Thesis Ref. No. ________________

DETEREMINATION OF OPTIMAL TIME OF VACCINATION AGAINST INFECTIOUS BURSAL DISEASE (GUMBORO) AND MOLECULAR DIAGNOSIS OF CLINICAL CASES IN CENTRAL ETHIOPIA

MVSc Thesis

BY

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JUNE, 2017

BISHOFTU, ETHIOPIA

DETEREMINATION OF OPTIMAL TIME OF VACCINATION AGAINST INFECTIOUS BURSAL DISEASE (GUMBORO) AND MOLECULAR DIAGNOSIS OF CLINICAL CASES IN CENTRAL ETHIOPIA

A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for Master's Degree in Veterinary Epidemiology

By

Samson Terefe Kassa

June, 2017

Bishoftu, Ethiopia

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by **Samson Terefe Kassa** titled: 'DETEREMINATION OF OPTIMAL TIME OF VACCINATION AGAINST INFECTIOUS BURSAL DISEASE (GUMBORO) AND MOLECULAR DIAGNOSIS OF CLINICAL CASES IN CENTRAL ETHIOPIA', and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree in Veterinary Epidemiology.

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BIOGRAPHICAL SKETCH

I, the author of this MVSc Thesis, was born in December 1986 in Nazareth, Ethiopia. I attended Elementary, Junior Secondary school at Merti Junior school and my High School Education at Arbegnoch Senior Secondary School. After a successful completion of my High School Education, I had joined Addis Ababa University and graduated with DVM degree in 2011. Soon after graduation, I was employed by the Agricultural Technical and Vocational Educational Training (ATVET) Department of the Ministry of Agriculture and Rural Development to teach at Agarfa ATVET College as Instructor. Then after, I was employed by Elfora agro-industries as a Poultry Health Manager. I was then employed by Ethiopian Institute of Agricultural Research as a Veterinary Researcher where I am still working. After 2015, I have joined the school of graduate studies of College of Veterinary Medicine and Agriculture of Addis Ababa University to pursue Master's Degree in Veterinary Epidemiology.

STATEMENT OF THE AUTHOR

I first, declare that this dissertation is my *bonafide* work and that all sources of materials used for this dissertation have been duly acknowledged. This dissertation has been submitted to the requirements for MVSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University's Library to be made available to borrowers under rules of the Library. I solemnly declare that this dissertation is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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ACKNOWLEDGEMENTS

Above all, I am indebted to merciful God almighty through Jesus Christ, the only source of power and hope to my soul.

Secondly, I sincerely acknowledge my academic advisor Dr. Teshale Sori for his time and patience to analyze the results, thorough editing and organizing this paper to be more informative and always ready to helped me through providing scientific suggestions for the completion of this work, and my co-advisor Dr. Fufa Abunna for his unreserved advice and his encouraging word 'Very good' for any effort I have done. My appreciation also goes to Dr. Berecha Bayissa (NVI) for giving me valuable comments and whenever we met, always interested to made our discussion epidemiological.

Thirdly, I would like to thank Shok Singh for the provision of all poultry equipments and his assistance in feeding, watering, vaccinating and collecting blood samples in the whole period of the experiment and equally interested on the experiment. Without him this experiment work would have not come to its completion.

I am also grateful to Addis Ababa University and Ethiopian Institute of Agricultural Research for partial funding the cost of the study and Debre Zeit Agricultural Research Center for facilitating the financial and technical activities. And to the National Veterinary Institute Research and Diagnostic Department for their permission to use their laboratory facilities during serology and molecular analysis of the samples.

Finally, words fail to express my gratitude and love to my family, for their fervent prayers, tremendous support and encouragement throughout my life.

LIST OF ABBREVIATIONS

AA	Amino acid
Ab	Antibody
I-ELISA	Indirect 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
ELISA	Enzyme linked Immunosorbent Assay
FAO	Food and agriculture organization
GALT	Gut-associated lymphoid tissues
GC	Germinal centre
IBD	Infectious Bursal disease
IBDV	Infectious Bursal disease virus
Ig	Immunoglobulin
IFA	Immunofluorescence Assay
IFN	Interferon
IIF	Indirect immunofluorescence
IL	Interleukin
ILTV	Infectious laryngo tracheitis virus
MDAB	Maternally derived antibodies

MHC	Major Histocompatibility complex class
NAHDIC	National Animal Health Diagnostic Investigation Center
NCD	Newcastle disease
NVI	National Veterinary Institute
OD	Optical density
OIE	International Animal Health Organization
PCR	Polymerase Chain Reaction
PI	Post infection
qRT-PCR	Quantitative real time RT-PCR
RE	Restriction enzymes
RNA	Ribonucleic acid IBD
RFLP	Restriction fragment length polymorphism.
RPM	Rotation per minute
RT-PCR	Reverse transcription polymerase chain reaction
SAN	Specific antibody negative
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
VP2	Virus Protein2
VP1-VP4	Virus Protein1-4
VvIBDV	Very Virulent Infectious bursal disease virus
WRL	World Referral Laboratory

