statistical agency
DFA	Direct Fluorescent-Antibody Assay
ELLISA	Enzyme linked Immunosorbent Assay
FAO	Food and agriculture organization
GALT	Gut-associated lymphoid tissues
GC	Germinal centre

GMEM	Glasgow minimum essential medium
HBSS	Hanks balanced salt solution
IBD	Infectious Bursal disease
IBDV	Infectious Bursal disease virus
Ig	Immunoglobulin
IFA	Immunofluorescence Assay
IFN	Interferon
IIF	Indirect immunofluorescence on section of infected organs
IL	Interleukin
ILTV	Infectious laryngo tracheitis virus
MAB	Maternal antibody
MDAB	Maternally derived antibodies
MHC	Major Histocompatibility complex class
NCD	Newcastle disease
NO	Nitric oxide
NVI	National Veterinary Institute
NCD	Newcastle disease
NOS	Nitric oxide synthesis
OD	Optical density
OIE	International Animal Health Organization
ORF	Open reading frames

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PI	Post infection
qRT-PCR	Quantitative real time RT-PCR
RE	Restriction enzymes Digestion with different
RNA	Ribonucleic acid IBD
RFLP	Restriction fragment length polymorphism.
RPM	Rotation per minute
RT-PCR	Reverse transcription polymerase chain reaction
SAN	Specifically IBDV antibody free
SNNP	Southern Nation and Nationalities of people
S/P	Sample positive
SPF	Specific pathogens free chickens
SsRNA	Single stranded RNA
TCID50	Tissue culture infective dose 50
USA	United States of America
VIBD	Virulent IBDV
VNT	Virus Neutralization Test
VP	Virus Protein VP
VP2	Virus Protein2
VP1-VP4	Virus Protein1-4

VvIBDV	Very Virulent Infectious bursal disease virus
WRL	World Referral Laboratory

ABSTRACT

Infectious bursal disease (IBD) is a highly contagious and economically important immunosuppressive viral disease of poultry that is found worldwide. The disease is caused by a dsRNA virus. This study was conducted in National Veterinary Institute from September 2015 to April 2015 on the objective of the isolation, identification and molecular characterization of infectious bursal disease virus (IBDV) on infectious bursal disease (IBD) suspected outbreak samples were collected from 2013 to 2015 from different parts in Ethiopia and to test the immunogenicity of four different live attenuated commercially available infectious bursal disease vaccines and also to select the appropriate infectious bursal disease virus vaccine through the selected different IBDV vaccine immunogenicity test and cross protection test against the local isolated VVIBDV challenge virus and also to determine the appropriate time of IBDV vaccine administering time on vaccinated progeny originated chickens. A representative outbreak samples were employed for virus isolation (cell culture) on chicken fibroblast cell (CFC), molecular characterization (touchdown PCR) and PCR positive isolates subjected to further sequencing. On the other hand the IBDV vaccine immunogenicity test was conducted by employed the four different live attenuated commercially available infectious bursal disease vaccines strain: Namely (one mild intermediate strain - D78, one invasive intermediate strain - Bursine-B2k, and two intermediate plus or hot strain IBD LC75 and IBD EXTREM) and infectious bursal disease virus maternal antibody (IBDV MAb) free specific pathogen free (SPF) chickens of vaccinated progeny and also the recently local isolated very virulent infectious bursal disease virus (VVIBDV) as a challenge virus. The IBDV MAb was screened the experimental SPF chickens before administering the experimental IBDV vaccines. The chickens sera was collected and examined post 14 days of primary immunization, post 7 days of booster dose or prior to challenge test and also 21 days post challenge test serologically by flock IBDV antibody screening ELISA kit to compute the immunogenicity and cross protection capacity against the challenge locally isolated VVIBDV. Among the study objectives and criteria's results were revealed as followed, Eighteen (95%) of bursa and four (80%) of the spleen samples suspensions were grown and develop visible cytopathic effect (CPE) on chicken fibroblast cell cultures (CEF), totally nineteen cell culture isolates (eleven 2013 and 2014 samples and seven 2015 samples) were revealed the expected VP2 gene amplified PCR band (645base pairs); the

eleven 2013 and 2014 positive PCR products sequence analysis were also confirmed the currently circulated IBDV were VVIBDV virus. On the other hand based on those computed parameters, the IBDV vaccine immunogenicity test studies were revealed that the IBDV vaccine administration appropriate scheduled on vaccinated progeny flocks should be at 21 and 35 days age for primary and booster dose vaccination respectively and also the superior immunogenic, potent and efficiently cross protective for recently local isolated VVIBDV was intermediate plus IBDV vaccine strain LC75. Therefore based on the above study results the following points were recommended producing this candidate live intermediate plus IBDV vaccine strain LC75 should be effective to prevent and control the currently circulated VVIBDV virus, commercial poultry farm owners should be screened the IBDV maternal antibody (MAb) of the flock before primary IBDV vaccine administration , and IBDV virus molecular epidemiology study with a planned interval should be conducted to assessed the antigenic diversity of the IBDV virus.

Key words*: Infectious Bursal Disease Virus (IBDV), IBD, IBDV vaccine, polymerase chain reaction, sequencing, IBDV maternal antibody*

1. INTRODUCTION

Livestock are important for human health and wellbeing; they sustain our populations due to their role as a source of essential protein and nutritious human diet through milk, eggs, and meat; source of income, source of employment, soil fertility, livelihoods, transport, agricultural traction, agricultural diversification and sustainable agricultural production, ritual purposes and social status Moyo and Swanepoel (2010) but also plays an important role in the utilization of non-edible agricultural by-products (FAO, 1997) and serve as store of wealth (Oxford., 2012). Additionally in the adverse climatic conditions and natural calamities like drought, flood etc., animal husbandry proved to be a boon for sustaining the livelihood of the landless and marginal farmers (Calpi, 2001). Therefore, sustained growth in the livestock sector has a significant beneficial impact in generating employment and reducing rural poverty.

Apart from livestock sector Poultry production, is one segment of livestock production, and also an important agricultural activity in almost all developing communities in Africa and elsewhere, and also it is the fastest growing components of global agricultural demands because it has a peculiar privilege to contribute to the sector as well as to improve the living standards of the poor livestock keepers (Fisseha, 2009). This is mainly due to their yield quick return on low investment with shorter generation interval (Tadelle and Ogle, 2001; Hailemariam *et al.,* 2006) and fast reproduction cycle compared to most other livestock and its well fitness with the concept of small-scale agricultural development. Moreover, it goes eco friendly and does not compete for scarce land resources and also providing one way of getting food and food security. Poultry production in tropical countries is based on the traditional scavenging system and chickens are the most important poultry species (FAO, 2000). In fact, 80% of the total poultry population in the world is in traditional village-based production systems, being "low input–low output" systems (Permin *et al*., 2000). Rural poultry is the dominant form of poultry production in the developing world including Ethiopia. In some of these countries, scavenging and backyard chicken production systems are more important than the modern intensive poultry production (Hailu *et al.,* 2009).

Ethiopia has the highest number of livestock population in Africa; the poultry population being 56, 866,719 of which according to the CSA (2014/15) Indigenous 54,510,523(95.86%); Exotic 770,052 (1.35%); Hybrid 1,586,144 (2.79) unfortunately however, the contribution of exotic poultry to the Ethiopian economy is significantly lower than that of other African countries (FAO, 2007). In Ethiopia, the major poultry products come from backyards chickens and also have contributed to the country's economy, but they are not productive and huge in number. Although now a day's Ethiopia like other developing countries is experiencing rapid growth in its poultry sector. This is being driven by rising incomes and the expanding middle class, together with the fact that poultry products are among the cheapest sources of protein, and also due to increasing population of the country with an increasing demand for the supply of food. Therefore, intensification and upgrading of the potential of birds will be inevitable to provide surplus products (Hailemariam *et al.,* 2006). However the industry is confronted with a variety of problems, particularly the diseases of viral origin.

Infectious bursal disease (IBD) and Newcastle disease (NCD) have remained as two most important infectious diseases threatening the village chicken and commercial poultry production in most parts of the world (El-Yuguda *et al.,* 2005; El-Yuguda *et al.,* 2009). In Ethiopia, accompanying the intensification of poultry farming; constraints associated with the prevalence of infectious diseases are challenging factors. Among these, infectious bursal disease is the one that become to cause frequent outbreaks and a serious threat and a challenge to the juvenile poultry industry in Ethiopia (Mazengia, 2012).

Infectious bursal disease and also known as Gumboro disease is acute highly contagious infectious and immunosuppressive (Rauf, 2011) viral disease of young susceptible chicks (Lukert and Saif, 1997 and Hair-Bejo *et al.,* 2004) and it affects the poultry industries worldwide (Toro *et al*., 2009) caused by an RNA virus ,infectious bursal disease virus (IBDV) (Mahgoub, 2012; Muller *et al*., 2012) with a primary target organ of the bursa of Fabricius (Abu-Tabeekh and Al-Mayah, 2009). IBD virus remains infectious for a very long period of time and has resistance to commonly used disinfectants (OIE, 2012). The emergence of antigenic variant as well as very virulent strains in vaccinated flocks considerably stimulated research efforts on

both, IBD and IBDV. The disease causes heavy economic losses in poultry industries due to immunosuppression in subclinical cases (Jackwood and Sommer-Wagner, 2010) and in acute cases; it is associated with mortalities, haemorrhages and also bursal damage (Jackwood *et al.,* 2009). The disease is mainly affects young chickens of between 3 and 6 weeks of age characterized by enlarged bursa of fubricus, watery diarrhoea, accumulation of urate in the urinary structure, and severe depression (Saif and Barnes, 2003). IBD is a clinical disease solely in chickens but also turkeys, ducks, guinea fowl and ostriches may be infected.

IBD first described in the USA near the town of Gumboro Delaware in 1962 (Cosgrove, 1962) and first reported in Ethiopia in 2002 at privately owned commercial poultry farm in which 45-50% mortality rate (Zeleke *et al*., 2003) subsequently, IBD has become a priority problem in commercial and backyard poultry production system despite regular vaccination practices (in some cases) using attenuated IBDV D78 vaccine and improved biosecurity measures (Zeleke *et al*., 200 5 a,b). 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). In recent years, highly pathogenic (hotter) strains are emerging via mutation and genetic re-assortment (Mardassi *et al.,* 2004).

The epidemiology of this disease is usually influenced by certain factors like host's immune status (MAb), wide host range, incubation period, thermo-stability and variation in strains of the causative viruses. Currently the emergence of antigenic variant as well as very virulent strains in vaccinated flocks considerably devastated the poultry industries. Improvement in the poultry industry should incorporate emphasis on the prevention and control of diseases that cause economic losses (Okwor *et al.,* 2009). It is therefore identify and characterize new IBDV isolates as soon as they appear and compare them with previously described viruses (Van den Berg, 2000; Van den Berg *et al.,* 2000), rational vaccination schedules and strict biosecurity measures were indicated in many reports as essential tools for the control of IBD (Farooq *et al*., 2003 ; Susan *et al.,* 2013).

Therefore, the objectives of the study were:-

- To isolate, identify and characterize the currently circulating IBDV virus in the country.
- Evaluate the efficacy and cross protection of mild intermediate, invasive intermediate, and intermediate-plus or hot IBDV vaccines through challenge with local VVIBDV virus.
- To select the appropriate IBDV vaccine strain by verifying the immune competence of the immunized chickens upon challenge test.
- To determine the appropriate IBDV vaccine administration time on vaccinated progeny chickens.

2. REVIEW OF THE LITERATURE

2.1. History and distribution of infectious bursal disease virus

Infectious bursal disease (IBD) was first recognized as a distinct clinical entity in 1957 (Cosgrove, 1962). Cosgrove initially described the malady as "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 was a nephropathogenic strain of infectious bronchitis virus due to similar gross changes observed in the kidney by (Winterfield and Hitchner, 1962). Subsequent studies (Pejkovski*, et al.,* 1979) however, revealed that IBV immunized birds could still be infected with the "infectious bursal agent" (IBA) and develop changes in their cloacal bursas specific for the disease. 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.

The immunosuppressive effects of infectious bursal disease virus (IBDV) infections were first disclosed by Allen *et al.* (1972). In 1980, a second serotype was reported (Mc Ferran, *et al.,* 1980). These factors, along with the high tendency for IBD infections to recur in successive flocks, emphasized the need for stringent measures of prevention and control. Prior to 1984, spread of both the clinical and subclinical forms of the disease was satisfactorily controlled by vaccination programs. However, in 1984 and 1985, a significant increase in mortality, condemnations, and vaccine failures were reported in the Delmarva Peninsula broiler growing area (Rosenberger, *et al* 1986). These newly emergent viruses were capable of breaking through maternal immunity against classic strains of IBDV (Rosenberger *et al.,* 1986). *In vivo* reciprocal cross-challenge tests showed that unlike classic or standard strains of IBDV, the field isolates

caused rapid atrophy and minimal inflammation of the cloacal bursa when inoculated into susceptible SPF leghorns (Rosenberger *et al*., 1987). Studies suggested that a major antigenic shift in serotype I viruses had occurred in the field (Snyder *et al.,* 1992).The IBDV field isolates were characterized as antigenic "variants" of serotype 1 IBDV, while the older serotype 1 viruses discovered prior to these newly emergent viruses were called classic strains of IBDV (Rosenberger *et al.,* 1986). Currently in the United States, clinical cases are rarely reported and these variant strains are the predominant viruses circulating in the field (Eterradossi and Saif, 2008). Outbreaks of very virulent IBDV (vvIBDV) were first reported in Europe in 1987 to 1988 (Eterradossi, *et al*., 1992). Highly virulent IBDV (vvIBDV) infections are characterized by a per acute onset of severe clinical disease and high mortality (Van den Berg, and Meulemans., 1991). Although these new serotype 1 viruses demonstrate increased virulence in their ability to break through the existing level of maternal immunity; they are antigenically similar to the classic strains of IBDV (Van den Berg, and Meulemans., 1991). Strains of vvIBDV have rapidly disseminated to every poultry-producing country, except Canada, Mexico, Australia, and New Zealand (Van den Berg, 2000). During the 63rd General Session of the Office International des Epizooties (OIE, 1995), it was estimated that IBD has considerable socio-economic importance at the international level, as the disease is present in more than 95% of the Member Countries (Eterradossi, 1995).

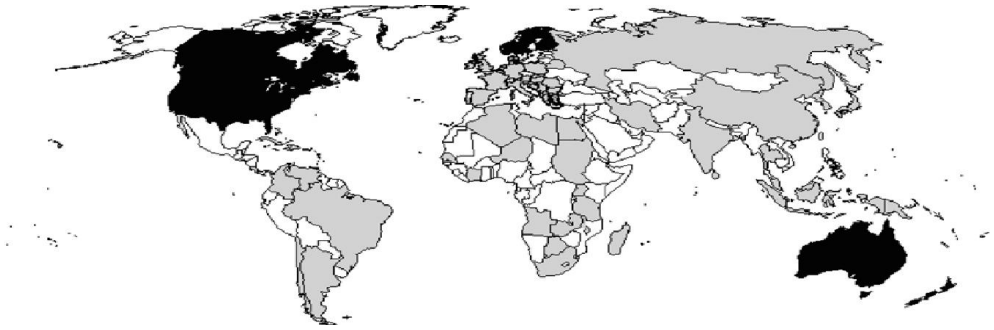


Figure. 1. Worldwide geographical distribution of the acute forms of IBDV**.**

In gray, countries where acute forms have been reported. In black, countries where no acute forms have been reported. In white, countries with no report (updated from Eterradossi, 1995). *Source:* (van den Berg, 2000)

2.2.Disease Definition

Infectious bursal disease also called as Gumboro disease (OIE, 2004) is economically important an acute, highly contagious immunosuppressive viral infection of young chickens (Rauf, 2011) caused by infectious bursal disease virus (IBDV) (Mahgoub, 2012; Muller *et al*., 2012), which belongs to a genus Avibirnavirus (Fauquet *et al*., 2005), of family Birnaviridae (Delmas *et al.,* 2004) that causes disease and mortality in young chickens mainly 3–6-week-old (Van den Berg *et al* 2000; Lukert and Saif, 2003) with a worldwide distribution (Sharma *et al*., 2000). Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. The main clinical signs include watery diarrhea, depression, ruffled feathers, anorexia, trembling, prostration and death after two to three days of clinical signs onset (OIE, 2012). The major post-mortem lesions may include dehydration of the muscles with numerous ecchymotic hemorrhages, swelling and discoloration of the kidneys, with urates in the tubules, inflammation, edema and bursal hemorrhages or atrophy (Chansiripornchai and Sasipreeyajan, 2009). Disease severity depends on the age and breed of the affected birds, the degree of passive immunity and the virulence of the strain of virus (Van den Berg *et al.,* 2000) and secondary infections associated with the immunosuppressive effects of the disease.

2.3.Etiology

Infectious bursal disease virus (IBDV) is an etiology of infectious bursal disease "Gumboro disease", (Mahgoub, 2012; Muller *et al*., 2012), which belongs to a genus Avibirnavirus (Fauquet *et al*., 2005), of family Birnaviridae (Delmas, 2011). It is a double strand an RNA virus (dsRNA) virus (Eterradossi and Saif, 2008) and a non-enveloped, icosahedral capsid with bi-segmented genome (Wu *et al.,* 2007; Zhu *et al.,* 2008). The larger segment, A, is 3261 nucleotides long and contains two open reading frames (ORF) and encodes four viral proteins designated as VP2, VP3, VP4 and VP5 and also the smaller segment B encodes only VP1 which has polymerase activity (Van den Berg, 2000; Lukert and Saif, 2003). The two viral proteins, VP2 and VP3 are structural proteins which form the viral capsid. The epitopes responsible for

the induction of neutralizing and protective antibodies are located on the VP2 protein (Abdel *et al.,* 2001).

2.3.1. Taxonomy of the Virus

IBDV is classified as a genus Avibirnavirus (Fauquet *et al*., 2005), family Birnaviridae (Delmas*,* 2011). The family includes 3 genera: *Aquabirnavirus* whose type species is infectious pancreatic necrosis virus (IPNV), which infects fish, mollusks, and crustaceans; *Avibirnavirus* whose type species is infectious bursal disease virus (IBDV), which infects birds; and *Entomobirnavirus* whose type species is *Drosophila* X virus (DXV), which infects insects (Delmas*.,* 2004).

IBDV has two serotypes of the virus. IBD virus serotype 1 and IBD virus serotype 2. IBD virus serotype 1 is an important pathogen of chickens (Muller *et al*., 2003; Van den berg *et al*., 2004). Serotype 2 viruses are immunologically distinct from serotype 1 viruses since vaccination with serotype 2 viruses did not confer protection against serotype 1 (Van den berg *et al*., 2004). 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 (Lukert and Saif, 2003). Serotype 1 IBD viruses can be classified in a number of ways, based on phenotypic traits (such as antigenicity and pathogenicity) and genetic molecular traits (nucleotide sequence of the gene coding for the viral protein VP2) (Lukert and Saif, 2003). Based on their phenotypic traits serotype 1 IBD viruses can be classified in increasing order of virulence as attenuated (vaccine strains), classical (standard), antigenic variant, and very virulent (also known as hypervirulent) strains (Van den Berg *et al*., 2000; Sapats and Ignjatovic, 2000; Muller *et al*., 2003). Currently, serotype I IBDV viruses are antigenically grouped as classic (also known as standard) and variant strains based on virus neutralization (Wu, *et al.,* 2007; Eterradossi and Saif, 2008). Antigenic variation two serotypes of IBDV are described and distinguished by cross neutralization and cross-protection tests.