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ABSTRACT

In spite of the intensive and varied vaccination procedures to control Infectious Bursa Disease (IBD), the emergence of a very virulent (vv) IBD pathotype in Ethiopia and post vaccination outbreak have led to high economic losses in the poultry industry. The objectives of this study was to determine optimal time of vaccination against IBD and molecular diagnosis of clinical cases in commercial poultry farms in central Ethiopia. The study was conducted from November 2016 to April 2017 on exotic breed chickens kept under semi-intensive and intensive poultry farms selected randomly by simple random sampling technique in Addis Ababa, Bishoftu, and Adama areas. It was a type of prospective longitudinal study where chickens were followed for a defined period of time until they reached 6 weeks of age for clinical cases. A total of 11 samples from 11 farms were collected from clinical cases of infectious bursal diease in chickens (5 samples from Bishoftu, 3 samples from Adama and 3 samples from Addis Ababa) were analyzed with RT-PCR. Of these 8 samples (72.73%; CI: 39.03 – 93.98) were positive for infectious bursal disease virus RNA. The RNA of IBDV was detected in 4 of 5 (80%) of samples from Bishoftu and 2 of 3 (66.67%) of samples from each of Adama and Addis Ababa. For vaccine experimental study, One hundred eighty, day-old Lohman brown chicks were reared and used for this purpose. The chicks were divided into three groups A, B, C. Groups A were vaccinated via drinking water route at 7th day whereas B were vaccinated at 14th day of age. Group C was acted as control. Blood samples were collected from wing vein of individual chicken at day 1, 7, 14, 21, 28, 40 and serum was harvested. Indirect Enzyme Linked Immunosorbent Assay (IELISA) was employed to measure antibody titration. The proportion of chicks in the unvaccinated group with S/P ratio greater than the protective level continuously fall from 0.90 on day 1 to 0.0 on day 14. At day 21 of age after hatching, the time IBD commonly occurred, 55% of the chicks in group A had protective antibody level with average antibody titre of 1064.61± 748.1621; whereas only 5% of the chicks in group B had protective antibody level with average antibody titre of 123.2321 ± 212.0105. It was concluded that in chickens with low MDA, the 1st vaccination should be given at 7th day and repeat after one week. The effect of vaccination programs on the immune response to IBD vaccine in the farms should be further investigated.

Key words: Commercial farms, Ethiopia, IBD, MDA, PCR, Poultry, Vaccine

1. INTRODUCTION

Chicken are important livestock resources with the potential to break the vicious cycle of poverty and malnutrition in developing countries. They are considered to be cheap sources of quality animal protein. As a result Ministry of Agriculture has identified poultry production as key sector to deal with food security issues (LMP, 2014). As the demand for food rises due to a growing population, it will be critically important to develop methods to produce more food with greater efficiency, while lowering the prevalence of infectious diseases that inflicts significant losses in poultry industry. The poultry flocks of Ethiopia are featured by lower inflow of flock than its outflow. Mortality contributed up to 56% of the number of chicken that moved out of flock, implying that disease control is a top priority intervention area. Seventy percent of the mortality is caused by diseases where as only limited households (6%) have been using vaccines and drugs (Hailemichael *et al.,* 2016). Researchers and development workers rate infectious bursal disease as one of the infectious diseases constraining efficiency of poultry production throughout the country.

Infectious bursal disease (IBD) is one of the highly contagious disease affecting mostly young chickens. Infected chickens do not properly respond to vaccination, develop strong post vaccinal reactions, and become susceptible to concurrent infections (Müller *et al.,* 2012). Moreover, highly virulent IBDV can cause high mortality in unprotected flocks. In all poultry producing regions of the world, infectious bursal disease virus (IBDV) continues to be a major constraint for poultry farmers. The characteristic consequences of immunosuppression associated with IBDV are vaccine failure and susceptibility of chickens to opportunistic pathogens. This means that IBDV-infected chicken may become a good propagator for other viral and bacterial pathogens. For example, low pathogenic duck adapted avian influenza virus becomes more virulent when serially passaged in IBDV-infected chickens (Butcher and Miles, 2012). This has important implications for zoonotic infections such as *E. coli*, *Cambylobactria* and *Salmonella* and could contribute to their occurrence, which in turn could impair public health and poultry business.

The main means to control the disease is by restricted biosecurity and vaccination with a suitable vaccine and at a proper age. Live vaccines are administered to achieve active immunity but interference of maternally derived antibody (MDA) is the crucial problem in determining a successful live IBDV vaccination schedule. Vaccinating chickens in the presence of high levels of MDA results in vaccine virus neutralization and no immunity (Moraes *et al.,* 2005).

Due to the ever-increasing farm size and the proximity of one farm to another, the disease status in any given location is constantly evolving. More virulent strains of pathogens have and continue to emerge in all aspects of poultry. Therefore, it becomes glaringly evident that no single vaccination programme will be suitable for all farms in all areas. In order to have chickens protected from IBDV field challenge, it is crucial to determine the optimal timing for IBD vaccine delivery. The optimal timing is often predicted based on serological data following detection of IBDV MDA by an ELISA system during the first week post hatch (Moraes *et al.,* 2005).

The first confirmed acute outbreak of IBD in Ethiopia was reported in the months of March and April, 2002 in Commercial poultry farms, Debre Zeit. Since then, unconfirmed post-vaccination outbreaks have been observed and many farms are closed in different areas of the country and becoming a threat to the poultry industry and no further investigation was carried out to know the nature of the virus and the distribution of the disease (Zeleke *et al.,* 2002).

Despite the growing importance of IBD vaccines in Ethiopia, it is being availed by importation from Intervet, Holland, and USA and distributed for customers (personal communication). This has its own drawbacks. It could re-assort with the field strains and end up with emergence new strain of the virus. Vaccine produced from a local isolate is an appropriate approach, which requires in depth understanding of the molecular epidemiology of the disease and its etiologic agent. These vaccines are produced from IBDV strains isolated from different production systems and because of this they may not be as effective as they are supposed to be (Natnael, 2015) also underlined the need for development of IBD vaccines from local IBDV strains for securing the efficacy of the vaccines being used. Imported vaccines are acquired from elsewhere via foreign currency and with higher transportation cost, and this makes them to be more costly. Besides, there is genetic diversity among strains from different countries and with the same countries making cross-protection incomplete. Development and production of effective IBD vaccine locally via isolation of IBD virus strains from the local production systems is mandatory. However, previously no studies have been conducted in Ethiopia by isolation and sequencing of the IBDV. Hence, the findings from this research are therefore steps forward to materialize the production of effective Gumboro vaccine from local isolate (Duguma *et al.*, 2006).

Small and large scale chicken farms are rapidly growing in Ethiopia. The chicken strains imported are temperate breeds that are less adapted to the heat stress and disease challenges in the country. Accompanying intensification of poultry farming, there is occurrence of epidemics of newly introduced diseases and/or epidemics of endemic diseases. One of the diseases that is of growing concern in poultry is Infectious Bursal disease (Gumboro disease). There are different reports and complains of disease outbreak from different parts of the country and many small scale poultry farms are closed. Although vaccination is given, the disease outbreak is common. Gumboro is the worst and number one problem which causes high mortality by concurrent diseases. Despite the fact that IBD incidences are increasing at alarming rate all over the country, little have been known about it to devise effective control strategies (Zeleke, 2005).

In Ethiopia IBD is prevalent in various areas (Zeleke *et al.*, 2005) causing high mortality ranging from 49.89% to 72% in chicken (Woldemariam and Wossene, 2007; Tesfaheywet and Getnet, 2012). Its control is constrained by lack of epidemiological information, absence of effective administration schedule and shortage of molecular diagnostic tools. Elsewhere in the world the use of molecular techniques to detect and identify IBDV strains has increased in recent years. RT-PCR has been used to amplify sections of the VP2 gene, which encodes for the major protective epitopes, contains determinants for pathogenicity, and is highly variable among strains (Natnael, 2015). Control through effective vaccines requires knowledge of the occurrence and distribution of the strains of IBDV. At present IBD vaccines are widely used in attempts made to control IBD in Ethiopia. However, there have been several reports of post-vaccination outbreaks. Maternally derived antibody is one of the hypothesized reasons for the occurrence of outbreaks after vaccination. Two different vaccination schedules are used in central Ethiopia, both of which are not supported by systematic evaluation of the effectiveness of the schedules. Therefore, there is a need for epidemiological assessment of the efficacy of the schedules. The objectives of this study are:

- To determine optimal time of vaccination in chicks from unvaccinated parent stock using randomized controlled trial
- To diagnose cases of IBD encountered during the study period using molecular methods

2. LITERATURE REVIEW

2.1. History of IBD

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).

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 (IBD) is caused by a virus that is a member of the genus Avibirnavirus of the family Birnaviridae. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two serotypes of IBDV are recognised. These are designated serotypes 1 and 2. Both serotypes can be differentiated by cross-neutralisation assays. Clinical disease has been associated with only serotype 1 and all commercial vaccines are prepared against this serotype. Antigenic variants of IBD serotype 1 have been described and these may require special vaccines for maximum protection. Very virulent strains of classical serotype 1 are now common and are causing serious disease in many countries (OIE, 2012).