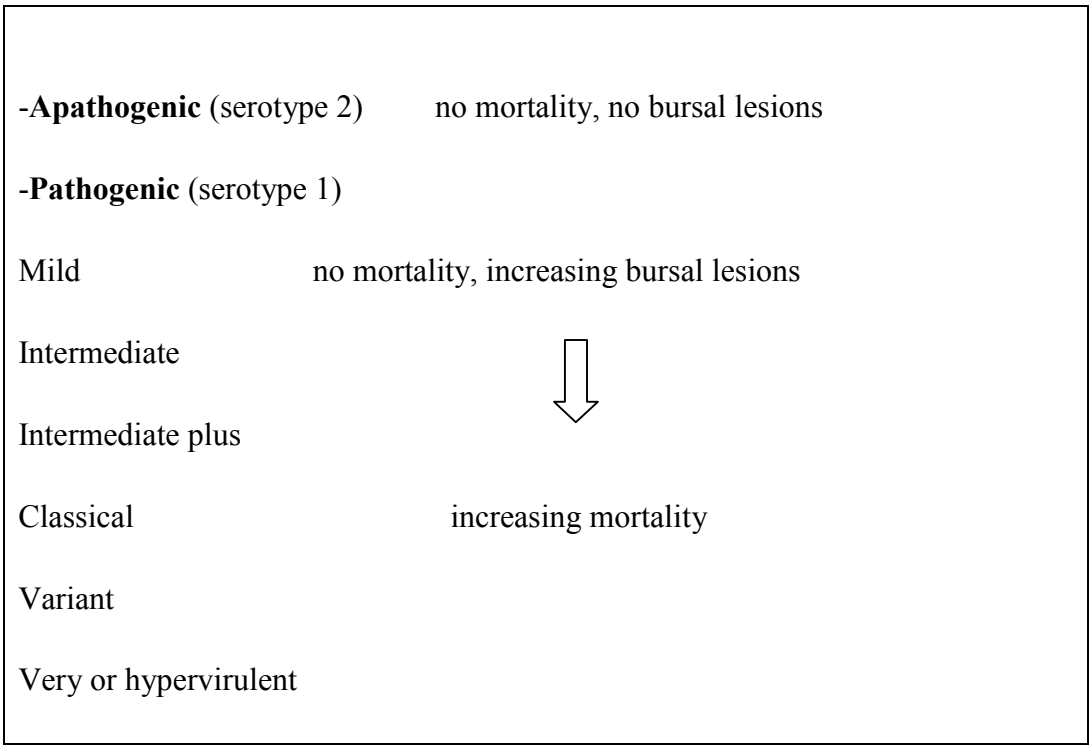


Figure 2. Classification of IBDV strains as pathotypes.

IBDV strains can be defined as apathogenic (serotype 2); mild, intermediate or ‘‘hot’’ (serotype 1 vaccines); classical virulent (IBDV), variant, or very virulent (serotype 1). Serotype 2 strains cause neither mortality nor bursal lesions in specified pathogen free birds. Serotype 1 vaccines cause no mortality but possess residual pathogenicity with bursal lesions varying from mild to moderate or even severe. Virulent serotype 1 strains induce both mortality and bursal lesions.

2.3.2. Morphology of the virus, structural protein and functions of IBDV proteins

Infectious bursal disease virus (IBDV) is a double strand RNA virus (dsRNA) and a non-enveloped, icosahedral capsid with bi-segmented genome (Wu *et al.,* 2007; Zhu *et al.,* 2008). The capsid shell exhibits icosahedral symmetry composed of 32 cashmeres 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 larger segment A encodes four viral proteins designated as VP2, VP3, VP4 and VP5 and also the smaller segment B encodes only VP1 which has polymerase activity (Van den Berg, 2000; Lukert and Saif, 2003). The two viral proteins, VP2 and VP3 are structural proteins which form the viral capsid. The epitopes responsible for the induction of neutralizing and protective antibodies are located on the VP2 protein (Abdel *et al*., 2001).

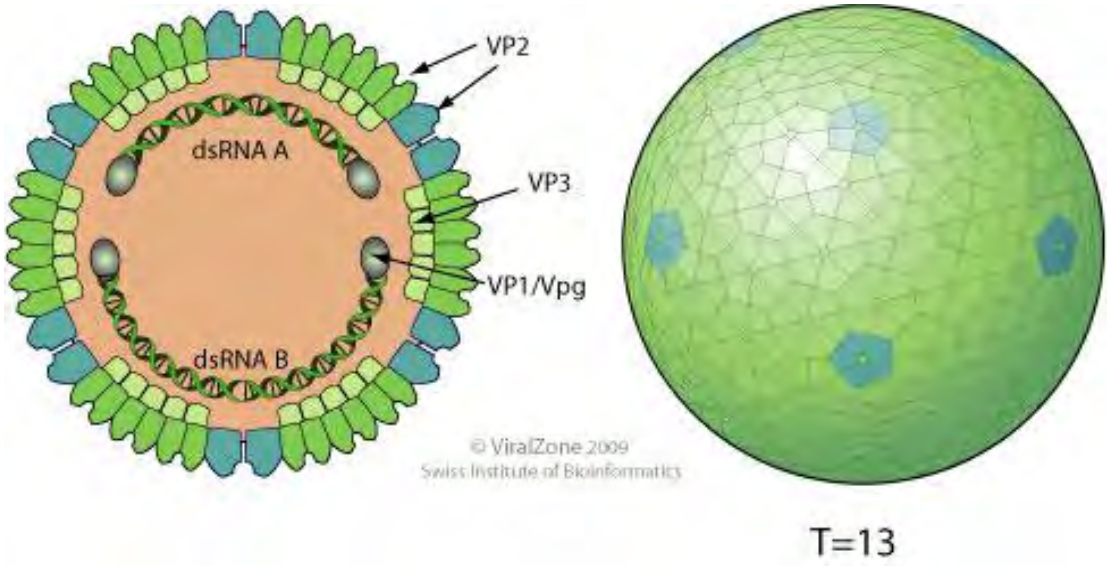


Figure 3. Morphology of the IBDV Virus.

Non enveloped, single-shelled T=13 icosahedral symmetry capsid of about 70 nm in diameter, composed of 260 trimers of VP2 that form spikes projecting radially from the capsid. The peptides derived from pre-VP2 C-terminal cleavages remain associated within virion. VP3 forms a ribonucleoprotein complex with the genomic RNA. Minor amounts of VP1 are also incorporated in the virion.

Source: (Viral zone, 2009)

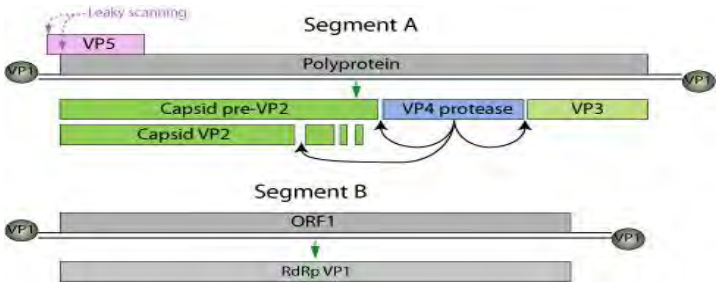


Figure 4. The structure of the segmented linear dsRNA genome.

2 segments (A, 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, 2009).

Table 1: Functions of IBDV proteins

Proteins	Functions	References
VP1	Viral polymerase	(Saugar *et al*., 2010)
	Virulence determinant	(lenouen *et al.,* 2012)
VP2	Host receptor binding	(Ogawa *et al.,* 1998)
	Contains neutralizing epitopes	(Azad *et al.,* 1987)
	Virulence determinant	(Brandt *et al.,* 2001)
	Cell culture adaptation	(Mundt, 1999)
	Apoptosis	(Fernandezarias *et al.,* 1997)
	Endopeptidase activity	(Irigoyen *et al.*, 2009)
VP3	Chaperone activity	(Chevalier *et al.*, 2004
	Antiapoptosis by interacting with PKR	(Busnadiego *et al.,* 2012)

	Suppresses hosts RNA silencing mechanism	(Valli *et al*., 2012)
	Transcriptional activator	(Tacken *et al.,* 2002)
	Forms ribonucleoprotein complex	(Luque *et al.,* 2009)
VP4	Viral protein processing (viral protease)	(Birghan *et al.,* 2000)
	Trans-activate VP1 synthesis	(Birghan *et al.,* 2000)
	Suppresses type I IFN by interacting with GILZ	(Li *et al.,* 2013b)
VP5	Early antiapoptotic effects	(Liu u. Vakharia, 2006)
	Late apoptotic effects	(Li *et al.,* 2012)

Source: (Negash, 2013).

2.4.Epidemiology of the virus

2.4.1. Host range

Clinical disease occurs solely in chickens tut Turkeys, ducks, and ostriches can be naturally and experimentally infected with IBDV serotypes I and II, as evidenced by serological response and isolation; however, the infections are apathogenic. Several other avian species including rooks, wild pheasants, crows, gulls, and falcons, were reported to be susceptible to infection or to possess antibodies against IBDV (Campbell, 2001). Serotype I viruses affect every breed of chicken, but the most severe clinical signs and lesions and the highest mortality rate have been observed in white leghorns (Eterradossi and Saif, 2008). In fully susceptible flocks, mortality associated with classic strain infections may range from 1-60%, with high morbidity of up to 100% (Muller *et al*., 2003; Eterradossi and Saif, 2008). In contrast, vvIBDV strains cause mortality of 50-60% in laying hens, 25-30% in broilers, and 90-100% in susceptible SPF leghorns (Van den Berg *et al.,* 2000). According to (Schat and Xing, 2000; Jarosinski *et al*.,

2005; Asif *et al.,* 2007) reports the induction of a high mortality rate after IBDV infection of susceptible chickens with virulent strains correlated with the ability of the bird to mount a rapid systemic cytokine-mediated immune response, which may lead to a shock-like syndrome followed by death.

2.4.2. Physico chemical nature of the virus

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).

2.4.3. *Route excretion and transmission of the virus*

Infected birds excrete virus in their dropping 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 then 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 (Van den berg *et al.,* 2000; Garriga *et al*., 2006). IBDV 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 (*Alphitobius diaperinus*) (Eterradossi and Saif, 2008). Viable vvIBD virus was recovered after 2 days from

the 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).

2.4.4. Molecular epidemiology and field evolution

The molecular epidemiology of IBDV has been studied in many geographical areas and IBDV evolution was well documented. Particularly, serotype 1 IBDV strains have been circulating in many poultry operations in North and South America, Europe, Asia and in African countries (Zierenberg *et al.,* 2000; Van den berg *et al*., 2004; Jackwood, 2005; Jackwood, 2007; Juneja *et al.,* 2008; Kasanga *et al.,* 2012; He *et al*., 2012b). High mutation rate of the RNA polymerase of RNA viruses generates a genetic diversification that could lead to emergence in the field of viruses, with new properties allowing them to persist in immune populations. In the case of IBDV, these mutations lead to antigenic variation and modification in virulence *in vivo* and attenuation *in vitro*. IBDV undergoes genetic variation during its evolution to adapt to new hosts and to escape the host immune responses. Different biological mechanisms may play important roles for the emergence of novel viruses, particularly in segmented RNA viruses, such as IBDV. Early IBDV isolates frequently showed mutations at the major hydrophilic domains particularly in the loops PBC and PHI, which affected the antigenicity of the strains and induced vaccination failure (Bayliss *et al.,* 1990; Heine *et al.,* 1991; Lana *et al.,* 1992; Dormitorio *et al.,* 1997). In the past few years, several field IBDV strains isolated from different geographic areas showed aa substitutions at the minor hydrophilic domains (Jackwood, 2005; Martin *et al.,* 2007; Duraira *et al.,* 2011; Jackwood , 2011). The VP2 protein has a high mutation rate and it contains the antigenic region responsible for induction of neutralizing antibodies and for serotype specificity. Comparisons of the immunogenic dominant IBDV VP2 protein sequences of the IBDVs offer the best evolutionary clue for vvIBDVs.

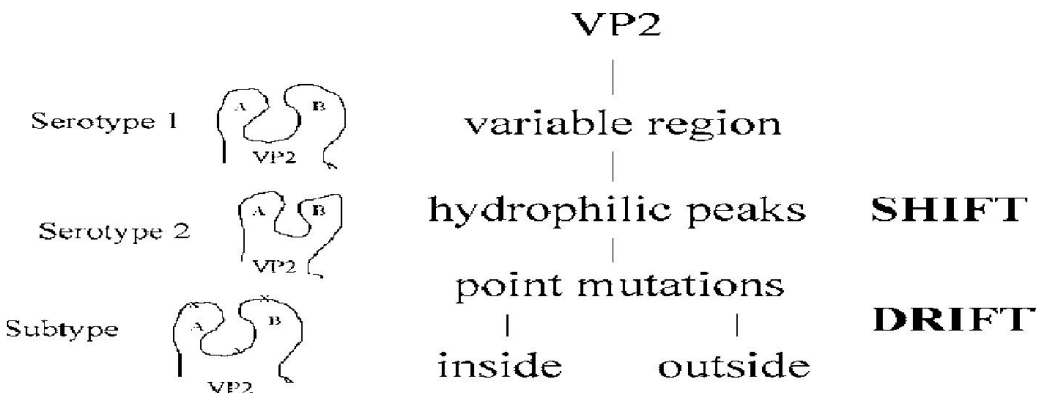


Figure 5. Molecular basis of the antigenicity of IBDV

Source: (Van den Berg, 2000).

Neutralizing antibodies have been shown to bind to VP2, within a minimal region, called the variable domain or vVP2, which is highly hydrophobic with a small hydrophilic region at each terminus. These two hydrophilic peaks are located at the surface of the virus and constitute the neutralizing epitopes. Important changes in the sequences of these peaks might determine an antigenic shift as described for serotype 2 viruses. Point mutations inside or outside these peaks might produce an antigenic drift, giving raise to sub-types such as the serotype 1 variant strains described in the US.

The first outbreak of infectious bursal disease (IBD) that had occurred in 1957 in a broiler farm near Gumboro, the Delaware area in the USA, was caused by the classical serotype 1 IBDV (Cosgrove 1962). The variant IBDV strains then emerged in the 1980's in IBDV-vaccinated farms in the Delmarva area and in the late 1980's, vvIBDV emerged in Europe (Chettle *et al.,* 1989) and rapidly spread across continental Europe and Asia (Lin *et al.,* 1993; Shcherbakova *et al.,* 1998), Middle East (Pitcovski *et al*., 1998), South America (Difabio *et al*., 1999), and Africa (Zierenberg *et al.,* 2000). However, there are significant differences between the African, and European and Asian vvIBDV strains, suggesting independent evolution (Van den Berg, 2000). Based on a recent study, it is expected that, worldwide, about 60 to 76% of IBDV isolates are of vvIBDV genotype (Jackwood, 2007; He *et al.,* 2012b). Most of these viruses have been identified from areas where the viruses have been circulating for a long period of time (Martin *et al.*, 2007). The rest of the isolates are classical and variant strains based on their hVP2

characteristics (Jackwood, 2007; He.*et al.,* 2012b). Molecular approaches allow the identification and differentiation of IBDV strains circulating in chicken populations and associate recent and past isolates (Le Nouen *et al*., 2005).

2.4.5. Antigenic variation of IBDV strains

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

In Ethiopia a recent countrywide study reported IBDV seropositivity rates in backyard chickens to be close to 92% (Chaka *et al.,* 2012; Jenbreie *et al.,* 2012) and IBDV isolates appear clonal and are very virulent. How the vvIBDV strains evolved in Ethiopia remains unclear. Literature suggests that international trade of live poultry and poultry products may facilitate the global spread of IBDV (Cobb, 2011). Almost all acute disease outbreaks in backyard chickens in developing countries remain undiagnosed. VVIBDV isolates from wild birds and backyard chickens were shown to be highly pathogenic for SPF chickens under experimental conditions and maintain virulence marker aa residues across their VP2 and VP1 genes (Hernandez-Divers *et al.*, 2008).

2.4.6. IBDV reassortment, recombination and reversion to virulence

In the case of RNA viruses, such as IBDV biological events including genetic reassortment or recombination alter the phenotypes and genotypes of circulating viruses and compromise their genetic stability. Genetic reassortment might be accountable for the emergence of vvIBDV in the late 1980's in Europe (Hon *et al.,* 2006). The most common reassortant of IBDVs contain segment A of vvIBDV and segment B from attenuated strains indicating the drawbacks of extensive application of live IBDV vaccines. The process of reassortment may be more complex in the field than expected and may involve the interactions of several factors: time, environment and vaccine pressure (Wei *et al.,* 2008).

The risk of live vaccines recombining to generate virulent natural recombinants have been well described, and disease outbreaks associated with these viruses have recently been described for infectious laryngotracheitis virus (ILTV) infections of chickens (Lee *et al.,* 2012). Natural homologous intragenic recombination apart from reassortment may lead to new variants of IBDV (Hon *et al.,* 2008; He *et al.,* 2009a) and also recombination may lead to antigenetically and genetically diverse IBDV populations and the emergence of novel vvIBDV groups (Hon *et al.,* 2008; He *et al*., 2009a). It has the potential to alter the interactions of IBDV proteins and the orientation of the capsid domains preventing neutralization by pre-existing Abs, which lead to vaccine failure.

The existence of RNA virus quasispecies may have a paramount contribution to virus evolution. An RNA virus population is made up of heterogeneous viruses, which share the consensus sequence but differ from each other by one or many mutations (Domingo *et al.,* 1985). In IBDV vaccine and field strains, the quasi species phenomenon has been described by real time RT-PCR and melting curve analysis (Jackwood, 2002; Hernandez *et al.,* 2006). Attenuated live IBDV vaccines are most frequently used to vaccinate commercial chickens. Reversion of these attenuated vaccinal strains to more virulent phenotypes under field and experimental conditions has been frequently reported (Yamaguchi *et al.*, 2000; Jackwood *et al.,* 2008) possibly due to a lack of IBDV polymerase fidelity during vaccine viral genome replication in the host cells. A tissue culture adapted IBDV generated by reverse genetics from a vvIBDV strain reverted

phenotypically and genotypically to the vvIBDV pathotype after inoculation into SPF chickens and maintained this pathotype afterwards (Raue *et al.,* 2004). Genetic reversion of vaccine strains is most likely to be one of the mechanisms that may contribute to the dissemination and persistence of virulent IBDV in the chicken population worldwide.

2.5.Immune response of IBD

IBDV infection in chickens activates all branches of the immune system. However, the level of activation varies depending on the virulence of infecting strains, age, immune status and genetic background of affected chickens. The immune response can be altered by maternal antibody, and the more virulent vaccine strains can override higher levels of antibody. Progeny of parent flocks vaccinated with classical strains of IBD virus may have poor maternal immunity against strains of the virus (Ignjatovic *et al.,* 2001). A high level of maternal antibodies will protect most young chickens against challenge by vvIBD virus for up to 3 weeks after hatching (Van den Berg, 2000). This is borne out by the excellent passive protection provided by maternal antibodies against immunosuppression, bursal lesions, or mortality. The half-life of the passive antibodies varies between depending on breeds, three days (for broilers) and five days (for laying hens) (Brandt *et al*, 2001). Thus, if the antibody titre of a chick at hatch is known, then the time of maximum flock susceptibility to the wild or vaccinal virus can be determined. This information is very important when establishing the timing of vaccination programmes (Van den berg, 2000).