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 bisegmented 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). 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.

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 (Muller *et al.,* 2003).

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 nonenveloped, 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).

2.4. Epidemiology of the virus

2.4.1. Host range

Clinical disease occurs solely in chickens but 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.

The first outbreak of infectious bursal disease (IBD) that had occurred in 1957 in a broiler farmnear Gumboro, the Delaware area in the USA, was caused by the classical serotype 1 IBDV (Cos grove 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. 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 defense 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 Tcell 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 antigenpresenting 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 induces 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).

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. 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).

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).

2.7. Clinical findings of the disease

In 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 diarrhea, ruffled feathers, severe prostration, vent picking, vent feathers soiled with urates, anorexia, dehydration, and elevated water consumption.

2.8. Laboratory Diagnosis

Clinical disease due to infection with the IBDV can usually be diagnosed by a combination of characteristic signs and post-mortem lesions. Laboratory confirmation of disease, or detection of subclinical infection, can be carried out by demonstration of a humoral immune response in unvaccinated chickens or by detecting the presence of viral antigen or viral genome in tissues. In the absence of such tests, histological examination of bursa may be helpful. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) based on plates coated with IBDV-specific antibodies have also been described for the demonstration of IBDV antigens in bursal homogenates. The reverse transcription polymerase chain reaction (RT-PCR) with specific primers may be used to detect viral genomic RNA in the bursa of Fabricius (OIE, 2012). Generally a preliminary diagnosis can usually be made based on flock history, clinical signs and post-mortem (necropsy) examinations.

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 qRTPCR 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 IBD 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

Vaccination failures occur when, following vaccine administration, the chicken do not develop the adequate antibody titer levels and/ or are susceptible to a field disease outbreak. This means that IBDV-infected chicken may become a good propagator for other viral and bacterial pathogens. For example, low pathogenic duck adapted avian influenza virus becomes more virulent when serially passaged in IBDV-infected chickens (Tadelle and Ogle, 2001; Hailemariam *et al.*, 2006). This has important implications for zoonotic infections such as E. coli, Cambylobactria and Salmonella and could contribute to their occurrence, which in turn could impair public health and poultry business. When a vaccination fail the natural tendency is to blame the vaccine. Although this is an important factor to consider, many other factors must be evaluated to determine the causes of the failure. The common factors responsible for vaccine failure are; vaccination program, vaccine administration deficiency, administration and handling of the vaccine, timing, maternal antibody, stress, immuno-suppression, management practice, vaccine quality, vaccine modification, and vaccine strain are the main ones (Saif, 2003).

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).

No one internationally effective vaccination programme can be designed. Certain regions with a long history of poultry production may require a programme with repeated vaccinations against numerous different pathogenic agents but new poultry farms, geographically isolated from other poultry operations, may have the opportunity to decrease the costs of production with a more limited vaccination programme. If the vaccination programme is not well studied and executed it can harm rather than assist the birds. If it is not known what diseases are prevalent in the region, you may be infecting the birds with an unnecessary virus when vaccinated. It is postulated that a vaccine, actually could have caused serious ILT outbreaks in different countries. A good vaccination programme is not enough when the vaccine used has not been delivered and handled properly. Live vaccines may become inactivated due to improper handling. Store and handle vaccines as recommended by the manufacturer. Once a vaccine is reconstituted, the “time clock is ticking.” Certain live vaccines, such as Marek’s disease vaccines, are easily killed and failure to follow the manufacturer’s recommended handling practices will result in the inactivation of the virus prior to administration. Infectious bronchitis (IB) vaccines lose 50% of its potency in warm conditions in under one hour (Mojtaba *et al.*, 2012).

Poor administration is the most common cause of vaccine failure in poultry. Planning and attention to details resulting in better administration that will improve disease control and therefore performance of poultry. As one poultry grower put it: “vaccines are no good if they do not get into the chicken.” Poor distribution of live vaccine administered by the water or spray route may result in chickens being “missed” in parts of the house. Relying on transmission of the vaccine from bird to bird is risky, and can result in excessive rolling – type reactions of long duration and delayed immunity in the flock. “Misses” with killed vaccines will result in chickens with no protection, as killed vaccines will not spread from bird to bird. Live vaccines administered in the drinking water can be destroyed before they reach the bird if water sanitisers have not been removed from the water prior to the addition of vaccine. Vaccines that are administered. The immune status of the breeder flocks also can be involved in a vaccine failure. If the breeder flock provides progeny with high levels of maternal antibodies, it may interfere with the multiplication of live vaccines, reducing the amount of immunity produced. For example, if a chick comes from a breeder hen with high levels of antibody against IBD, the chick will typically have high levels of maternal antibodies for several weeks. If vaccination is attempted in the presence of these antibodies, some of the vaccine virus will be inactivated and a decreased response to the vaccine results. On the other hand, doing vaccination too late may leave them susceptible to field challenge and can cause very strong reaction for the mentioned disease (Mojtaba *et al.*, 2012).

Vaccination itself is a stress. Stress may reduce the chicken's ability to mount an immune response. Stress could include environmental extremes (temperature, relative humidity), inadequate nutrition, parasitism and other diseases. Vaccination of sick birds not only interferes with the response to vaccination but also interferes with the birds' ability to overcome the disease challenge present. Delay vaccination until the birds are healthy. Chickens may already be incubating the disease at the time of vaccine administration. Despite proper administration, the birds become diseased because time is needed for antibody production to begin and reach protective levels. Worthy to mention that after first exposure to a live virus – type vaccine, antibodies M, G and A are first detected approximately 4 to 5 days following exposure. Additional days are required for titers to reach protective levels (Mojtaba *et al.*, 2012).

The immune status of the flock is an important factor which must be considered. Chickens may be immunosuppressed due to infection with IBD, CIA or Marek's disease viruses or from consumption of feed with high levels of mycotoxins. The term immunosuppression refers to circumstances where the noncellular (antibody) and cellular components of the immune system are not functioning properly. This may result in the development of limited protection from the vaccination and an excessive vaccine reaction with morbidity and mortality. Management measures play an important role in vaccine failures. If infectious disease agents are allowed to build up on a farm over successive flocks without clean-out and disinfection, it is possible that the challenge dose of a particular infectious agent will be so great, or so soon, that a normally effective vaccination programme will be overwhelmed. However, there is no substitute for good management. Vaccines should be used as part of a good management programme, not as a replacement. It is obvious that if there would exist a vaccine that could be administered to alleviate our managerial deficiencies, it would be top selling. Usually if a vaccine fails to stimulate the generation of sufficient antibody titers or when a disease outbreak occurs, the first thing crosses everybody's mind is that the vaccine is of poor quality. But does it really mean that the vaccines can be the culprits? Evidence shows that in the great majority of cases, if not all, the vaccines are of excellent quality since they are usually manufactured in reputable and highly regulated pharmaceutical companies that take pride in their R&D department and have an excellent quality control programme. In order to save money never try to stretch vaccines by underdosing or overdosing. By underdosing, all the birds would not get enough of vaccine to produce sufficient antibodies and by overdosing you may stress the birds by overwhelming them. The use of vaccines that have been over – attenuated can lead to a lack of immunogenecity and enhanced susceptibility to field challenge. On the other hand, vaccines that are not sufficiently modified can cause disease and may result in prolonged reactions and increased susceptibility to secondary bacterial infections (Mojtaba *et al.,* 2012).

Many infectious agents have several different strains/serotypes. The vaccine may not contain the proper strains or serotypes of organism required to stimulate protective immunity against the agent causing the field challenge. Although the vaccine is administered properly and uniform/adequate antibody titers are present, the chickens still break with the disease, particularly with IB and more recently with IBD. Most IB vaccination programmes include the Massachusetts and Connecticut serotypes of IB virus in the vaccine. If the chickens in the field are challenged with variant serotypes, the disease may occur. Likewise, if protective levels of antibodies against IB virus are present, it does not suggest the chickens are necessarily protected against ND or other diseases. Antibody titre levels must be determined for each disease. In some cases, the field strain of an organism is of very high virulence and the vaccine strain is highly attenuated. In this situation, the flock may be effectively immunised, but the immunity is insufficient to protect against disease completely. Vaccines administered properly, at the correct time and with the appropriate antigen content do not guarantee 100% and there is a great need for continuous evaluation. In many situations, vaccinations may only be employed as a means of minimising the economic impacts of a disease rather than total prevention in 100% of the flock. Strict attention to details can mean the difference between protected birds and those that remain susceptible. However, a well – designed, well – timed and soundly executed vaccination programme coupled with good management, nutrition and biosecurity will go a long way to helping maintain a healthy and productive flock (Mojtaba *et al.,* 2012).

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).

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).