2.5.1. Innate immunity

Influx of macrophages, heterophils and mast cells in the bursa of Fabricius constitutes the early innate immune response to IBDV (Khatri *et al*., 2005; Palmquist *et al.,* 2006; Rautenschlein *et al.,* 2007; Wang *et al.*, 2008). The influx of these cells may be mediated by chemokines (IL-8, iNOS) (Khatri *et al.,* 2005; Eldaghayes *et al.,* 2006; Palmquist *et al.*, 2006; Rautenschlein *et al.,* 2007; Rauw *et al.* 2007; Rauf *et al.* 2011a). The release of these cytokines was suggested to be tightly regulated by NF-κB, whereby its expression was found to be elevated in the bursa during the early phase of IBDV infection (Guo *et al.*, 2012). Nitric oxide released by macrophages may

constitute an early host defence against IBDV and promotes the killing of IBDV-infected and possibly virus-free cells (Khatri *et al.*, 2005; Palmquist *et al.,* 2006; Khatri and Sharma, 2009a).

2.5.2. Humoral immunity and cellular immunity

Humoral immunity plays a significant role in protection against IBDV. All classes of Igs can be produced, but the Ab response may not protect chickens from antigenetically different IBDV strains. Neutralizing Abs is directed against the conformation dependent neutralizing epitopes of VP2 (Snyder *et al.,* 1992). Significant titers of systemic IBDV specific-Abs have been detected in the convalescent sera of chickens that are naturally or experimentally infected with IBDV (Eterradossi and Saif, 2008). Although Ab mediated immunity is crucial against IBDV, Maternal antibody (MAb) provides passive protection in the first few weeks after hatch (Alnatour *et al.,* 2004). MAb positive chickens developed significantly less bursal lesions than Ab negative chickens after IBDV challenge supporting the role of passive immunity in protection (Hassan *et al.*, 2002; Aricibasi *et al.,* 2010). MAb may interfere with the development of an active immune response after IBDV vaccination (Rautenschlein *et al.,* 2005a). Live and inactivated IBDV vaccines may induce vigorous Ab responses in the first few weeks post vaccination (Maas *et al.,* 2001; Aricibasi *et al.,* 2010). Compared to cell culture derived strains, bursal and embryo derived strains induce higher neutralizing Ab titers (Rodriguezchavez *et al.* 2002).

During acute IBD, while bursal follicles are B-cell depleted, T-cells accumulate at the site of virus replication (Kim *et al.,* 2000; Sharma *et al.,* 2000). A notable influx of CD4+- and CD8+ T-cells was detected as early as 1 dpi and peaked at around 7 dpi (Kim *et al.* 2000). Although viral Ag was cleared by week 3 pi, T-cell influx and activation continued to week 12 pi. No T-cell depletion was detected from the bursa during IBDV infection. However, IBDV particles were detected in intra bursal T-cells (Mahgoub *et al.,* 2012). Infiltrating T-cells in the bursa show markers of activation such as up regulated IL-2, major histocompatibility complex (MHC) class II molecules, and IFN-γ mRNA expression (Rauw *et al.,* 2007; Rauf *et al*., 2011b). T-cells are not only involved in bursal recovery by killing virus infected cells, but also contribute to bursal lesions. An important role of the cell mediated immunity (CMI) is suggested by several groups

(Rautenschlein *et al.,* 2002a; Yeh *et al*., 2002). Results of recent investigations on the role of cell-mediated immunity (Yeh *et al.,* 2002) and the significance of virus-specific antibodies (Rautenschlein *et al.,* 2002b) indicate that antibody alone is not adequate in inducing protection against IBDV and that T cell involvement is critical for protection.

2.5.3. Immunosuppression mechanism of IBD

Apart from its immunosuppressive effects, both humoral and cellular arms of the immune system are compromised during the IBDV infection due to lysis of the B cells and altered antigen-presenting cells. Since the virus has a predilection for actively dividing B cells as compared to the mature B cells (OIE, 2012). So that IBDV causes severe immune suppression in young chickens by its lympho cytolytic effects on surface IgM bearing B-cells (Sharma *et al.,* 2000). Although exposure to IBDV compromises humoral and cellular immunity as well as innate immunity in chickens (Khatri *et al*., 2005; Palmquist *et al.,* 2006).

The adverse effect on antibody responses is due to the damage to the B cells in the bursa and the blood. The destruction of Ig-producing B cells by IBDV may be one of the main inhibitors of humoral immunity; however, the involvement of other mechanisms such as altered antigen-presenting and helper T cell functions has been proposed (Sharma *et al,* 2000). Chickens infected with IBDV at 1 day of age were found to be completely deficient in serum immunoglobulin G and produced only a monomeric immunoglobulin M (IgM) (Van den berg, 2004). IgG levels varied depending on the age at the time of infection, virus replication during the acute lytic phase results in a dramatic reduction in circulating IgM + cells and a prolonged suppression of the primary antibody response (Sharma *et al,* 2000). The number of B cells in peripheral blood was reduced after infection with IBDV but T cells were not appreciably affected or T cells are resistant to infection by IBDV (Kim *et al.,* 2000; Sharma *et al.,* 2000).

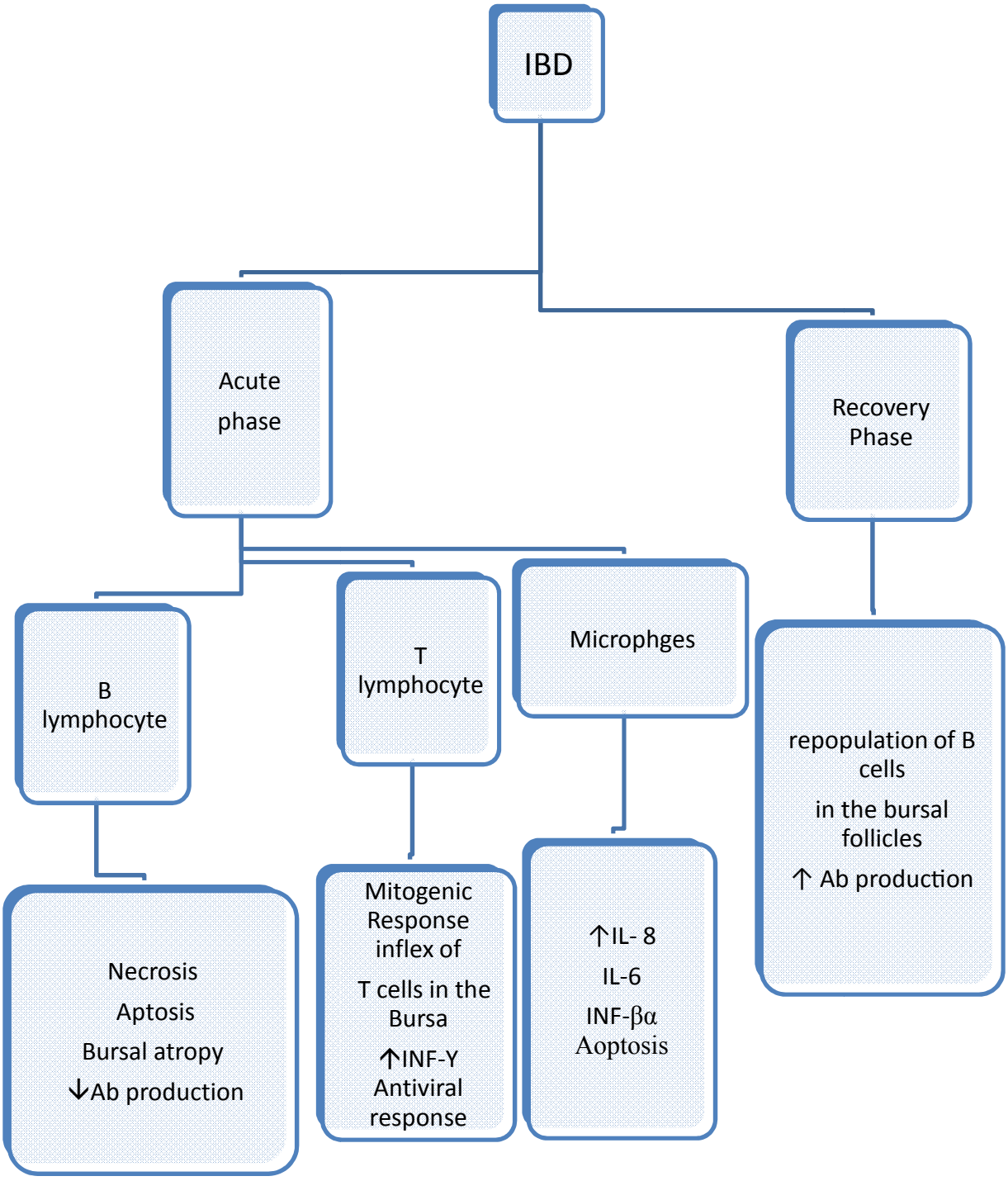


Figure 6.An outline of the pathogenic and immunosuppressive aspects of IBDV.

Source: (Sharma, 2000)

2.6.Pathogenesis and pathological lesions

Following host entry via oral ingestion or inhalation, IBDV may bind to host cell proteins such as N-glycosylated polypeptide(s) expressed on the cell membrane of immature IgM+ B-cells during viral entry process (Luo *et al.,* 2010). A pore forming peptide of the virus (pep46), which is associated with the outer capsid of the IBDV particle, may facilitate viral entry into the cytoplasm of infected cells (Galloux *et al.,* 2007; Galloux *et al.,* 2010). A lipid draft mediated endocytic mechanism was suggested based on the results of an in *vitro* study to support entry of attenuated IBDV to the cells (Yip *et al.,* 2012).

IBDV initiates infection and replication in lymphocytes and macrophages of the gut-associated lymphoid tissues (GALT) (Muller *et al*., 1979). This stage of viral replication marks the primary viremia. Infected macrophages transport the virus to the bursa of Fabricius (BF), the prime target organ for extensive IBDV replication in the cytoplasm of intra bursal IgM+ B-cells (OIE, 2004). After 16 hr post-infection a second viraemia occurs leading to disease and death (Van den Berg, 2000) or the virus destroys the lymphoid follicles in the bursa of Fabricius as well as the circulating B-cells in the secondary lymphoid tissues such as GALT (gut-associated lymphoid tissue), CALT (conjunctiva), BALT (Bronchial) caecal tonsils, (Van den Berg, 2000). Virus dissemination to other lymphoid organs such as to the thymus, bone marrow, spleen, Peyer's patches, cecal tonsils, and Harderian glands may take place mainly during vvIBDV infection of susceptible chickens (Eterradossi and Saif, 2008). As early as 48 hr pi, IBDV infection induces prominent inflammation in the BF. By day 3 to 4 pi all bursal IgM+ B-cells are infected and show cytolytic changes (Cheville, 1967). Clinical signs and death may result from the acute phase (7-10 days) of IBD.

Virus replication during the acute lytic phase results in a dramatic reduction in circulating IgM + cells and a prolonged suppression of the primary antibody response (Sharma *et al,* 2000). Acute disease and death is due to the necrotizing effect of these viruses on the host tissues. If the bird survives and recovers from this phase of the disease, it remains immunocompromised that inhibits protective responses to widely used vaccines against other pathogens and renders chickens susceptible to opportunistic infections (Van den berg, 2004).

The virus preferentially affects actively proliferating and differentiating B lymphocytes, which leads to an age-dependent immunosuppression mainly chicks infected less than one-week of age suffer severe and they may lose the entire bursal B-cells, permanent B-cell immunosuppression which result in permanent immunologic damage (Van den Berg *et al*., 2000; Withers *et al.* 2005).The mature and competent lymphocytes will expand as a result of stimulation by the virus whereas the immature lymphocytes will be destroyed. The bursa is infiltrated by heterophils and undergoes hyperplasia of the reticuloendothelial cells and of the inter follicular tissue (OIE, 2004). The virus infects and destroys actively dividing IgM-bearing B cells in the bursa of Fabricius, this have revealed that replication of IBDV in the bursa is accompanied by an influx of T cells during infection (Kim *et al.,* 2000; Sharma *et al.,* 2000). In addition to B cells, there are reports that macrophages and monocytes may be susceptible to infection with the virus (Khatri *et al*., 2005). IBDV-infected Macrophages have been proposed to serve as virus carriers from the site of infection in the gut to the bursa and other peripheral tissues (Van den Berg *et al*., 2000). Other cells like the bone marrow-derived mesenchymal stem cells may be infected with IBDV (Khatri and Sharma, 2009b). The reticular cells of mesenchymal origin, which reside in the bursal cortex, and periarteriolar lymphoid sheaths, germinal center (GC) and red pulp of the spleen were relatively resistant to IBDV (Biro *et al.,* 2011). Bursal follicular dendritic cells disappeared during IBDV infection probably due to lack of an intact B-cell microenvironment (Kabell *et al.,* 2006).

In addition, activated macrophages may secrete chemotactic, proinflammatory, and other immunoregulatory cytokines that recruit heterophils, T cells, and macrophages to the site of infection. Elevated levels of these inflammatory mediators and chemokines during the acute phase can enhance local inflammation and tissue destruction (Khatri *et al,* 2005; Palmquist *et al.*, 2006). In addition to necrosis, IBDV induces apoptosis or programmed cell death, in chicken peripheral bursal lymphocytes, chicken embryos, chicken embryo fibroblasts, and Vero cells (Tham and Moon, 1996). Moreover, apoptosis has been reported in lymphoid cells and tissues of young chickens and embryos experimentally infected with pathogenic serotype I IBDV strains (Rodríguez-Lecompte *et al.*, 2005). Two viral proteins of IBDV, VP2 and VP5, are suspected to play a role in the induction of apoptosis (Yao and Vakharia, 2001).

Infection with IBDV causes the production of proinflammatory mediators and cytokines in macrophages, which peaks during the early phase of active virus replication (Khatri *et al.,* 2005; Palmquist *et al.,* 2006). IBDV induces expression of the cytokines and cytokine genes: interleukin (IL- 12), interferon (IFN)-g, IL-1b, IL-6 and CXCLi2 in bursal cells (Eldaghayes *et al*., 2006; Rauw *et al.,* 2007), and expression of IL-1β, IL-6, IL-18 and inducible nitric oxide synthase (iNOS) in spleen cells (Palmquist *et al.,* 2006).

Nitric oxide (NO), which is produced by activated macrophages, may promote cellular destruction of both virus-infected and virus-free cells (Yeh *et al.,* 2002). T cells are resistant to infection with IBDV (Kim *et al.,* 2000) may modulate the pathogenesis by limiting viral replication in the BF during the early phase of the disease at 5 days pi, by promoting bursal tissue damage and delaying tissue recovery, possibly through the release of cytokines and their concomitant cytotoxic effects (Rautenschlein *et al.,* 2002a).

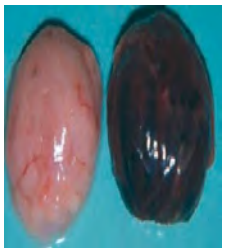
The role of T cells in IBDV-induced immunopathogenesis, and tissue recovery or Depletion of B-cells in the bursa is accompanied by an influx of activated both CD4+ and CD8+ T cells infiltrate the BF reaching maximal levels at 7 days post infection (Kim *et al.,* 2000; Sharma *et al.,* 2000). T-cells are resistant to infection and replication of IBDV. However, IBDV infection can severely decrease the in *vitro* proliferative response of T cells to mitogens, indicating that cellular immune responses are also compromised (Sharma. *et al,* 2000). Evidence suggests that T cells may modulate IBDV immunopathogenesis by limit viral replication in the bursa in the early phase of the disease, but also may enhance bursal tissue destruction, suppress immunity, and delay recovery of bursa follicles through their release of cytokines and cytotoxic effects (Rautenschlein *et al.,* 2002a). At the same time, T-cells may promote clearance of IBDV (Sharma *et al,* 200; Kim. *et al* 2000; Rautenschlein. *et al,* 2002). The role of macrophages and the significance of cytokine release in IBD pathogenesis has been reviewed recently (Van den Berg, 2000).

Generally, the sequellae of IBDV infections such as severity of clinical signs, organ lesions and immunosuppression correlate with the status of immunity, age and genetic background of

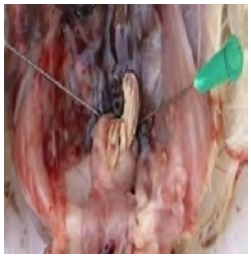
affected chickens and with the virulence of the infecting virus strain (Van den Berg, 2000). SPF chickens infected with vvIBDV develop an earlier onset of mortality and more severe bursal lesions compared to broiler chickens with MAB and vaccinated chickens (Aricibasi *et al.,* 2010). A massive mast cell influx detected in the bursa of SPF chickens infected with vvIBDV may aggravate bursal lesions as typical indicators of acute hypersensitivity responses were observed in the bursa of such chickens (Wang *et al.,* 2008; Wang *et al.,* 2012a). These cytokine mediated bursal lesions may result in an early onset of severe immunosuppression in younger chickens (Rautenschlein *et al.,* 2007). Highly virulent virus strains could also cause depletion of lymphoid cells in the thymus, spleen and bone marrow (Corley *et al.,* 2001). In long standing cases, there is an increased connective tissue mass in the interfolicular areas replacing the depleted lymphoid tissues (Sharma *et al.*, 2000; Negash, 2004).

According to the virus virulence and pathogenicity, IBD cause more severe or less severe lesions on the bursa of Fabricius and other organs such as: spleen, thymus and kidneys, and may induce immunosuppression and mortality in birds (Sharma, 2000; Van den berg, 2004; Eterradossi and Saif, 2008). Macroscopic lesions are observed principally in the bursa which presents all stages of inflammation following acute infection (Muller, 2003). Autopsies performed on birds that died during the acute phase (three to four days following infection) the bursa reveal initially hypertrophic, oedematous and haemorrhagic and its colour turns from white to cream and a yellow transudate covers its serosa early in infection(figure -A and B). The most severe cases are characterized by a major infection of the mucous membrane and a serous transudate, giving the bursal surface a yellowish colour and often accompanied by petechiae and haemorrhages. By the fifth day, the bursa reverts to normal size and by the eighth day becomes atrophied to less than a third of the normal size. Moreover, in the acute form of the disease caused by hyper virulent strains, macroscopic lesions may also be observed in other lymphoid organs (thymus, spleen, caecal tonsils, Harderian glands, and Peyer's patches) (Eterradossi and Saif, 2008). On post-mortem examination the affected animals have hypertrophic and whitish kidneys containing deposits of urate crystals (Figure- C) and cell debris, severely dehydrated carcasses, often darkened pectoral muscles with many petechiae haemorrhages masses in the thigh and pectoral muscles may be present(Figure- D) and are frequently observed, probably due to a coagulation

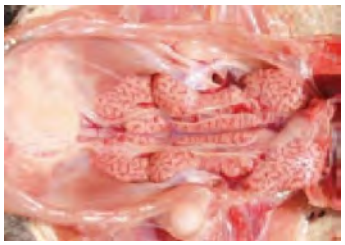
disorder (Skeeles *et al*., 1980). Mucus may also be present within the intestines. Liver appears pale, bile stained and grey foci may also be present on an enlarged spleen (Figure- E).



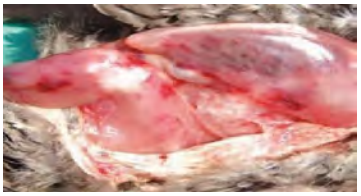
A- 2 affected bursae. Hemorrhagic to severe hemorrhagic



B- Caseous exudates bursa of Fabricius



C. Severe urate diathesis. serous lesions of kidneys



D- Haemorrhages in the affected pectoral, And thigh Muscles

E- Liver appears pale, bile stained

Figure 7. Different post mortem pathological lesions on IBD infection.

Source: *(Lucien Mahin, 2008)*

2.7. Occurrence, courses and clinical findings of the disease

Infectious bursal disease virus infection severity of clinical signs and immunosuppression correlate with the status of immunity, age and genetic background of affected chickens and with the virulence of the infecting virus strain (Van den Berg, 2000). IBD occurs in both layer and broiler birds and although it has been found in turkeys (Eterradossi and Saif, 2008). The age of maximum susceptibility is between three and six weeks (Muller, 2003) corresponding to the period of maximum bursa development, during which the acute clinical signs are observed.

Infections occurring prior to the age of three weeks are generally subclinical and immunosuppressive. Some studies have shown that age of infection is directly related to the degree of immunosuppression, (Ivanyi and Morris., 1976) demonstrated that no immunosuppressive response after 3 weeks of infection despite the manifestation of a clinical disease. Variant IBDV strains do not produce overt clinical signs, but cause immunosuppression, which is the most significant economic losses, result from subclinical infections and may cause mortality due to secondary opportunistic infections in immune compromised birds (Van den Berg *et al.,* 2000; Rodriguez, 2002; Eterradossi and Saif, 2008). In contrast, vvIBDV strains cause mortality of 50-60% in laying hens, 25-30% in broilers, and 90-100% in susceptible SPF leghorns (Van den Berg, 2000). In fully susceptible flocks, mortality associated with classic strain infections may range from 1-60%, with high morbidity of up to 100% (Van den Berg, 2000; Muller, 2003).

The disease has an acute and per acute course. The incubation period is very short 2-3 days. However, (OIE, 2004) recommends an incubation period of 7 days for regulatory purposes. Virus excretion can begin as early as 24 hours after infection. Mortality will peak and recede usually in a period of 5-7 days (OIE, 2012). Accompanying symptoms include the disease has been described worldwide (Van den Berg *et al.,* 2000) as acute onset of depression, trembling, white watery diarrhoea, ruffled feathers, severe prostration, vent picking, vent feathers soiled with urates, anorexia, dehydration, and elevated water consumption.

2.8.Diagnosis

Generally a preliminary diagnosis can usually be made based on flock history, clinical signs and post-mortem (necropsy) examinations. Necropsy examination will usually show changes in the bursa of Fabricius such as swelling, oedema, haemorrhage, the presence of a jelly serosa transudate and eventually, bursal atrophy. Pathological changes, especially haemorrhages, may also be seen in the skeletal muscle, intestines, kidney and spleen; however, definitive diagnosis can only be achieved by the isolation and/or specific detection and characterization of IBDV (OIE, 2012).

2.8.1. Serological diagnosis

Serological tests such as AGID, ELISA, and VNT for detecting antibodies are used for monitoring vaccine responses and might be additional information for diagnosis of infection of unvaccinated flocks (OIE, 212). The enzyme linked immuno sorbent assay (ELISA) is the most commonly used test for the detection and quantification of IBDV antibodies to check response to vaccination, natural field exposure and decay of maternal antibody titer (Lukert and Saif, 2003). It is economical, simple, and quick tests a large number of samples at the same time and is adaptive to automation to computer software (Lukert and Saif, 2003).

Viral antigens can be demonstrated by the agar-gel precipitin assay or by the antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) (Islam *et al.,* 2001a). The VN titers accurately correlate with protection of chickens against IBDV (Knoblich *et al.,* 2000). Differentiation of classic and variant strains has been made by using ELISA and monoclonal antibodies (Sapats *et al.,* 2005). However, these methods may not be as rapid and sensitive as molecular methods (Jackwood, 2004).

2.8.2. Virological diagnosis

Infectious bursal disease virus may be detected in the bursa of Fabricius of chicks in the acute phase of infection, ideally within the first three days following the appearance of clinical signs (OIE, 2012). The viral antigens specific to IBDV may be detected by direct and indirect immunofluorescence (Abdel-Alim and Saif, 2001) or by immuno peroxidase staining (Cho *et al.*, 1987) in the bursal follicles of infected chickens between the fourth and sixth day after infection, Immunofluorescence (Macdonald, 1980) and electron microscopy (McNulty *et al*., 1979) of the infected cell culture or embryonated eggs are valuable tools for monitoring the growth of IBDV particularly those strains lacking pronounced cytopathic effect (CPE).

2.8.2.1.Embryo inoculation

The inoculation of bursal homogenates from IBDV infected chickens per the chorioallantoic membrane of 9-10 days old embryonated SPF (Specific-pathogen-free) chicken eggs is the most sensitive diagnostic method for virus isolation. The most sensitive route of inoculation is the CAM; the yolk sac route is also practicable (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 (Brandt *et al.,* 2001; Islam *et al.,* 2001c) but are not readily adapted to grow in CEF (Chicken embryo fibroblasts) or CEK (Chicken embryo kidney) (Lee and Lukert, 1986). Variant viruses however, do not kill the embryos but cause embryo stunting, discoloration, splenomegaly and hepatic necrosis (Lukert and Saif. 2003).

2.8.2.2.Cell culture

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). 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).

2.8.3. Molecular characterization

The classical methods for molecular characterization and differentiation of IBDV field isolates include reverse transcriptase polymerase chain reaction (RT-PCR) and restriction fragment

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

The molecular differentiation of IBDV strains using VP2 has been improved by use of labelled probes in real-time RT-PCR (Jackwood and Sommer, 2005). In recent years detection of nucleotide variation has been facilitated by application of melt curve analysis. A TaqMan qRT-PCR and melting curve analysis can be used to trace mutations in the hVP2 region (Jackwood *et al.,* 2003) this method allows comparing sequences between field and vaccinal strains (Jackwood and Sommer., 2002; Gao *et al.,* 2007). It determines a single nucleotide polymorphism in VP2 (Wu *et al*., 2007a). Genetic typing according VP2 sequence of IBDV has been widely used as a means of tracing the spread of IBDV and virulence change (Jackwood and Sommer-Wagner., 2007).

2.9.Treatment, prevention and control

2.9.1. Treatment

No therapeutic treatment has been found to have an effect on the course of the viral infection; however birds may be helped with drugs to treat symptoms so as to control secondary agents and the effects of immunosuppression (Muller, 2003).

2.9.2. Prevention and Control

2.9.2.1.Management and hygiene procedures

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

2.9.2.2.Vaccine and Vaccination

Immunization of chickens with high quality vaccines is the primary method of control of many poultry infectious diseases; However IBDV is resistant to a large variety of disinfectants and is environmentally very stable but mainly controlled by vaccination (Van den Berg, 2000; Muller *et al.,* 2003; Dacic *et al*., 2008) with a proper vaccination schedule. Vaccines developed against the Gumboro disease were effective for about 25 years. The first vaccines to prevent IBD in broilers and replacement pullets were prepared by adaptation of field isolates in embryonated eggs (Edgar and Cho, 1965; Edgar and Cho 1973).

Rational vaccination schedules and strict biosecurity measures were indicated in many reports as essential tools for the control of IBD (Farooq *et al.,* 2003). Vaccines and vaccination programmes vary widely, depending on several local factors (e.g. type of production, level of biosecurity, local pattern of disease, status of maternally derived antibodies (MDAbs), vaccines available, costs and potential losses). Many previous studies proved the role of the MDAbs in protection against IBDV in chicks (AI-Natour *et al.,* 2004). In *vivo* cross-protection studies, vaccination-challenge studies, and progeny challenge studies are frequently performed for assessment of IBDV vaccine efficacy and to determine the pathogenicity and antigenic

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

Traditionally, breeder flocks are hyper immunized by priming with live vaccines and boosting with killed vaccine prior to laying in order to confer high titers of MAb to their progeny (Van den Berg *et al.,*2000; Maas *et al.,* 2001; Muller *et al.,* 2012) and is applied in some countries. This passive immunity protects chicks against early immunosuppressive infections for 1 to 3 weeks; however, protection may be extended to 4 or 5 weeks by boosting the immunity in breeders with oil-adjuvanted vaccines (Eterradossi and Saif, 2008). Serological monitoring of the antibody level in a breeder flock or its progeny can aid in determining the right time to vaccinate (Eterradossi and Saif, 2008; OIE, 2012). According to literature (Van den Berg, 2000) oral, nasal or ocular mild vaccines were effective only in immunizing chicks that had passively acquired neutralizing antibody titers lower than 100.

2.9.2.3.Type of vaccines

Most of the commercially available IBDV vaccines against IBDV are live attenuated and inactivated and also IBD Immune complex (IBD-ICX) vaccines

Live IBDV vaccines

Live vaccines are produced from classical and variant IBDV strains by passaging these viruses in tissue cultures or embryonated chicken eggs (Jackwood and Sommer-Wagner., 2011). Several live-attenuated virus vaccines that differ according to their virulence and antigenic characteristics are available commercially. With regard to virulence or residual virulence for SPF chickens, and

the level of attenuation vaccine strains are classified as mild, mild intermediate, intermediate, intermediate plus, or "hot," (Van den berg *et al.,* 2004; Eterradossi and Saif., 2008; OIE, 2012). Live-attenuated vaccines are administered via drinking water application or nebulisation between the ages of 7 days and 2 or 3 weeks (Van den Berg *et al.,* 2000; Eterradossi and Saif, 2008).

Live vaccines are favourable for mass application through drinking water and can induce strong humoral and cellular immunity (Muller *et al.,* 2003; Muller *et al.,* 2012). The proven reversion to virulence (Yamaguchi *et al.,* 2000) and their residual immunosuppressive effects (Rautenschlein *et al.,* 2005b; Rautenschlein *et al.,* 2007) are major safety concern of their extensive field applications.

Killed IBDV vaccines

Killed-virus vaccines in an oil adjuvant are often used to boost levels of maternal antibodies and confer longer-lasting immunity in breeder hens. 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). These vaccines are not ideal for stimulating a primary antibody response; therefore, they tend to be most effective in chicks that have been "primed" with a live virus vaccine or naturally infected through field exposure to IBDV (Eterradossi and Saif. 2008). Currently, many oil-adjuvant vaccines contain both classic and variant IBDV strains (Eterradossi and Saif. 2008). Killed-virus vaccines are administered by subcutaneous or intramuscular injection at sixteen to twenty weeks of age (Van den Berg *et al.,* 2000).

IBD Immune complex (IBD-ICX) vaccines

IBD immune complex (IBD-ICX) vaccines are found to be safe and efficacious for in *ovo* and post hatch vaccination of broilers (Giambrone *et al.,* 2001; Ivan *et al.,* 2005) is commercially available in some country. They are prepared by combining an IBDV-hyper immune serum with live intermediate plus IBDV (Johnston *et al.*, 1997). The viruses are released from the ICX when the levels of MAB declined to induce specific humoral immune responses that protect chickens

against challenge virus. A recombinant neutralizing Ab has been evaluated for formulation of an IBD-ICX vaccine (Ignjatovic *et al.*, 2006).

2.9.3. *Vaccination failures and potential causes*

In general vaccine efficacy highly depends on the dose and strains of the vaccine and challenge viruses, as well as, the route of administration, the appropriate vaccination time, and the levels of maternal antibodies (OIE., 2012).The potential causes that affect the outcome of an IBDV vaccine are largely based on the gap on correlation between strains of the vaccine with pathogenicity and antigenicity type of the circulated virus, the appropriate vaccination time, the age and the breed of the bird, and the presence or absence of neutralizing antibodies (MAB) and the vaccination history of the progeny of parent flocks, which determine the efficacy of IBD vaccination. In addition to this vaccination is not usual practice in smallholder poultry and control is further complicated by the regular emergence of new strains that may not be covered by existing 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).

The causes of failure of live-virus vaccinations are numerous. The most trivial cause's are interference from MAB (AI-Natour *et al.,* 2004) is one of the most frequent causes of failure. The duration and uniformity of this immunity may be influenced by the concentration and antigenic specificity of the vaccine strain (Van den Berg *et al.,* 2000). Therefore it requires continuous monitoring of the antibody level in a breeder flock or its progeny to aid in determining the right time to vaccinate (Eterradossi and Saif, 2008). Classical live attenuated vaccines may induce broad, lifelong protection, but they also carry residual pathogenicity and the potential to revert to virulence (Van den Berg *et al.,* 2000) but inactivated vaccines failure is rare, but may occur, either due to the absence of previous contact of some of the birds with a live virus (vaccine virus), or to the existence of antigenic variants not present in the vaccine .These vaccines are not ideal for stimulating a primary antibody response; therefore, they tend to be

most effective in chicks that have been “primed” with a live virus vaccine or naturally infected through field exposure to IBDV (Eterradossi and Saif. 2008).

2.10. Economic Importance

The economic impact of IBD in fowl is serious and influenced by strain of virus, susceptibility and breed of flock; inter current primary and secondary pathogens, and environmental and managemental factors. Clinical IBDV leads to direct losses due to high mortality, in addition, condemnation of carcasses due to skeletal muscle, thigh and pectoral muscle haemorrhages can be an important cause of economic losses (Mc.Ferran, 1993, van den berg, 2004). Indirect losses in Gumboro disease arise due to the severe immunosuppression of broilers and egg laying hens and their increased predisposition for other diseases and vaccination failure (Van den berg, 2004). Thereby, as a consequence, they result delayed growth, reduced weight gain, greater food conversion, longer fattening, lesser production values, increased mortality and lower quality of products observed (Sharma, 2000). The occurrence of vvIBDVs has increased the economic importance of the disease. Until 1987, the strains of the virus were of low virulence, causing less than 2% mortality, and vaccination was able to satisfactorily control the disease. However, the occurrence of vvIBDV has led to vaccination failures, and increased mortality and morbidity (van den Berg, 2000). In 80% of the OIE member countries, acute clinical disease due to IBDV has been reported (van den Berg, 2000).

The presence of disease may also limit opportunities in the market place, either locally or internationally, and hinder the adoption of improved technologies, be they improved breeds, better management systems or more efficient processing and marketing methodologies. There would be further loss of income for an extended period because of the stamping-out policy. The disruption to the flow of product and decreased production may cause job losses on farms and in service and associated industries, depending on the time it takes to bring the outbreak under control. Even a small outbreak would result in dislocation of the industry and its normal marketing patterns. An uncontrolled outbreak would markedly increase production costs because of the impact of the disease and the need for continuing control measures.

2.11. The status of infectious bursal disease (IBD) in Ethiopia

Infectious Bursal Disease is a newly emerging disease of chicken in Ethiopia, as described by Zeleke *et al.*, (2005) the disease has been speculated to be introduced concurrent with the increased number of commercial state and private poultry farms flourishing in the country. Research and case reports coming from various regions of the country indicated that viral diseases are posing a growing threat to the young poultry industry flourishing in the country (Alemgotr, 1987; Zeleke *et al*., 2002; Zeleke *et al*., 2005, Zeleke *et al*., 2005a, b; Woldemariam and Wossene, 2007; Mazengia *et al.,* 2009). Therefore infectious diseases like IBD are becoming real threats to chicken production (Alamargot 1987; Zeleke *et al.* 2005). Frequent outbreaks and occurrence of new strains of infectious bursal disease became a challenge to the juvenile poultry industry in Ethiopia (Mazengia, 2012). Over the past few years, 25 to 75% of the deaths/losses in exotic and cross chickens have been associated with infectious bursal disease (Zeleke *et al*., 2002; Zeleke *et al*., 2005a; Woldemariam and Wossene 2007).

Gumboro disease was first reported in 2002 in Ethiopia at privately owned commercial poultry farm in which 45-50% mortality rate was documented (Zeleke *et al*., 2003) and diagnosed first in commercial poultry (Zeleke *et al.,* 2005b) and thereafter in a government-owned poultry multiplication center (Woldemariam and Wossene, 2007) and a commercial broiler farm (Chanie *et al.*, 2009) with serological tests. In addition to the above different serological studies molecular characterization of the Ethiopian IBD virus isolates was done for the first time in 2005 from the samples collected from Kombolcha Poultry Multiplication Center, and in commercial and breeding poultry farms in Ethiopia between 2009 and 2011 (Jenbreie *et al.,* 2012). In both cases the samples were processed at the National Veterinary Institute, Ethiopia, for virus isolation using chicken fibroblast cell culture, and the positive isolates were submitted to OIE-IBD Reference Laboratory, AFSSA, France, for further antigenic and genomic characterization, and were identified as virulent classical viruses and very virulent IBD virus. In all cases the situation of the disease at small scale commercial flocks, and back yard poultry farms indicate the disease is widely distributed in the country, More over chicken traders also suffer from huge financial losses due to IBDV mortality in chicken, particularly those who buy young aged chicken and rear them for several weeks after purchase (Zeleke *et al*., 2005).

Prior to February 2006, the health measures at the government owned poultry multiplication and distribution centres with the exception of Bonga and Bedelle, all the centres were devastated by the outbreak of Infectious Bursal Disease i.e. Gumboro disease (Yilma, 2007) and a commercial broiler farm (Chanie *et al.,* 2009**).** On the other hand most of the researchers (Zeleke *et al.,* 2005a and b ; Degefu *et al*., 2010, Mazengia *et al.,* 2012) in Ethiopia performed serological surveyed in different parts of the country and documented results indicates that IBD is a threat on both backyard chickens and commercial chickens. Currently, IBD is the most important threat to poultry production in the country (Zeleke *et al.,* 2005b) and widely distributed in all regions in the backyard chickens, commercial farms and poultry multiplication centres. The disease has since spread to all investigated commercial farms and multiplication centres occurring at an average outbreak rate of 3-4 farms per year (Ethiopia animal health yearbook, 2011).

The studies summarized above indicates the presence of the disease in many parts of the country and is despite the fact that IBD incidences are increasing at alarming rate all over the country where commercial poultry production is intensified and even in the backyard chickens, and also a serious problem for the poultry industry of Ethiopia. This disease has incurred considerable economic loss to the country and has been posing a challenge especially for the success of vaccines used at this time (Wit and Baxendale, 2004). A good example is failure of NCD vaccination in areas where there is no integrated approach for the control of IBD (Wit and Baxendale, 2004; Woldemariam and Wossene, 2007). 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). Considering the significant economic losses associated with IBDV, the development and evaluation of new generation IBDV vaccine are important to minimize the effects of these agents and design suitable preventive and control measures this tendency of growing poultry industry (Mazengia, 2012).

3. MATERIALS AND METHODS

3.1.Study Area

Accordingly, clinical materials (Bursa and Spleen) were collected from IBD suspected chicken reared under semi-intensive and intensive poultry farms located at Tigray (Mekele), Amhara (Kombolcha and Bahirdar in and around), Southern Nation and Nationalities of people (SNNP) (Wollita, Dilla); Addis Ababa, Oromia (Bishoftu, Ada, Haramaya University) (figure. 8) for IBD outbreak investigation. In addition to this infectious bursal disease (IBD) outbreak investigation sample collection areas, the study of IBD vaccine immunogenicity test and the investigation of those samples were conducted at National Veterinary Institute (NVI) which is located at the side of Addis Ababa University, Collage of Veterinary medicine and Agriculture, Debre Zeite. Addis Ababa has an altitude of 2300 meter above sea level with subtropical high climate. The average annual rainfall and average maximum and minimum temperature for the area are 1180mm, and 22.8^{o}c and 10.6^{o}c, respectively (CSA, 2009). Debre zeite is located 45 Km south east of Addis Ababa. The area is located at 9^{o}N latitude and 40^{o} E longitude at an altitude of 1850 maximum above sea level with annual rainfall of 866 mm of which 84% is in the long rainy season(June to September)(NMSA, 2010).Haramaya is located an altitude 2000m above the sea level. Although have 900mm annual rainfall and 18 ^{o}c and 65% humidity (CSA, 2007). Wollita Sodo is located at 80^{o} 50’ N latitude 37^{o} 45’ E longitude and an altitude 1100- 2950m above the sea level. Although have 450mm-1446m annual rainfall and 11.4 ^{o}c -26. 6^{o}c average temperature (CSA, 2003). Dilla is located at 6^{o} 24’ 30’ N latitude 38^{o} 18’30’E longitude, and an altitude 1570m above the sea level (CSA, 2007). Tigray (Mekele) is located at 12323^{o}N latitude 393832^{o}E longitude, and an altitude 2270 meter above the sea level. Although have 579-628.8 mm annual rainfall and 11.5^{o}c to 30^{o}c average temperature (TRHDA, 2007). Bahirdar is located at between 11^{o} and 36^{o} N latitude, 37^{o} 23’E longitude, and an altitude 1038m above the sea level (BBOA, 2006). Kombolcha is located at 11^{o} 07’ N latitude, 39^{o} 44’E longitude, and an altitude 1864 m above the sea level. Although have 1038 mm annual rainfall and 18 ^{o}c average temperature (ARAAI, 2008).