Table 1. Factors which interfere with vaccine efficacy in poultry

Type of factors	Impact on vaccine efficacy
Factors associated with the vaccine itself	
Virus serotype	Many infectious agents (e.g. infectious bronchitis virus) have different serotypes, and vaccine antigens do not provide protection against all field strains
Level of protection	Field strain of very high virulence, and/or highly attenuated vaccine strains
Factors associated with vaccine administration	
Handling	Certain live vaccines (e.g. live cell-mediated Marek's disease vaccines) are easily killed if mishandled
Diluent used	Viable vaccines administered in drinking water are destroyed if water sanitisers are not removed
Route	Vaccines administered by injection fail if vaccinators do not deliver the vaccine to the appropriate vaccination site Mass vaccination (drinking water and aerosol) tends towards lower uniformity than individual administration
Associations	Administration of certain combinations of live virus vaccines affects the single virus response if theyhave the same target tissues
Factors associated with the bird/flock	
Maternal immunity	In presence of high levels of maternal antibodies, live vaccines administered during the first two weeks of life may be neutralised
Immunosuppression	Stress, certain infectious agents (e.g. infectious bursal disease, infectious anaemia and Marek's disease in chickens, haemorrhagic enteritis in turkeys), mycotoxins (in particular aflatoxins) impair immune response
Sanitary status	The birds are already infected (incubation period) with the pathogen against which the vaccination is directed
Genetic factors	Different vaccine responses with respect to species or commercial hybrids
Management conditions	
Hygienic practices	Without clean-out and disinfection over successive flocks, the challenge dose might be too high or infection might occur too soon

(Marangon and Busani, 2006).

2.10. Economic Importance of IBD

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 (Van den berg, 2004).

2.11. The status of 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; 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; 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 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, Moreover, 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 (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.,* 2005) 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 Areas

This study has two components. The molecular study was conducted in semi-intensive and intensive poultry farms found in Addis Ababa, Bishoftu, and Adama (Figur 1). The experimental study was conducted in Bishoftu. These areas are selected due to the presence of many commercial poultry farms. Addis Ababa has an altitude of 2300 meter above sea level with subtropical highland 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). Bishoftu is located 45 Km south east of Addis Ababa. The area is situated 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). Adama is located in East Shoa zone of Oromia regional state of Ethiopia. Absolute location of East Shewa Zone extends from 70 33′0″ to 9008′56″N and 38024′10″E to 400 05′ 34″E which indicate that this zone is located in tropical climatic zone though the climate is influenced by altitudinal variation. The total number of cattle found in East Shewa Zone is 1,147,173 and the Zone covers approximately 9,633.52km2. The altitude ranges from 500 to 4307 meter above mean sea level. The Zone can be categorized under rift valley system of Oromia since about 93% of the total area of the zone is completely located in rift valley system. The mean annual temperature varies between 18ºC and 30ºC and its mean annual rainfall is 410mm-820mm (CSA, 2015).

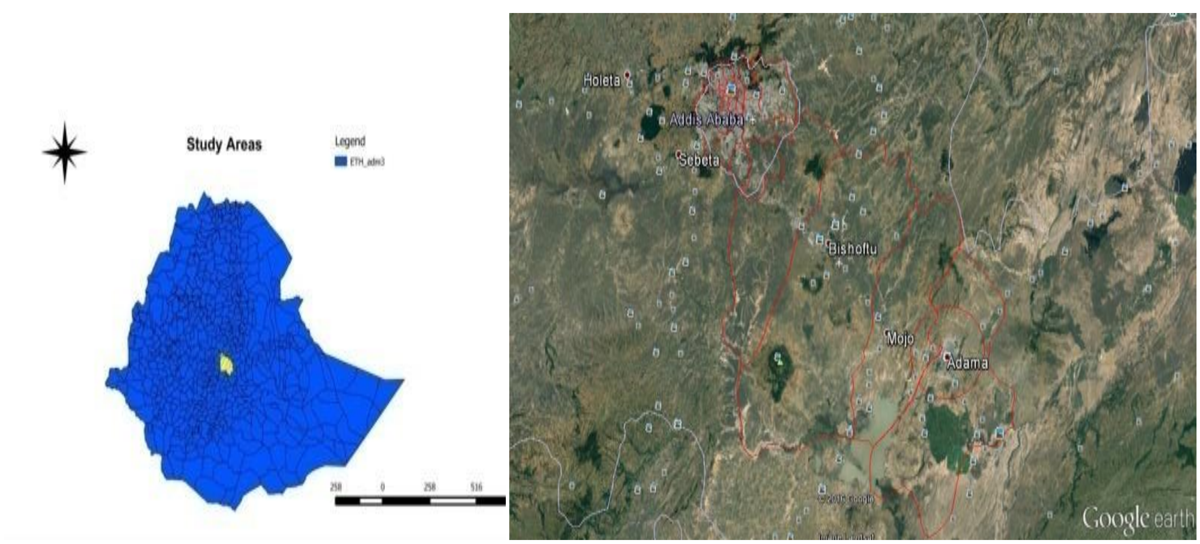

Figure 1. Map of Ethiopia showing the study areas

3.2. Study Population

The study population for the molecular diagnosis consists of exotic layer chickens kept under semi-intensive and intensive production systems in the study areas. However, special attention was given to young chickens aged between 3-6 weeks. For on farm vaccine experimental study, One-day-old Lohman Brawn chicks (Lohmann, Germany) were used in this study. The parent flock was raised at the Debre Zeit agricultural research Center, Poultry farm Station.

3.3. Study Design and Sampling Method

List of poultry farms in each town were obtained from respective agricultural offices and poultry producers' associations. Twenty poultry farms for the study were selected from each study area. The study was a type of prospective longitudinal study where chickens on the selected farms were followed until they reached two months of age in a dribbling way from Nov, 2016 - Apr, 2017 for cases of IBD. Samples of bursa and spleen from IBD suspected chickens were collected for molecular diagnosis of the cases. Samples were placed in sterile and labeled universal bottles and transported to National Veterinary Institute, Bishoftu for molecular diagnosis. The samples were processed in the laboratory immediately after submission. Whenever immediate processing was not possible, they were stored at -80^{o}C until processing (OIE, 2012).

For the vaccine effectiveness experimental study, at day one the chicks were randomly assigned to one of three experimental groups consisting of 60 chickens per group. One group of chickens were vaccinated on day 7 with CEVAC^{R} GumboL and day 14 using CEVAC^{R} IBDL Winterfield 2512 G-61 strain. This group was designated group A. These two vaccines were obtained from Alema Poultry farms, Bishoftu, Ethiopia and the vaccination schedule was based on the recommendation of the provider. One group of chickens were vaccinated with Bursitis virus strain LC75, produced at National Veterinary Institute (NVI), Bishoftu Ethiopia. This group was designated as group B and chickens were vaccinated on day 14 and 21 by drinking ground water taken from Maranata poultry farm. The third group served as unvaccinated control. Blood samples were collected on day 0, 7, 14, 21, 28 and 40 from the wing vein by using sterile disposable 3 ml syringe with 23 gage needle. After leting the blood to clot in the syringe for 8 hours, the serum was harvested from blood samples and stored at -20^0C until tested (OIE, 2012).

3.4. Study Methodology

3.4.1. Observation of clinical manifestation and post mortem examination

The selected chicken farms were followed for clinical and post-mortem examination. Signs of infection in a flock was the tendency for some birds to pick at their own vents, soiled vent feathers, whitish or watery diarrhea, anorexia, depression, ruffled feathers, trembling, severe prostration, and finally death. Affected birds became dehydrated and, in terminal stages of the disease, had a subnormal temperature. Post-mortem lesions include: dehydration of the muscles with numerous ecchymotic haemorrhages, enlargement and discoloration of the kidneys, with urates in the tubules. The bursa of Fabricius showed the main diagnostic lesions. In birds that died at the peak of the disease outbreak, the bursa was enlarged and turgid with a pale yellow discoloration. Intrafollicular haemorrhages were present and, in some cases, the bursa was completely haemorrhagic giving the appearance of a black cherry. Peribursal straw-coloured oedema was present in many bursae. Confirmation of clinical disease or detection of subclinical disease was done by using Molecular technique from the bursa.

3.4.2. Sample collection, transportation and preparation

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 Nov, 2016 to April, 2017. On the other hand, for IBDV vaccine immunogenicity experimental study, blood samples were collected and the serum was harvested. The serum samples were preserved at -20 ^{oc} until processed (OIE, 2012).

Tissue samples were collected from chickens displaying typical clinical signs for IBD or suspected of being infected with IBD in the eleven selected farms for observational study. Two sick birds per farm were sacrificed for the collection of specimens (Bursa of Fabricius) in the early stages of the disease. The samples were collected as aseptic as possible for virus isolation and molecular characterization after being processed in the Virology laboratory. Chop the bursae using two scalpels, add a small amount of peptone broth containing penicillin and streptomycin (1000 μg/ml each), and homogenise in a tissue blender. Centrifuge the homogenate at 3000rpm for 10 minutes. Harvest the supernatant fluid for use in the investigations described below. For virus isolation, bursa and spleen samples were collected aseptically from IBD suspected clinically sick chickens after killing in the farms. 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^{oc} a waiting processing (OIE, 2012).

The BF tissue samples collected from the farms were processed and cultured on vero cell monolayer during the study period where about 1 gram of each BF tissue specimens were taken and washed three times using phosphate Buffered Saline (PBS) on petridish. Washed tissue was transferred to mortar and cut into small pieces using scissors and minced by scalpel blade. The minced tissues were then ground and homogenized using pestle. 9 ml of phosphate Buffered Saline (PBS) was added to the ground and homogenized tissues and mixed. Filtration through a 0.22 μ filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titre. The homogenized tissues were transferred to test tube and centrifuged at 3400 rpm for 10 min. 0.5 ml of the supernatant was inoculated on to confluent vero cells and incubated at 37 ºC for 1 hour. Following incubation, the inoculated cell lines were washed using PBS and about 10 ml of 2% GMEM added and incubated at 37 ºC to see the development of cytopathic effect (CPE),

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 samples were 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 sent to Molecular Laboratory.