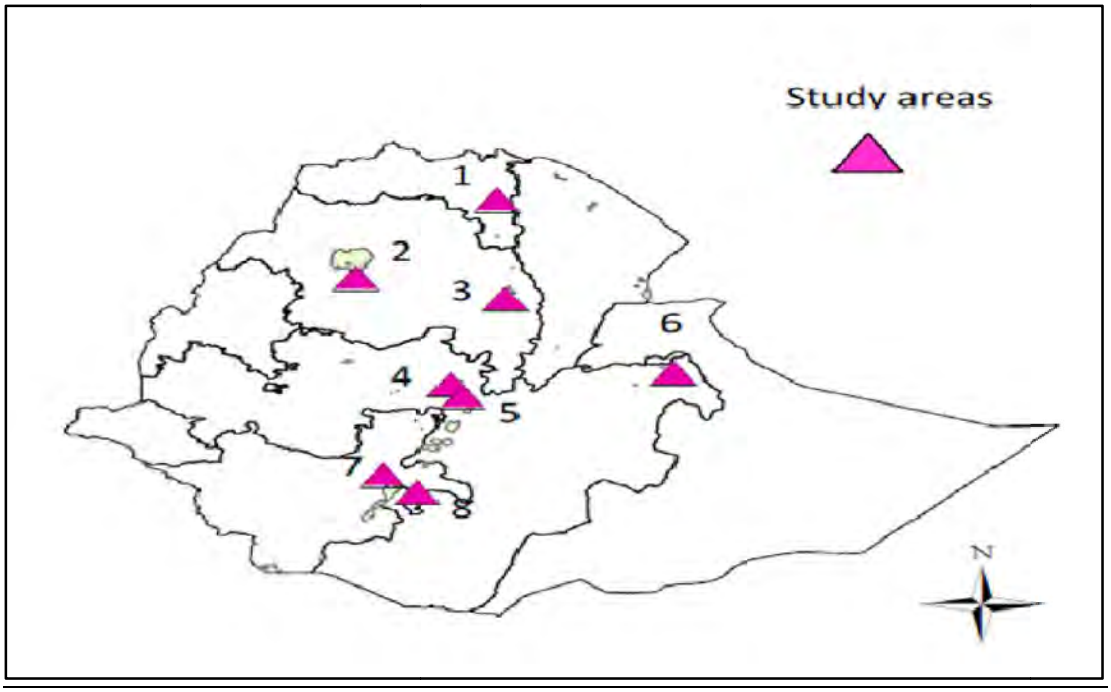


Figure 8. Map of Ethiopia showing the study areas where IBD outbreak samples were collected from clinically diseased chickens.

Where 1= Mekele, 2=Bahirdar, 3=Kombolcha, 4= Addis Ababa, 5= Bishoftu, 6= Haramaya, 7= Wollita; 8= Dilla

3.2.Study animals and study designs

The study animals are chickens for two study designs. Cross sectional study deign on chickens of all ages and breeds reared under semi-intensive and intensive production management systems that had experienced out breaks of Infectious bursal disease were used for outbreak investigation and molecular characterization of infectious bursal disease. On the other hand, complete random study design on healthy specific pathogens free (SPF) chickens of specifically IBDV antibody free (SAN) were employed for IBD vaccine immunogenicity test.

3.3.Sample collection and transportation

Two types of samples were collected accordingly to meet the two study designs. The bursa and spleen samples from IBD suspected chickens were collected for IBD outbreak investigation started from 2013 to March 2015. On the other hand serum samples were collected from semi SPF chickens for IBD vaccine immunogenicity test during the experiment work at 2015. Samples for IBD outbreak investigation were collected in two ways, i.e., either from clinically sick chickens suspected of infectious bursal disease and brought to NVI (National Veterinary Institute) Research and Diagnostic Laboratory by the poultry owners or attendant for disease diagnosis or from field based on outbreak reports. Suspected clinical samples were collected following careful examination of individual's cases. For virus isolation, bursa and spleen samples were collected aseptically from IBD suspected clinically sick chickens after killing in the post mortem facilities of the institute. Samples were placed in sterile and labelled universal bottles and transported using cold-chain to the diagnostic virology laboratory of the NVI. In the laboratory samples were either processed immediately or kept at -80^{O}c a waiting processing (OIE, 2012). On the other hand, for IBDV vaccine immunogenicity experimental study, blood samples were collected and harvested the serum. Although the serum samples were preserved at -20 ^{o}c until processed (OIE, 2012).

3.4.Laboratory investigation of IBD outbreak samples

3.4.1. Sample preparation

The viral antigen was prepared from directly during the collection of suspected bursae of fubricus samples and also frozen bursal samples were thawed and taken out from the labelled sample bottle in the class II Biosafety cabinet and took 1 gram of tissue aseptically. The tissue sample was chopped into small pieces using a sterile scalpel blade, and scissors, and also minced using a mortar and pestle. A 10% (W/V) suspension of each bursa sample was prepared in sterile phosphate buffer saline solution supplemented with penicillin and streptomycin (1000 µg/ml each). The suspension was transferred into sterile centrifuge tube and centrifuged at 3000 rpm

for 10 minutes. The upper aqueous phase (supernatant) fluid was harvested aseptically to sterile test tubes and stored at - 80^{o}C and used in the investigations described below.

3.4.2. Cell culture preparation

Primary chicken embryo fibroblast (CEF) cells were prepared inside the Biosafety cabinet class II from 11 day old fertile embryonated specific pathogen free (SPF) eggs (Delany *et al.,* 1998; Tan *et al.,* 2008). Accordingly embryonated SPF eggs were disinfected with 70% ethanol and shell opened to remove embryo. After removing the visera and the extremities (the head and legs) the body was washed three times with sterile phosphate buffer saline (PBSA) to remove the cells debris (RBCs) and also chopped by using sterile scissors. A 10ml volume of 37^{o}c pre warmed, 0.25% trypsin solution was added on the chopped material (tissue fragments) and placed in to a 37^{o}c incubator for 15 minute with slow agitation. Suspension were collected in a mesh filtered flask containing 5% fetal bovine serum and remaining tissue fragments were retrypsinized for 5 minutes in 10ml of 2x trypsin. Collected cell suspensions were transferred to sterile centrifuge tube and centrifuged at 1000 rpm for 10 minutes. The supernatant of trypsin solution was discarded and Pelleted cells were resuspended with Glasgow minimum essential medium (GMEM) supplemented with antibiotics (Penicillin G 100 IU/ml, Streptomycin Sulphate 100 µg/ml) and 10% calf serum. The cell suspension was transferred in to 25cm^2 sterile plastic tissue culture flask and kept at 37^{o}c incubator with 5% CO_2.

3.4.3. Virus isolation

The prepared and harvested 10% tissue suspension supernatants were employed for two methods of virus isolation (cell culture based virus isolation and embryonated egg inoculation).Cell culture virus isolation was performed as followed: 11 day pre incubated (embryonated) specific pathogen free (SPF) eggs were inoculated 0.2 ml of 10 % bursa suspension with via chorioallantoic membrane route according to (OIE, 2012). The inoculated embryonated eggs were candled daily one week and mortality was recorded. Any mortality within the first 24 hours post-inoculation was considered non-specific and the eggs were discarded. Embryos that were

died after this time post-inoculation (PI), the eggs were chilled at 4°C for 24 hours and the embryos were examined for gross IBD lesions. And although 10% tissue suspension was inoculated on to pre conflented primary chicken fibroblast cell (CFC) with in adsorption techniques (OIE, 2012). The inoculated Cultures after 1 hour adsorption were maintained in GMEM containing 2% bovine calf serum and incubated at 37 °C. Cultures were observed microscopically for up to seven days for the presence of cytopathic effect (CPE) characteristic of IBDV. After seven days, samples with no CPE were blindly passed further three times following two cycles of freeze–thawing. Samples which did not develop any CPE after the third blind passage were considered as negative, where as samples revelled characteristic CPE were considered as positive and kept at -20^{o}c for further analysis by molecular techniques (OIE, 2012).

3.4.4. RNA extraction and reverse transcription

RNA extraction and reverse transcription were conducted in the molecular biology laboratory of the National Veterinary Institute. Extraction of RNA from 10 % (w/v) tissue sample suspensions and/or cell culture homogenates was carried out using PureLinK^{TM} RNA Mini Kit Cat no.12183-018A, based on the manufacturer protocols. Accordingly, 400µl tissue suspension was transferred in to 1.5 ml micro centrifuge tube and 400µl lysis buffer with 2-mercaptoethanole was added to each tube and mixed by votexing and incubated at 56^{o}cfor 30 minutes (until the cell is dispersed and appear lyzed). The lysate was transferred in to a clean homogenization tube, and homogenized at maximum speed for 45 second. The homogenate was centrifuged at 26,000 rpm for 5 minutes and then transferred the supernatant in to a clean RNase free tube. Four hundred µl 70% ethanol was added to the cell homogenate and vortexed to mix thoroughly and to disperse any visible precipitate that may form after adding ethanol. The homogenized suspension was transferred to labelled spin cartridge with collection tube and centrifuged at 12,000 rpm for 15 seconds at room temperature. This step was repeated until the homogenate sample was transferred completely through with discarded the flow and reinsert spin cartridge in to the same collection tube. Finally the viral nucleic acids released were remained bounded to the silica membrane and the fluid part passed through the membrane down to the collection tube. The spin

cartridge that contained the nucleic acids bound to the membrane was placed in collection tube and washed by using two wash buffers as followed: 700 µl wash buffer I was added and centrifuged at 12,000 rpm for 15 seconds at room temperature followed by discarding the flow through with collection tube and placed the spine cartridge in to new collection tube and then 500 µl wash buffer II with ethanol was added to the spin cartridge and centrifuged at 12,000 rpm for 15 seconds at room temperature and discarded the flow through. This step was repeated to dry the membrane with bounded RNA. Finally, the spin cartridge was transferred in to a labelled recovery tube and 40 µl RNase free water was added to the centre of spin cartridge and incubate at room temperature for one minute. The nucleic acids bound to the silica membrane was eluted into a labelled recovery tube by centrifuge RNase free water contained one minute pre incubated the spin cartridge at 12,000 rpm for 2 minutes at room temperature and the eluted RNA was used for cDNA synthesis.

Complementary DNA (cDNA) was generated from RNA temple using the reverse transcriptase RevertAid™ (Fermentas) by two step cDNA synthesis method. A 10 µl volume reaction mix was prepared first from 3 µl RNase free water, 1µl Oligo(dT) 20, 1 µl 10 Mm dNTP mix and mix by vortex and then 5 µl template RNA was added and incubate at 65 ºC was 5 minutes and place at + 4^{o}c, and also a 10 µl volume cDNA synthesis mix was prepared from 1 µl DEPC treated water,2 µl 1X RT buffer, 4 µl of 25 mm $MgCl_2$, 2 µl of 0.1 MDTT, and 1 µl superscript IIIRT enzyme, by incubated those cDNA synthesis mix at 55^{o}c. The synthesized cDNA and the cDNA synthesis mix were rewarmed, mix the reaction gently and incubate at 55^{o}c for 50 minutes. The reactions was terminated at 85 ^{o}c for 5 minutes and chill on ice and then collect by brief centrifugation and added 1 µl RNase H to each tube and incubate for 20 minutes at 37 ^{o}c and finally the obtained cDNA was used for PCR (Polymerase Chain Reaction).

3.4.5. Polymerase chain reaction (PCR)

Reverse transcriptase polymerase chain reaction is a molecular tool frequently applied in IBDV diagnosis (Zierenberg *et al;* 2001; OIE, 2012). Reverse transcriptase Polymerase chain reaction (RT-PCR) was performed following three steps: extraction of nucleic acids using the

commercially available RNA extraction kit, reverse transcription (RT) of IBD virus RNA into cDNA using reverse transcriptase. 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 primers, Forward primer IBDF3 design (5' to 3' TGT AAA ACG ACG ATG GCA TGC GG ATG TGA GGC TTG GTG AC) and Reverse primer design IBDR3 (5' to3' CAG GAA ACA GCT ATG ACC GAA TTC GAT CCT GTT GCC ACT CTT TC) 645 bp amplification capacity, with negative and positive control reactions.

Touchdown PCR was carried out in a final reaction volume of 20µl using 200 µl capacity thin wall PCR tube containing 5 µl 5X PCR Buffer with $MgSO_4$ (Fermentas), 1µl 10 mM dNTPs (Thermo Scientific), 6 µl RNase free water,1U Taq DNA polymerase, 2 µl of each primers and 3 µl of cDNA template. PCR reactions were carried out for 1 cycle at 95 °C for 5 minutes, for initial denaturation; and 95°C for 30 seconds, 60 for 30, 72 for 45 seconds for 15 cycles and also at 95°C for 30 seconds, 56 °C for 30 seconds, 72°C for 45seconds for 20 cycles and finally extension at 72 °C for 7 minutes.

3.4.6. Agarose gel electrophoresis of polymerase chain reaction products

PCR products were analyzed by 1.5 % (w/v) Agarose electrophoresis gel stained with gel red and 1X TAE buffer. Briefly, 5 µl each PCR products was mixed with 1 µl 6X loading buffer and loaded in to separate well of the pre-prepared gel and 100bp DNA molecular marker was also added onto the first lane and run at 120 volt for about 1:20 hours in electrophoresis apparatus. The PCR products band was visualized by gel documentation under UV-lamp camera and the size of the PCR products was estimated by comparing with the band size of the molecular marker 1Kb ladder (marker) that was loaded on a separate lane (OIE, 2012).

3.4.7. Sequencing and phylogenetic analysis

PCR positive product was purified individually using Wizard® SV Gel and PCR product purification kit (promega, Germany) following the manufacturer's instruction. Amplicons were extracted using QIAGEN Gel extraction kit; with the concentration of extracted DNA was determined spectrophotometrically using micro volume a Thermo Scientific Nano Drop Spectrophometer; model Nano Drop 2000c, USA. The concentration of the quantified Purified PCR product was adjusted following the requirements set by sequencing company. The quantified DNA, and also a IBDV Vp2 gene specific forward and reverse sequencing primers were added in to the separated labelled eppendorf tubes. The eppendorf tubes containing DNA and primer were sent to the sequencing service company. The raw sequence data was edited and phylogenetic tree was construed including the reference strain.

3.5. Infections bursal disease vaccine immunogenicity experimental study

3.5.1. Ethical statement.

All animal work was conducted according to Addis Ababa University College of Veterinary Medicine and Agriculture Home Office guidelines.

3.5.2. Experimental animal and management

Two hundred seventy five (275) vaccinated progeny originated, unvaccinated COOB 500 broiler chickens obtained from a commercial hatchery were used for IBD vaccine immunogenicity and pathogenicity test. Chickens were placed into separate sterile cages at the experimental house of the research and diagnostic department laboratory animal handling and safety room, in National Veterinary Institute under strict hygienic and standard management conditions with *ad libitum* access to food and water until the end of the experiment. All experiments were performed in

animal facilities according to Addis Ababa University College of Veterinary Medicine and Agriculture Home Office ethical guidelines on animal welfare.

3.5.3. Experimental design and methods

The chickens were divided randomly in to five groups; four treatment and one control groups. Blood samples were collected from wing vein using 3 ml syringe and harvested the serum at age of 14 days and 21 days (before the experiment was started) and the serum sample were examined and checked the IBDV MAb level of the flock by flock screening Enzyme Linked Immunosorbent Assay (ELISA) test. The serological test, ELISA technique was performed as described by the manufacturer, ELISA kit (X-ovo flock screen IBD antibody ELISA kit, Cat No.V090/ V094/V095) obtained from Carnegie Campus, United Kingdom and also ELISA reader and IBD antibody. Based on the examined serum ELISA test result the primary and booster dose vaccination; and the challenge dose administered schedule were determined and also was scheduled at days 21, 35 and 42 respectively.

Experimental design: Those IBDV Specific Antibody Negative (SAN) chickens were randomly assigned and allotted into 5 groups, which were 55 chickens per group. Each of four groups in the experiment was immunized at age of day 21 in intraoral route with commercial live IBDV vaccines, namely (A, B, C and D vaccines) listed on (Table.2) The fifth group (control group) was not vaccinated and serves as challenge control. The blood samples were collected before booster dose vaccination and challenge virus administration from each vaccinated groups and also at 21 days post challenge test (at day 35, 42 and 63) to harvest serum, and the serum sample was stored at -20^{o}C until tested (OIE, 2012). One week after the last vaccination, 30 birds in each group including control group were challenged using vVIBDV, local field isolated of 0.2 ml of 10 $^{5.4}$ $TCID_{50}$ dose in intraoral route. All birds were monitored for overt signs of disease (e.g. depression, inappetence, diarrhoea) and mortality over 21 days post challenge test. The effects of used IBD vaccines (A, B, C, and D) and locally isolated VVIBDV challenge virus were assessed in terms of percent of protection calculated as morbidity and mortality rate and seroconversion data.

Table 2. Experimental design summary

Groups	No of chicks	Vaccine type	* Challenge virus	Vaccine Schedule
A	55	D78	VVIBDV /ETH	1st dose =Day 21
B	55	B2K	VVIBDV /ETH	booster=day35
C	55	LC75	VVIBDV /ETH	challenge=day42
D	55	EXTREM	VVIBDV /ETH	
E	55	unvaccinated Control	VVIBDV/ETH	

Name of vaccines from group (A-E) A= Intermediate vaccine; B= Invasive Intermediate; C and D= Intermediate plus E= unvaccinated challenge control; VVIBDV/ET= very virulent IBDV Ethiopian field isolate.

3.5.4. *Chicken embryo fibroblast (CEF) and cell culture media*

CEF was prepared from 11 days pre incubated embryonated SPF eggs, and used for propagation and preparation the locally isolated challenge IBDV virus and titration of challenge IBD virus and IBD vaccines according to (CFR-USA, 2012). SPF embryonating chicken eggs were obtained from the SPF chicken egg production farm the VALO BioMedia GmbH Germany. Eggs were kept in egg incubator at 37^{o}c with humidity 40-60% for 11 days to be embryonated. Trypsin- version solution that was prepared according to Hanks balanced salt solution (HBSS), GMEM was prepared according to the manufacturer's instructions, and Bovine Serum was Mycoplasma free and virus screened, Gamma sterile "Moledinar. LTD, Australia" were the media and solutions used for cell culture preparation and virus titrations

3.5.5. IBDV vaccines

Four commercially available live attenuated infectious bursal disease vaccines (IBDV vaccines) were used. Namely: one Intermediate: Nobilis Gumboro IBD D78, Bursal Disease Vaccine, and Batch No. IBD 04/14 which is actively produced at National Veterinary Institute (NVI) and used in Ethiopia: one invasive intermediate strain: Indovax-BURSA B2K infectious bursal disease vaccine, Batch No GP1414, Manufactured by Indian INDOVAX PVT.LTD; Two Intermediate plus: Bursitis virus strain LC75 Bursal Disease Vaccine, Batch No. D037411 Manufactured by Lohmann Animal Health GmbH, Germany, and Avipor-IBD Xtreme Bursal Disease Vaccine; Batch No.3506N, Manufactured by U.S.A. LOHMANN ANIMAL HEALTH INT, LTD, with cell culture origin.

3.5.6. The challenge viruses and method of propagation

One very virulent infectious bursal disease virus (VVIBDV) pathotype was used to undertake challenge test. The virus used in the challenge were isolated in field cases from Kombolcha at the Amhara region of Ethiopia during an IBD outbreak in 2014 and identified by PCR and sequence analysis, and have gene accusation number (JF826453). This local field isolate (VVIBDV) was prepared on Infectious Bursal disease outbreak bursa sample and propagated using 11-day-old SPF embryonating eggs chicken fibroblast cell (CFC) obtained from the SPF production farm the VALO BioMedia GmbH Germany as (OIE,2012) and with titer$10^{5.4}$ TCID /ml calculated according to the method of (Reed and Muench, 1938).

3.5.7. Titration of IBDV vaccines and challenge virus

IBDV experimental Vaccines and challenge virus was tittered according to OIE (2012) and infected dose (TCID50) was calculated according to (Reed and Muench, 1938). Both IBDV vaccine strains and field isolate challenge virus were titrated by tenfold dilution starting with 10^{-1} by mixing 1 ml of virus in 9 ml of diluents (GMEM base medium) and subsequent transfer of 1ml of previous virus dilution to the next using sterile pipette. Fifty micro litters (100µl/well) of

each virus dilution (10^{-1} to 10^{-10}) were distributed in to the wells of their respective rows of flat bottom microtitre plates containing established cell line of (CFC). Then all the wells were cover over flown by dispensing hundred µl/well GMEM medium with 2% calf serum and incubate at 37^{o}c with CO_2 for seven days. The titer for each vaccine and challenge virus was determined by use of Reed and Muench formula; Log10= $((xo - (\frac{d}{2}) + \frac{d(\varepsilon ri)}{ni}$

Where; x_o= Log 10 of reciprocal of the lowest dilution at which all set monolayer's are positive,

d=Log 10 of the dilution factor that is the difference between the log dilution intervals. ri= number of positive test monolayer's out of ni.

Ɛ (ri/ni) = Ɛ (P) sum proportion of the tests beginning at the lowest dilution showing 100% positive result**.**

The summation is started at dilution X_o. (Reed and Muench, 1938).

3.5.8. Serological detection of infectious bursal disease virus antibody

All five groups of experimental chickens before administering different experimental IBDV vaccines along the corresponding groups at age of day 14 and 21 and four groups of SPF chickens through the experiment at age of 4th and 5^{th} weeks (after primary and booster dose immunization respectively), and also at age of 9^{th} weeks (21 days post challenge test) antibody responses were assessed by measuring specific antibodies using ELISA. The ELISA technique was performed as described by the manufacturer of ELISA kit (X-ovo flock screen IBD antibody ELISA kit, Cat No.V090/ V094/V095) obtained from Carnegie Campus; ELISA reader and infectious bursal disease antibody. According to the methods described by the ELISA kit serum samples that preserved at - 20^{o}C, the antigen coated plate that consisted of 96 wells and the ELISA kit reagents that preserved at 2 - 4^{o}C were adjusted to room temperature of 22 - 27^{o}C prior to the test and record samples and control locations on 12 x 8 temple sheet. Briefly; 50 µl undiluted control and diluted samples was added in to the appropriate wells and covered with an adhesive cover and incubated at 37^{o}c for 30 minutes with gentle agitation. The adhesive cover was removed and washed the plate four times with wash buffer and wells were inverted and

taped firmly on absorbent paper to dry. After which 50µL of the conjugate reagent (sheep anti-chicken) was added into each well and incubated at 37^{o}c for 30 minutes by mix on a plate shaker. The adhesive cover was removed and washed 4 times with wash buffer (300 µl for each well) and the plate was inverted and taped firmly on absorbent paper to dry. ELISA substrate reagent of 50 µl was added into each well and covers the plate with adhesive cover and also incubated at 37^{o}c for 15 minutes. 50 µl ELSIA stop solution was added in to each well after removing the adhesive cover. Finally the test result was read by using microtitre plate reader at 550nm absorbance having the first blank on air. The reader, connected to the computer loaded with Excel packages, was used to automate the reading of optical density (OD) value. The percentage positivity (pp) for test samples in relation to the negative and the positive controls was calculated as per the formula give by the kit manufacture.

$$\text{SP value} = \frac{OD\ sample - OD\ Negative}{OD\ positive - OD\ Negative}$$

Where: OD samples was optical density of the test sera,

OD negative was optical density of negative control,

OD positive was optical density of positive control.

The cut off value provided by the manufacturer was used to determine the percentage positivity. For estimating the maternal antibody level was likely to have fallen below the target titer, calculate the ELISA titer value as followed:

Log10 Titer= 0.557x (Log10 S/P) +3.6845 Titer =Antilog of log10 Titer

The IBD S/P ratio and/or ELISA titer value of the samples may be interpreted using the following guide (N.B. 'Hot' vaccines can be administered at much higher titers the target titer of 500 is intended only as a guide).

S/P	IBD Titer	IBD Antibody
$\leq$ 0.306	0.2501	can be vaccinated
>0.306	2501 or greater	Too high vaccination

3.5.9. *Challenge infection and protection efficacy against VVIBDV challenge*

As an additional measure of vaccine efficacy, a challenge test was performed. Chickens were challenged with a vvIBDV local isolate at the age of 6weeks, 1weeks post booster dose vaccination and after blood sampling. 6 week-old birds were inoculated by oral route with 0.2 ml $10^{5.4}$of local isolated VVIBDV strain and observed for 21 day post-challenge. The morbidity and mortality rate were recorded. Clinical signs as described in (OIE, 2004) were observed and classified as positive or negative and also the mortality rate was calculated as a percentage of the initial number of the birds. All the dead and sacrificed birds were examined for gross lesions and Post mortem gross lesions observed in every dead and scarified bird in each group were recorded and subjectively graded as normal (0), mild (1), mild to moderate (2) moderate (3) moderate to severe (4) and severe (5) based on the severity of the lesions on the bursa of Fabricius, body skeletal muscles surfaces, kidneys, and haemorrhages of the mucosa of proventriculus (OIE 2012).

4. DATA ANALYSIS

The data of ELISA IBDV antibody result were subjected to a one-way analysis of variance (ANOVA). Statements of statistical significance were based on $p < 0.05$. Variations among groups were determined using Turkey's comparative test (Myra, 1989).

5. RESULTS

5.1.Results on IBD outbreak investigation

5.1.1. Field clinical examination

A total of 60 chickens : 22 from Bishoftu, 10 from Bahirdar, 4 from Addis Ababa,5 from Kombolcha, 10 from Tigray, 5 from Dilla and 4 from Wollita were examined and representative bursa and spleen were collected (Table. 3). The diseased chickens showed clinical signs of severe depression, watery diahorrea discharge, and reduced feed and water intake, ruffled feather, and enlarged hemorrhagic bursa, accumulation of urate in kidney, haemorrhage in thigh and pictorial muscle during post mortem examination (figure. 9). The age of the chickens varied from 35 to 56 days. Mortality varied in all flocks, except in one flock where mortality was 60%. Most of flocks were vaccinated against classical IBD vaccine which was currently produced and used in Ethiopia (two dose vaccination approaches).

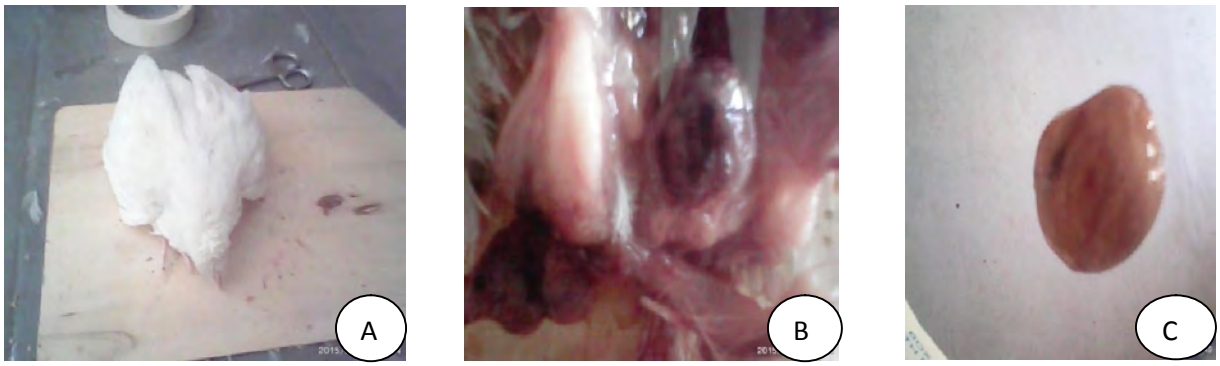


Figure 9. Clinically diseased chicken and post mortem lesion

Clinically diseased chickens suspected of IBD infection (figure A) and enlarged hemorrhagic bursa during post-mortem examination (figure B and C).

Table 3. Place, date of examination, farming system and organs of clinically diseased chickens examined for IBD

Town	Date of collection	Type of organ		Farming system
		Bursa	Spleen	
Addis Ababa	24/06/2013	3	2	Semi intensive
Bishoftu	25/09/2013	5	2	Intensive
Bishoftu	30/9/13	3	1	Intensive
Tigray	01/10/2013	10	2	Intensive
Dilla	30/05/2014	5	2	Intensive
Kombolcha	16/04/2014	5	2	Intensive
Bahir Dar	02/06/2014	3	2	Semi intensive
Bahir Dar	12/11/14	3	2	Intensive
Debre Zeite	12/11/2014	5	2	Semi intensive
Haromaya	3/1/205	4	-	Intensive
Bishoftu	29/01/2015	4	2	Semi intensive
Bishoftu	11/02/15	4	1	Semi intensive
Addis Ababa	11/02/2015	2	2	Semi intensive
Bahir Dar	29/3/15	2	2	Semi intensive
Wollita Sodo	30/03/2015	1		Intensive
Total		**60**	**26**	

5.1.2. Infectious bursal disease virus isolation

A total of 86 samples (60 Bursa and 26 spleens) were collected from 60 chickens of different IBD outbreaks. 20 Bursa and 5 spleen pooled samples were employed virus isolation. From 20 pooled bursa samples employed for virus isolation 18 (95%) virus were isolated. Eighteen of the isolates were grown and develop visible CPE on cell cultures starting from the 4rd days of primary inoculation and 3rd days of 3rd passage. An entirely CPE was seen as small round cells reflective and synctia form later that detached from the wall of cell culture flask (Figure.10) and also some of the isolates were caused embryo death on the 4th day of inoculation and also the

affected embryos had edematous distension of the abdomen, petechiae and congestion of the skin and ecchymotic haemorrhages in the cerebrum or head and neck region were observed on embryonated egg inoculation method of virus isolation.

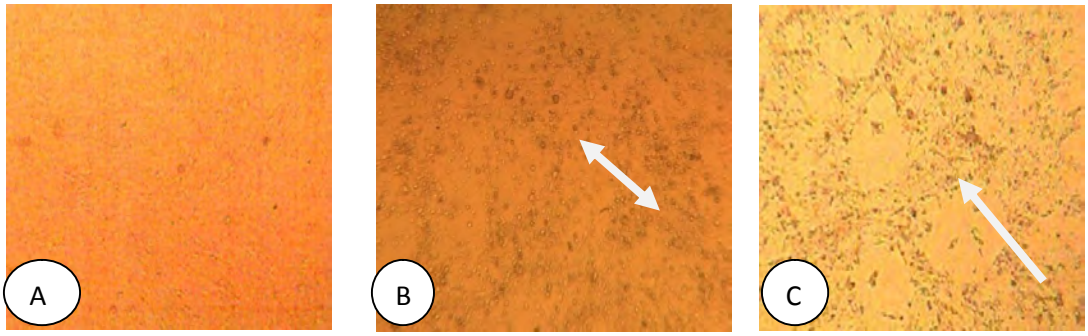


Figure 10. IBD virus growth on chicken embryo fibroblast cells.

Where (A) confluent monolayer of chicken embryo fibroblast (CEF) cell grown in GMEM growth medium after 48 hours of culture; (B and C) characteristic cytopathic effect of IBD virus white arrow on CEF cell developed after 4^{th} days post primary inoculation and 3^{rd} days post infection of third passage on B and C, respectively..

5.1.3. Detection of viral RNA by reverse transcriptase polymerase chain reaction (RT-PCR)

Polymerase chain reaction was performed to amplify a 645 bp fragment of VP2 gene of IBDV using forward primer IBDF3 and reverse primer IBDR3. This step allowed us to reach a definitive diagnosis of the causative agent of the outbreak. A totally of twenty samples (eleven 2013 and 2014 outbreak samples and nine 2015 outbreak samples) tissues as well as cell cultured suspensions were tested for IBDV genome amplification. Eighteen samples showed the presence of 645bp PCR product and the remaining two samples were negative RT-PCR test results as cell culture test (figures. 11 and 12).

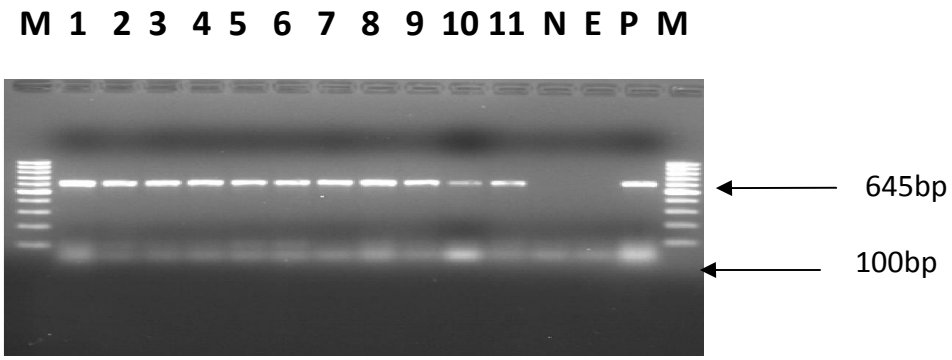


Figure 11. 2013 and 2014 IBD suspected outbreak tissue suspension samples RT-PCR test result gel picture.

Where M- Molecular marker started 1000bp (Fermentas)

N- Negative control without template-No amplification.

E- RNase free water extraction control- No amplification.

P- Positive control NVI Vaccine seed D78 strain - Positive around 645bp.

1. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Dilla 1/14

2. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Dilla 2/14

3. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Dilla 3/14

4. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Dilla 4/14

5. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Dilla 5/2014

6. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Kombolcha 6/2014

7. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Kombolcha 7/2014

8. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Kombolcha 8/20114

9. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Tigray 4/2013

10. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Tigray 5/2013

11. IBD ISO/IEC 17025:2005 NVI QMS reference no MB Tigray 6/2013

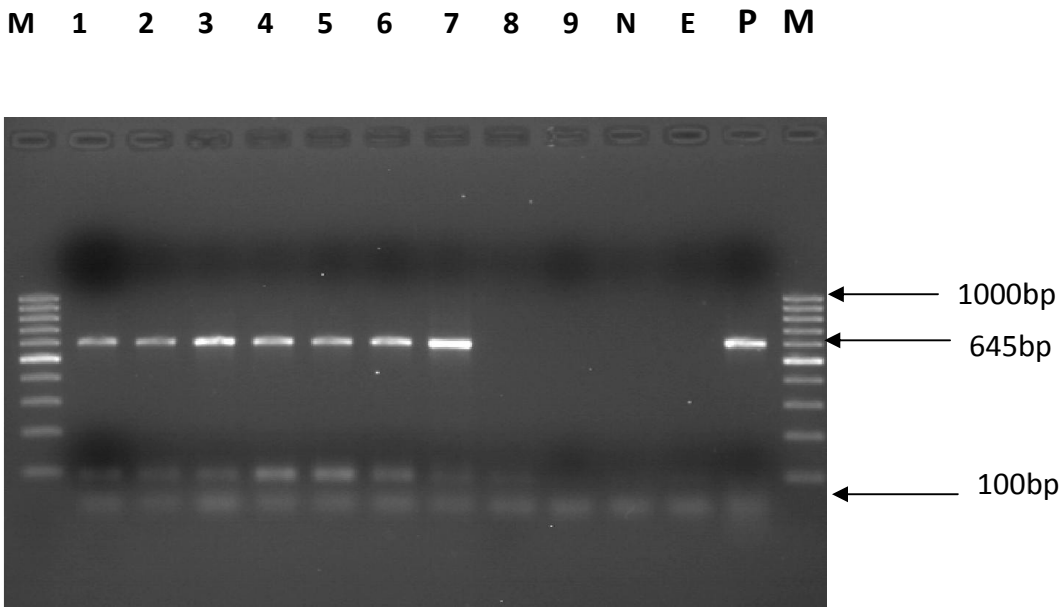


Figure 12. 2015 IBD suspected outbreak tissue suspension samples RT-PCR test result gel picture.

Where: N- Negative control without template-No amplification.

E- RNase free water extraction control- No amplification.

P- Positive control NVI Vaccine seed D78 strain - Positive around 645bp.

1. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 567/15

2. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 702/15

3. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 716/15

4. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 751/15

5. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 756/15

6. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 758/15

7. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 630/15

8. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 335/15

9. IBD ISO/IEC 17025:2005 NVI QMS reference no MB 672/15

Table 4. Outbreak samples which revealed positive result for IBDV tested by gene specific RTPCR

		Result	
Region /Town	No of samples tested	Positive	Negative
Tigray	7	7	-
Amhara (Bahirdar	4	4	-
and Kombolcha)			-
SNSS	4	4	-
(Wollita Sodo, Dilla)			
	2	2	-
Bishoftu			
Addis Ababa	1	1	-
Haromaya	2	-	2
Total	**20**	**18**	**2**

From a total of 20 samples processed for molecular characterization 18 of the samples was positive.

5.1.4. Sequence and phylogeny tree analysis

Nucleotide sequencing of RT-PCR products based on VP2 nt sequences is widely used for further characterization of IBDV strains (Sapats and Ignjatovic, 2000; Zierenberg *et al.,* 2000; Islam *et al.,* 2001a; Liu *et al.,* 2002; Viswas *et al.,* 2002). Characterization of the IBDV strain in Ethiopia that was isolated in this Study confirmed that vvIBDV pathotype is circulating in Ethiopia

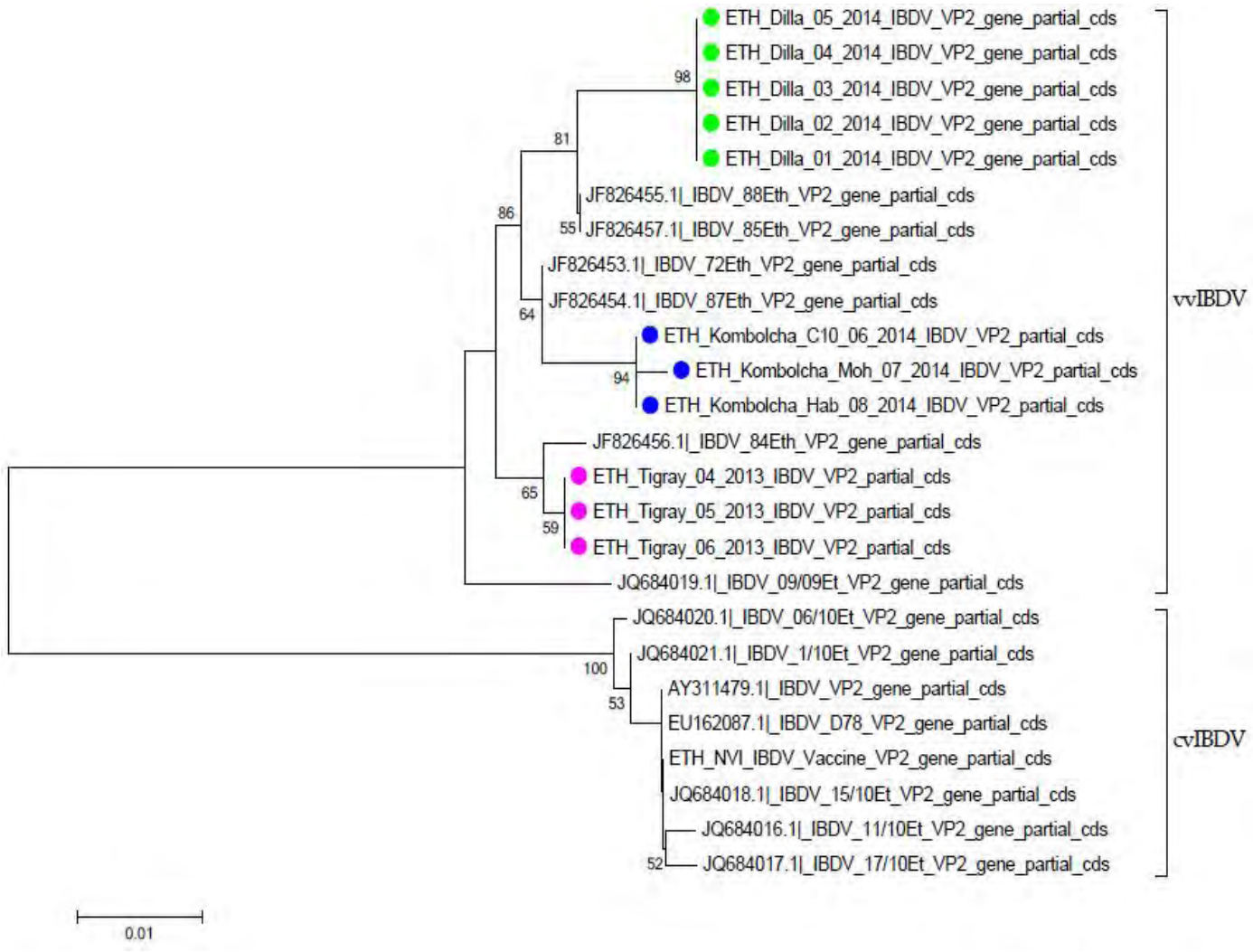


Figure 13. Phylogenetic tree analysis of sequence data.

Phylogenetic tree analysis of 25 IBD viruses based on nucleotide sequences of hyper variable coding (VP2) gene partial sequence (410bp). IBDV field isolates, classical/attenuated vaccine strains and reference sequences of classical and very virulent strains retrieved from the Genbank database were included in the analysis. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution with the pair wise deletion option was computed using the Kimura 2-parameter method of using MEGA6 software (Tamura, 2013). The percentages of bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The current eleven field isolates are marked with colored circle. Infectious bursal disease virus isolates grouped phylogenetically into classical virulent (CV) and very virulent (vv) strains.

5.2.Results on experimental studies

5.2.1. Detection of IBDV vaccine viral identity and titration

All the four IBD vaccines used in this experiment were identified by using: RT-PCR: IBD virus VP2 specific primers to give positive result at 645bp amplification band and titrated in chicken fibroblast cells. The results indicated that vaccines, tested were $10^{3.5}$TCID50 / dose for all cell cultured adapted IBDV vaccine strain. So the results of titration were judged according to the parameters of (OIE, 2012) in which IBDV titers must be not less than $10^{3.5}$ TCID50/dose in CEF.

5.2.2. IBDV Antibody (MAb) detection of experimental chickens before immunization

Antibody titers against IBDV were screened the non vaccinated chickens sera collected at 14 and 21-day-old. There was showed a significant reduction of IBDV MAb on day 21 examined serum than on day 14 and the Significant difference between the two days was ($p<0.05$) (0.422101 ± 0.140542, and 0.04976 ± 0.000987 mean S/P ratio respectively). Based on the ELISA kit protocol and the serum ELISA screened tests result, the experimental IBDV vaccine administered schedules were determined as primary and booster experimental IBDV vaccine dose was administered at day 21 and 35. The screened results of ELISA for IBD virus MAb antibodies S/P ratios before vaccination are given in (Table 5).

Table 5. Results of the geometric mean S/p ratios for antisera collected at day 14 and day 21 prior to experimental vaccination measured by ELISA.

Age	*Number of chickens sera*	*S/P ratio*	*Percent positive*
14 days	*275*	*0.422101±0.140542*	*56/275*
21 days	*275*	*0.04976± 0.000987*	*5/275*

*** Positive cut off S/P = 1.084, N = Negative= 0.073 *** S/P sample/absorbance of positive,***the ELISA kit instruction "S/p $\leq$ 0.3 is considered as negative and recommended to vaccinate and also S/P > 0.3 is considered as positive, too high to vaccinate''.

Based on the ELISA kit protocol and the sera MAb screening test result the primary and booster dose vaccination schedule was at age of day 21 and 42 respectively.

5.2.3. IBDV antibody detection of experimental chickens before and after immunization

Antibody titers against IBDV were measured on serums that was collected at age 21 days before vaccination and also at age of 35 days (14 days post primary immunization); and at 7 days post booster dose (at 42 days, just before challenge) on vaccinated groups to determine the experimental IBDV vaccinal strain immunogenicity. Results of ELISA IBDV mean S/P ratio and percent positivity for IBDV vaccine are given in (Table 6).

Table 6. Results of Means ELISA IBDV antibody titer S/P ratio before and after vaccination and positivity.

Group/ vaccine	Mean S/P ratio along different days					
	Before vaccination		After vaccination			
	Day 21		Post 7 days of 1st dose (at 35 day)		Post 7 days of booster dose (at 42 day)	
	S/ P	% positive	S/ P ratio	% positive	S/ P ratio	% positive
G-A	0.04976± 0.000987	1.6	0.470*± 0.01852	98	0.43003*± 0.021828	100
G-B	0.04976± 0.000987	1.6	0.61545± 0.014616	100	0.67035± 0.024885	100
G-C	0.04976± 0.000987	1.6	0.57761± 0.021617	100	0.65385± 0.035310	100
G-D	0.04976± 0.000987	1.6	0.64414± 0.019091	100	0.64625± 0.039149	100
G-E	-0.04976± 0.000987	1.6	-	-	-	-

***** The mean difference is significant at the 0.05 level ($p < 0.05$)**.**The data are S/P value ± (SE) and percent positivity, * SE-Standard Error, *S/P-sample positivity

The sera ELISA test S/P ratio result on day 35 and 42 along the treatment groups and also within a group along the treatment days was measured and compared. The sera ELISA test result on day 35 and 42 was indicated, the treatment group A was developed significantly low S/P ratio (0.470± 0.01852 and 0.43003±0.021828), respectively and also its sample positivity percent ratio on day 35 was lower by one (% positivity = (44/45) as compaired to the other treatment groups. Although the sera ELISA test S/P ratio result of group A on day 42 was lower than day 35 (0.470± 0.01852 and 0.43003±0.021828) but its sample positivity percent ratio was increased by one on day 35 results.

5.2.4. Detection of cross protection against challenge virus and seroconversion

Cross protection of the challenge virus and seroconversion rate on challenge control and vaccinated groups were evaluated and compared by ELISA test S/P ratio on the sera collected at age of 35 and 42 days before challenge and at age of 63 days (21 days post challenge test) on challenged groups of vaccinated and non vaccinated (challenge control) chickens (Table 7).

Table 7. Results of Means of ELISA IBDV antibody S/P ratio and percent positivity of vaccinated and challenge control groups prior and post 21 days of challenge test.

	IBDV mean ELISA antibody S/P ratio along different days							
Group	Before challenge test (14 days post 1st dose and 7dayspost 2nd dose of vaccination				After challenge test (21 days post challenge test)			
	Day 35		**Day 42**		**Day 63**			
	S/P ratio	%positivity	S/P ratio	%positivity	S/P ratio	Morbidity rate	Mortality rate	%protection
G-A	0.4702*± 0.018516	49/50	0.43003*± 0.02188	45/45	2.58867± 0.040826	7/30 (23.3%)	0/30 (0%)	30/30 (100%)
G-B	0.61545± 0.014616	50/50	0.67035± 0.024885	45/45	2.68563± 0.019183	3/30 10%	0/30 0%	30/30 (100%)
G-C	0.57761± 0.021617	50/50	0.65385± 0.035310	45/45	3.20194*± 0.06743	0/30 0%	0/3 0%	30/30 (100%)
G-D	0.64414± 0.019091	50/50	0.64625± 0.039149	45/45	2.06667*± 0.046321	0/30 0%	30/30	30/30 (100%)
G-E	-	-	-	-	2.83333*± 0.0112376	30/30 100%	18/30 60%	12/30 (40%)

***** The mean difference is significant at the 0.05 level ($p < 0.05$)**.**The data are S/P value ± (SE) and percent protection, * SE-Standard Error, *S/P-sample positivity,

G-A= IBD D78, G-B= IBD B2K, G-C= IBD LC75, G-D IBD Extreme and G-E= Control

The sera ELISA test S/P ratio and percent positivity result on day 35 and 45 before challenge and also S/P ratio and percent protection on day 63, 21 days post challenge along the treatment groups and also within a group along the treatment days was measured and compared.

The sera ELISA test S/P ratio result on day 63 was indicated, the treatment group A and B was developed insignificant and lower the S/P ratio as compaired to the non vaccinated challenge control group (2.589± 0.041, 2.686± 0.0192) but group C was developed significant and higher S/P ratio as compaired to vaccinated groups as well as non vaccinated challenge groups (3.202± 0.067). The seroconversion rate after challenge test along treatment group on day 63 was reflected increased amount of S/P ratio than day 42 except group D (2.067± 0.046) which is significantly low S/P ratio. There was morbidity on treatment group A and B (23.3% and 10%), respectively but not on group C and D along the treatment groups and also 100 % morbidity on non vaccinated challenge control groups. There was 100% protection (no mortality) on vaccinated groups but there was 60% mortality on non vaccinated challenge control groups (Table 7).

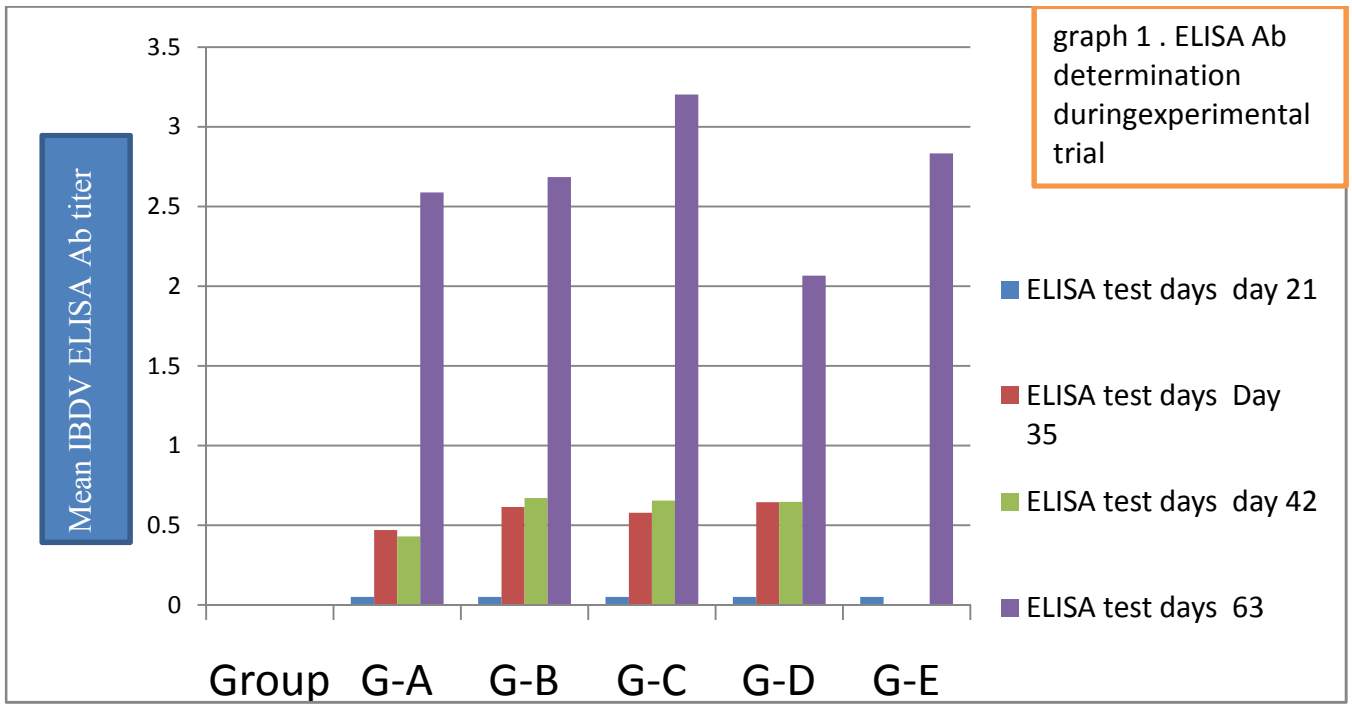


Figure 14. Summary of Mean ELISA IBDV vaccine Ab & challenge determination result**.**

5.2.5. Challenge infection and protective efficacy test of different strains of anti-IBDV vaccines against VVIBDV challenge virus

Challenge infection and Protective efficacy of different strains of experimental anti-IBDV vaccines against $_{VV}$IBDV challenge virus was assessed as in terms of morbidity rate, mortality rate and percent protection post 21 days of challenge test (Table 7) and also gross pathological lesions scoring (Table. 8).

Table 8. Results of gross pathological changes scoring in treatment and challenge control group along the treatment days

Age (days)	**Number of chickens per group**	**Experimental groups**				
		G-A	G-B	G-C	G-D	G-E
28	5	2	1	2	2	0
35	5	1	1	0	0	0
42	5	1	1	0	0	0
45	5	3	3	1	1	5
49	5	4	4	2	2	5
63	30	5	3	0	0	5
Total	55	16/55 (29.09%)	13/55 (23.63 %)	4/55 (7.27 %)	5/55 (9 %)	15/37 (40.54 %)

*gross pathological changes scoring subjectively graded as normal (0), mild (1), mild to moderate (2) moderate (3) moderate to severe (4) and severe (5) based on the severity of the lesions on the bursa of Fabricius, body skeletal muscles surfaces, kidneys, and haemorrhages of the mucosa of proventriculus (OIE, 2012).

The chickens was observed two times a day through the experiment time , there was obvious IBD clinical signs on all challenge control (unvaccinated birds) and in some chickens on Group A and B but there was no any clinical sign of disease or any other health disorder were recorded

in Group C and D. Necropsy finding on survived birds on 21 days Post challenge test: haemorrhage on pictorial and thigh muscle, on some Enlarged blood stringed bursa, on some atrophied gray to yellowish bursas with slight haemorrhages on proventriculus and urate in kidney was observed on vaccinated group A and B and non vaccinated control groups but there was no any lesion on Group C and D. There was no any mortality on vaccinated groups but the challenge virus induced 60% mortality in control groups at 47 days of ages, from 4 days post challenge test. The dead birds on control groups before death were showed a pathognomic clinical signs of IBD (intense prostration, inappetence, watery faeces or pasting vents and anorexia: ruffled feathers, depression, head between the shoulders and death and also At necropsy, bursas showed intense oedema, hemorrhagic and deposition of serofibrinous material on the muscular wall, enlarged spleen, pictorial and thigh muscle haemorrhage was observed (Figure 15 and 16).

Figure 15. Clinical signs and dead birds on challenge control group

Clinical signs on challenge control group on A and B; C some of dead birds on challenge control group during challenge test

Figure 16.pathological lesions on control groups during challenge test.

Bursa of fubricus lesion on control groups during death post 4 days of challenge test (figure A). B and C post mortem findings on 21 days post challenge test.

6. DISCUSSION

This study demonstrated the isolation and molecular characterization of infectious bursal disease virus from clinically diseased chickens reared under different production systems and parts in Ethiopia and the four different live attenuated IBDV vaccine immunogenicity and cross protection against the local isolated $_{VV}$IBDV challenge virus test result. The virus was indeed confirmed by using the (OIE, 2012) recommended diagnostic techniques like cell culture, polymerase chain reaction and sequencing of VP2 of the IBDV isolates.

There are many reports on the existence of Infectious bursal disease virus in commercial and back yard chickens and it becomes a serious threat on the juvenile poultry industry in Ethiopia (Zeleke *et al.,*2005a, b; Tesfaheywet *et al.,* 2012, Mazengia *et al.*, 2012, Jenbreie *et al.*,2012). Some of those reports were supported by virus isolation and molecular analysis data and some of the remaining reports were supported by serological data. From the recent years farm owners and farm employer professionals has been continually reporting the suspicious of occurrence of infectious bursal disease virus infection in the poultry farms reared in different geographical areas of the country under different production systems to the NVI despite the utilization of the intermediate IBDV vaccine strain D78 currently produced by NVI.

Based on the customers disease report the present study was designed to investigate the circulating IBDV virus pathotype among the chicken population using the recommended diagnostic laboratory techniques and to compare efficacies between different strains of live IBD vaccine in the poultry farms as method of controlling the Gumboro disease problem in Ethiopia and all over the world and although select the appropriate IBDV vaccine that protects the currently circulating IBDV virus pathotype through different live attenuated IBDV vaccine immunogenicity and challenge test and also to determine the IBDV vaccine administration appropriate time on vaccinated progeny flocks.

6.1.Infectious bursal disease outbreak investigation and characterization

Isolation of virus on chicken fibroblast cell culture revealing characteristic infectious bursal disease virus cytopathic effect of reflective rounding of cells after third blind passage in agreement with (Boot *et al.,* 2000) who stated that IBDV was isolated using CEF Primary cell cultures from most samples collected from clinically diseased chickens. And also Isolation of virus on inoculation of 9-10 days old embryonated SPF chicken eggs in chorioallantoic membrane revealing death of embryo on third and fifth days post inoculation and pathognomic gross pathological lesions of oedematous distension of the abdomen, petechiae and congestion of the skin and ecchymotic haemorrhages in the toe joints and cerebrum. (OIE, 2012) which stated that CAM route inoculation is the most sensitive route IBDV virus isolation.

The PCR analysis is successfully amplified the virus gene and revealed that the chickens were infected with infectious bursal disease virus by producing the expected band size on agarose gel electrophoresis analysis. This PCR finding is in agreement with the previous report of Jemberie *et al.,* (2012) that the same PCR product band size obtained using the same amplification primers.

In the present study the sequence analysis revealed the existence and circulation of $_{VV}$IBDV in Ethiopian chicken population and responsible for the frequently occurring outbreaks of IBD. Accordingly the current eleven field isolates was collected on different representative areas in Ethiopia IBD field outbreak samples were confirmed interestingly a single pathotype $_{VV}$IBDV strain.

Currently in this study the assessment of IBD outbreaks in country level and identification of the circulating virus pathotype at molecular level was answered efficiently the problems of the frequent occurrence of IBD outbreak despite using intermediate live attenuated IBDV vaccine strain D78 and also provide an option to make a decision on the selection of safe and protective vaccine strain. The present laboratory based findings will contribute more information to the

scientific communities on the existence of very virulent infectious bursal disease virus in Ethiopia.

6.2.Infectious bursal disease vaccine immunogenicity test

The epidemiology of the disease is usually influenced by certain factors like host's immune status, wide host range, thermo-stability; variation in strains of the causative viruses and its very resistantness to inactivation. Although vaccination of chickens has remained as the principal method to control these diseases (Okwor *et al.,* 2013; Susan *et al.,* 2013). Some important factors determining the success of vaccination include the time of vaccination, vaccine type, maternal antibodies in the chicks and pathogenicity of the offending virus (Hair-Bejo *et al.,* 2004). The time-point of vaccination is crucial as persisting maternally derived antibodies might neutralize the vaccine. The titers may vary considerably within a flock and revaccinations may be necessary. It has also to be taken into consideration that vvIBDV will break through immunity provided by highly attenuated vaccine strains. On the other side, it is well known that less attenuated strains ("hot vaccines") may cause lesions in the bursa follicles results in, immunosuppression even in vaccinated birds.

Progeny of vaccinated flocks have variable and declining passive immunity to IBD for several weeks after hatching. A high level of maternal antibodies will protect most young chickens against challenge by vvIBD virus for up to 3 weeks after hatching (Van den Berg, 2000). In addition, the persistence of the endemic serotype 1 virus between flocks and subsequent infection should provide most chickens older than 3 weeks with active antibodies for protection against clinical disease. Progeny of parent flocks vaccinated with classical strains of IBD virus may have poor maternal immunity against eav strains of the virus (Ignjatovic *et al*., 2001).

In an attempt to understand the epidemiology of the current IBD situation in Ethiopia, we have characterized local IBDV strains at the antigenic and molecular level. Characterization of a number of recently isolated strains between 2013 to 2015 and their comparison with existing

vaccine strains revealed some of the features of local IBDV strains previously unknown. We aimed also to correlate these changes with vaccinal immunity in commercial flocks in order to provide insurance that the best possible strategies are being applied for control of endemic strains.

Different types of vaccines are mostly available for the prevention of IBD. These are live attenuated vaccine, egg adapted or tissue culture one. However, some viruses that are immunosuppressive in nature may interfere with chicken's immune responses to other vaccine viruses which may lead to vaccine breaks (Phong *et al.,* 2003; Hair-Bejo *et al*., 2004). Some vaccines are capable of inducing similar or more severe bursal lesions than those caused by field virus strains. Although selecting the potent and safe IBDV vaccine to protect the currently isolated and circulated $_{VV}$IBDV in the country and all over the world poultry rearing and this $_{VV}$IBDV occurring areas through different live attenuated IBD virus vaccine immunogenicity test performed, and then computed and decided by on the degree of elevation of antibodies (ELISA) and protection efficacy against $_{VV}$IBDV as parameter.

The experimental animal was vaccinated progeny originated specific pathogen free chickens. During these experimental studies maternal antibody (MAb) titer was done prior to experimental vaccination at day 14 and day 21. The sera collected and examine at chickens age of day 14 were developed the mean S/P ratio *(*0.422$\pm$ 0.141) and 56/275 coefficient of variation which was above the negative control value (0.067) of the flock screening ELISA kit used during the test and they were almost reacted positively. Because of the first sampled sera ELISA test result increment rechecking of the IBDV MAb level was done again on day 21. There was showed reduction of IBDV MAb on day 21 examined serum than on a day 14 and the significant difference between the two days was ($p<0.05$) (0.422$\pm$0.141, and 0.05 $\pm$ 0.001 mean S/P ratio, respectively) and also the percent coefficient of variation was 5/257 (1.8%). Based on the kit protocol and the serum ELISA screening test result, the experimental IBDV vaccine administered schedules were determined as primary and booster experimental IBDV vaccine dose was administered at day 21 and 35 respectively (Table 5) and also the challenge test was conducted 7 days post booster dose, at age of day 42. This result agreed with finding of Hsieh *et*

al., (2010) who had reported interference of maternal antibodies in the establishment of the vaccination schedule. On the other hand (Kumar *et al.,* 2000) considered 21 days old as the ideal age for vaccination, since maternal antibodies were not detectable anymore and could not interfere with the replication of the vaccine virus and oppose the finding of (Zaheer *et al.,*2003) who had reported MAb as minimal and its protective efficacy diminished after one week of age. Although there is variability in the persistence of maternal antibodies in the progeny, antibody levels at the first day of age can be known according to the breeder immunity, and it is thus possible to estimate antibody half life and establish the most appropriate period for prime vaccination Alam *et al.,* (2002).

The seroconversion of the experimental vaccines was serologically tested on the sera were collected at 14 days post primary dose of vaccination (at and just before booster dose, 35 days) and prior to challenge, 7 days post booster dose at day 42 along the groups, the treatment group A was developed significantly low S/P ratio (0.470$\pm$ 0.02 and 0.43$\pm$0.022) respectively and also its sample positivity percent ratio on day 35 was lowered by one (% positivity = (44/45) as compared to the other treatment groups. Although the sera ELISA test S/P ratio result of group A on day 42 was lower than day 35 (0.47$\pm$ 0.02 and 0.43$\pm$0.022) but also its sample positivity percent ratio was increased by one on day 35 results. This result was in line with Butcher *et al.,* (2011) who stated that performances of chickens that can be influenced by vaccination are feed conversion, body weight gain, and sero-conversion uniformity, and these parameters are very important especially in broilers.

The remaining treatment groups B, C, and D was developed high mean S/P ratio as compaired to group-A but not significant difference each other; the P value were >0.05% (Table 6). In this study except group A similar pattern of antibody titer levels were observed as a study by Ahmad *et al*.,(2005) who had reported the antibody titer at 8, 10 and 14th days of PI resulted in a gradual increase of GMT values in all inoculated groups.

All chickens were determined the antibody level before challenge at 42 days of age and challenged with $10^{5.3}$TCID50 (0.2ml) of locally isolated vvIBDV virus via the oral route and

observed for 21 day post-challenge. Seroconversion and challenge virus protection was demonstrated by ELISA on chicken sere collected at day 21 post challenge and significantly high and superior antibody (ELISA S/P ratio) was developed by group C (3.20194± 0.06743) as compaired to vaccinated and challenge control groups E (2.83± 0.012) but significantly low antibody (ELISA S/P ratio) was developed by group D (2.067± 0.046) and also insignificant variable antibody levels were found at the experimental groups A, and B, (2.589± 0.041 and 2.686± 0.0192) respectively as compaired to the survived challenge control groups (2.83\± 0.012) (Table. 7). This result was in line with (Abd El-Aziz., 2000)who stated as vaccination at 2 weeks of age resulted in better immune response in vaccinated group with intermediate plus strain (228E).

There was no mortality was observed in any of the vaccinated chickens This agrees with findings of (Hassan *et al* .,2004) who reported that white Leghorn chickens vaccinated with a live intermediate vaccine were protected when challenged 10 days later with vvIBDV and also those of (Van den Berg *et al*.,2000) who demonstrated that chickens vaccinated with intermediate or intermediate-plus vaccines were fully protected from challenge with vvIBDV strains. According to Abdel and Saif (2001), vvIBDV are antigenically related to attenuated or mild vaccine type virus (70 to 80% homology), and the immunity induced by vvIBDV protects 100% against challenge with standard VVIBDV. Despite this, Zaheer *et al.,* (2003) observed outbreaks of IBD in flocks vaccinated with a variety of vaccines. Therefore, it appear that other factors such as immune status of chicks at the time of vaccination, immunosuppression by other agents, poor handling and administration of vaccines or inappropriate vaccination schedules may affect the success of immunization (Hair Bejo *et al* ., 2004). But also there was 60 % mortality on challenge control groups after 4 days post challenge test, resulted with haemorrhages in the bursa and in the thigh muscle, edematous swollen bursa, mucus in the intestine. This result was in line with (Baxendale, 2002) has also been reported that the vaccine did not give full protection to the birds even when the birds were kept on balance ration and in good conditions.

Although there were 100% sign of morbidity on challenge control groups and also variable amount of clinical signs after challenge test on vaccinated group A and B (23.3% and 3% respectively) and there was no any sign of morbidity on groups that was vaccinated by hot strain vaccines (group C and D). This result agrees with (Van den Berg, 2000) who stated that vvIBDV can be successfully controlled only if hot vaccines are used. The challenge virus causing the disease sign on vaccinated groups may be the (VVIBDV) has a potential for antigenic heterogenicity which result in frequent outbreaks in the field even in flocks vaccinated against IBDV (Hassan *et al.,* 1998), still one of the significant component of the control of IBD. The clinical signs were depression, reluctance to move, ruffled feathers, loss of appetite, white watery diarrhoea and death 2-3 days after the clinical disease has been diagnosed as stated by (Chansiripornchai and Sasipreeyajan, 2005).

7. CONCLUSIONS AND RECOMMENDATIONS

Poultry pathogens change their nature in response to intensified poultry production and present complex challenges to the poultry health and productivity. Very virulent IBDVs, which emerged in the late 1980s can cause mortality of up to 70% and induce severe immunosuppression. Since IBDV is ubiquitous and extremely resistant to environmental conditions, most of the efforts to control the disease are focused on vaccination programs, there is a high variation in the genetic properties between strains, these variations may play a role determining the antigenic and pathological characteristics of the viruses present in the field. In the case of RNA viruses, biological events including genetic reassortment or recombination alter the phenotypes and genotypes of circulating viruses and compromise their genetic stability.

It is clear from the present findings that IBD is one of the major poultry viral diseases causing very high mortality in chickens and prevalent in different production systems of poultry in Ethiopia and also the pathotype of the infectious bursal disease virus circulated in Ethiopia is very virulent infectious bursal disease virus ($_{VV}$IBDV). How the vvIBDV strains evolved in Ethiopia remains unclear. Literature suggests that international trade of live poultry and poultry products may facilitate the global spread of IBDV. The high tenacity of the virus and its resistance to several disinfections and virucidal procedures may contribute to the rapid distribution of the virus. Vaccination failure associated with the application of plaque purified and cloned live IBDV vaccines during and in subsequent IBD outbreaks in Ethiopia may be due to the lack of cross protection due to amino acid (aa) exchanges at the hydrophilic domains of field isolates compared to the vaccinal strains.

Improvement in the poultry industry should incorporate emphasis on the prevention and control of diseases that cause economic losses. This is the most comprehensive study on molecular typing of IBDV strains circulated and detected in the country and also IBDV vaccine immunogenicity test and concluded that this study confirmed the circulation of ($_{VV}$IBDV) in the country and also vaccines currently in use in Ethiopia might not effectively protect

chicken against highly virulent infectious bursal disease currently circulating locally. Therefore the following points are recommended as they are important to design strategies to control and prevent these diseases:

- The immunogenic candidate live intermediate plus IBDV vaccine strain LC75 should be produced and used vaccinate chickens to prevent and control the currently circulated VVIBDV virus, commercial poultry farm owners should be screened the IBDV maternal antibody (MAb) of the flock before primary IBDV vaccine administration.
- Village chickens should be vaccinated against most infectious diseases including IBD.
- The persistence time of IBDV maternal antibodies (MAb) on unvaccinated SPF progeny flocks should be investigated to design an optimum vaccination schedule.
- Infectious bursal disease (IBDV) virus molecular epidemiology should be studied with a planned interval to assess the antigenic diversity of the IBDV virus.

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9. ANNEXS

Annex. I: 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
H_2O (bi distilled)	1000ml

Dilute and adjust t 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 +4^{o}C

Preparation of maintenance cell culture medium

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

Preparation of maintenance cell culture medium

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

Annex .II: Preparation of Balanced Salt Solution (PBSA)

Phosphate buffer saline (PBSA) without calcium and magnesium

Sodium Phosphate Dibasic (N_a2HPO_4)	1.60 gms
Potassium Phosphate (KH_2PO_4)	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. III: 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. IV: Preparation of Chicken Embryo Fibroblast (CEF) Cell Cultures

- Use 9-11 day old embryos. The technique described here is for 3-5 embryos.
- Place all media, solution and trypsin in 37°c water bath.
- Spray eggs with Bio guard disinfectant (70% ethyl alcohol) place in hood. Using sterile technique, open shell and remove embryo with blunt ended curved forceps.

- Place embryos in Petri dish and cut off heads. Removal of limbs and viscera.
- Transfer bodies to new Petri dish or beaker containing PBS with antibiotics.
- Wash with PBS 3-4 times to remove red blood cells and extra debris
- In the beaker, the bodies can be fragmented by carefully chopping them with sterile scissors.
- Pour tissue fragments into trypsinization flask containing magnetic stirring bar. Add about 50 ml pre-warmed (37^{o}C) trypsin solution (0.25%) and put on stir plate at slow speed into 37 ^{o}C incubator for 10-15 minutes.
- Pour off supernatant into centrifuge tube with calf serum.
- Add 50 ml Trypsin solution and stir slowly in 37 ^{o}C incubator for 8 minutes. This may be repeated 1 more time for a total of 2 trypsinization.
- Centrifuge 10 min. at 1500 rpm. Note the amount of Pelleted cells obtained.
- Pour off Trypsin solution and resuspend cells complete cell culture medium (10% calf serum and tryptose phosphate broth contained GMEM or MEM. The cells may be counted or diluted 1:200and dispensed with in tissue culture flask and incubate at Co_2 connected 37 ^{o}C incubator.

Annex. V: Procedure for Inoculating Preformed Monolayer's

- Place all media and solution in 37°c water bath.
- Swirl T.C flask to resuspend as many RBC's and debris as possible and then decant and discard growth medium.
- Wash monolayer gently with 2-3times of pre warmed PBS and discard.
- Add 1 ml sample inoculums to the small T C flask (25 cm^2)or 2 ml for the larger size
- Rock each plate gently to distribute inoculums evenly over the cell monolayer.
- Incubate inoculated cultures in 37°c incubator for 45 minutes to 1 hour to allow virus to adsorb.
- Rock tray once or twice during incubation if possible.
- Add 20 ml maintenance medium to each small T C flask (25 cm^2).

NOTE: Maintenance media 2% calf serum.

Incubate at 37°c. Check plates daily for cytopathogenic effect (CPE) and condition of cells.

To harvest samples, freeze-thaw 2-3 times and collects.

Annex VI. Sample Preparation for Virus Isolation

- Sample received should be either fresh or frozen. If frozen, allow to thaw.
- Completely freeze and thaw sample(s).
- Take small pieces of sample (1gm) and wash 2 t0 3 times with PBSA
- Chop the sample with sterile scissors and grind I by using sterile mortar and pestle.
- Mince in Tryptose Phosphate Broth (TPB) or phosphate buffer saline solution (PBSA) with antibiotics.
- Transfer sample in centrifuge tubes and Centrifuge at 1500 rpm for 10 min.
- Collect the supernatant in appropriate screw cupped test tube, Label and use to inoculate embryos or cell cultures. (If bacterial contamination is a problem, the sample may be further filtered through a sterile 0.22 μm syringe filter).
- Long term storage of sample should be placed into a -80^{o} C freezer.

Annex. VII: Procedure of IBD Indirect Enzyme Linked Immunosorbent Assay

Assay procedure

- Remove the pre-coated plates from their sealed bags and record samples and control locations on a 12x8 template sheet. Each sample should be run in duplicate for optimum result. The positive and negative controls should always be run in duplicate.
- Add 50μl of the undiluted controls and diluted samples to the appropriate wells. Diluted samples should be retained at +4^{o}c until successful results are confirmed. Cover the plates with an adhesive cover and incubate at +37^{o}c for 30 minutes. Mix on a plate shaker or by gently tapping the side of the plate.
- Remove the adhesive cover and wash the plate 4 times with wash buffer (300 μl per well), invert and tap firmly on absorbent paper. N.B. to reduce the possibility of sample

carryover, it is recommended where possible, that the plate washer is programmed to wash each strip individually four times before washing the next strip.

- Add 50 µl of enzyme conjugate reagents to each well. Mix on plate shaker or by gently tapping the side of the plate.
- Cover the plates with the adhesive cover and incubate at +37^{o}c for 30 minutes.
- Remove the adhesive cover and wash the plate four times with wash buffer (300 µl per well), invert and tap firmly on absorbent paper.
- Add 50 µl ELISA substrate Regents to each well. The reagent must be at room temperature to achieve maximum colour development. Mix on a plate shaker or by gently tapping the side of the plate.
- Cover the plates with adhesive cover and incubate at +37^{o}c for 15 minutes. Colour development is pale pink, which deeper on addition of ELISA Stop solution.
- Remove the adhesive cover and add 50 µl ELISA solution to each well. Mix on a plate shaker to obtain full colour development.
- Wipe the under surface of the plate of dust etc. with a soft tissue. Read the plate using a microtitre plate reader at 550 nm having first blanked on air. In order to obtain optimum results the plate should be read immediately after adding the ELISA stop solution
- Stop solution must be added.
- Read with Spectrophometer and interpret the result according to the kit instruction

Annex VIII. 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 45^{o}c 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

  1 µl loading buffer

  5 µl PCR product

Prepare molecular weight marker

0.5 µl ml molecular weight markerVI (Bioehringer)

1 µl loading buffer

4.5 µl H_2O

- 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.

Annex IX. Test procedure for RT-PCR

Test procedure for RT-PCR

Extraction of RNA from IBD virus containing samples

Protocol: viral RNA is extracted from each sample using RNase spine column according to the pure link RNA extraction as follows:

i. Put 400µl of the sample is added to 1500 µl in an eppendorf tube and add equal volume of lysis buffer RLT to the sample and

ii. Mix the homogenate by votexing until the cell dispersed and centrifuge the homogenate at 12500rpm for 3 minutes.

iii. Proceed to RNA purification as followed

a). Add one volume of 70% ethanol alcohol to each volume of cell homogenate i .e 400 µl

b).vortex to ix thoroughly to dispersed any visible precipitate

c). Transfer up to 700 µl of homogenate sample to the spine cartridge with collecting tube

d). Centrifuge 12500rpm for 30 seconds at room temperature and discard the flow through

e) Repeat procedure d until the sample homogenate is completed

f). Add 700 µl wash buffer I to the spine cartridge and centrifuge at 12500rpm for 3min.

g) Place the spine cartridge in to new collection tube and add 500 µl wash buffer II and then centrifuge as above indicated

h). Repeated centrifugation at 13400 rpm for 3 minute to avoid the remaining ruminants and to dry and transfer the spine cartridge in to new collection tube

i). Add 40 µl RNase free water to the centre of the spine cartridge and incubate for 1 min.

j). Centrifuge the spine cartridge at 3400rpm for 3 min at room temperature to elute the RNA from the membrane in to the recovery tube

k). Label (the virus name and day of extraction) and preserved at -20^{o}C for further cDNA synthesis

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 42oc for 5 minute and add 1 µl RNase H in to each PCR tube incubate at 37oc for 20 min chill on ice

Finally preserve cDNA product at -20oc. or run PCR amplification