3.4.3. Molecular Analysis of the Samples

Extraction of the genetic materials and detection of RNA of infectious bursal disease RNA in the clinical samples collected was carried out RNeasy® Mini Kit (cat. nos. 74104 and 74106) (Qiagen, 2011) at the Molecular Biology Laboratory of the National Veterinary Institute. Reverse transcription PCR was used to RNA of IBDV from 10 % (w/v) tissue sample suspensions and/or cell culture homogenates using PureLinKTM RNA Mini Kit Cat no.12183-018A, based on the manufacturer protocols. Briefly, 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 vortexing and incubated at 56^{o}C for 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 seconds.

Amplification of the genetic material was done using Primers:
IBD3-Fow -5'TGTAAAACGACGGCCAGTGCATGCGGTATGTGACGCTTGGTCAC-3'
IBD3-REV-5'CAGGAAACAGCTATGACCGAATTCGATCCTGTTGCCACTCTTTC-3'.
The amplification was done using First-strand cDNA synthsis kit Catalog no-18080-051. A two step RT-PCR was done for cDNA synthisis (for one reaction) (Annex 1). Visualization of the amplification products was done using 1.5% agarose gel. Positive control (RNA from IBD vaccine virus) and negative control (PCR water) were used during the analysis.

3.4.3.1. RNA extraction and reverse transcription

RNA extraction was done by using Qigein RNeasy® mini Kit Procedure. RNA was elutied by using RNase free water and stored -20^{o}c. The cDNA was used superscript™ III First- strand Synthisis system (Invitrogen), cat.no 18080-051 for RT-PCR. According the Kit procedure mentioned in the above, two steps RT-PCR cDNA synthesis was conducted. The components of 50µM oligo(dT)(1µl),10mM dNTP mix(1µl),RNase free water (DEPC-treated water)(3µl) and RNA Template (5µl) were added for one reaction.Then it was incubated at 65^{o}c for

5minutes and placed on ice for at least 1 minute. The cDNA synthesis mixture compounds 10X RT buffer (2 µl), 25mM $MgCl_2$ (4 µl), 0.1 DTT (1 µl), RNase OUT^{TM} (40U/ µl) (1 µl) and Superscript ^{TM} III RT (200U/ µl) (1 µl) were used. 10 µl cDNA synthesis mixure was added in to each sample (the first reaction) and briefly centrifuge and incubated at 50^{o}c for 50 minutes. Then the reaction was terminated at 85^{o}c for 5 minutes and chilled on ice and briefly centrifuged. 1 µl RNase H was added and incubated at 37^{o}c for 20 minutes. Finally the prepared cDNA was used immediately for PCR based on the routine procedures done by National Veterinary Institute SOP manuals.

A total of 20 µl master mix was prepared by using 3µl of RNase free water, 5 PM/ µl -2ul of forward primer, 5 PM/ µl 2ul of reverse primer, 10 ul of IQ super mix and 3ul of Template (cDNA). A set of primers were used for the RT-PCR reaction and for the subsequent sequence analysis using forward and reverse PCR primers for amplification of 645 bp fragment IBDV on VP2Gene. The following primers were used for RT-PCR reaction.

Forward primer:
5'TGTAAAACGACGGCCAGTGCATGCGGTATGTGACGCTTGGTCAC-3'
Reverse primer:
5'CAGGAAACAGCTATGACCGAATTCGATCCTGTTGCCACTCTTTC-3'
(OIE, 2016).

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 PureLinKTM 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 56ocfor 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 + 4oc, 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 MgCl2 , 2 µl of 0.1 MDTT, and 1 µl superscript IIIRT enzyme, by incubated those cDNA synthesis mix at 55oc. The synthesized cDNA and the cDNA synthesis mix were rewarmed, mix the reaction gently and incubate at 55oc for 50 minutes. The reactions was terminated at 85 oc 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 oc and finally the obtained cDNA was used for PCR (Polymerase Chain Reaction).

3.4.3.2. Polymerase chain reaction (Touch down PCR)

PCR was conducted using the conventional method that involved initial denaturation at 95ºC for 5 minutes to 1 cycle, followed by 15 cycles of 1st denaturation at 95ºC for 30sec, annealing at 60ºC for 30sec, extension at 72ºC for 30sec and again followed by 20 cycles of 2nd denaturation at 95ºC for 30sec ,annealing at 56^{0}c for 30sec ,extension at 72^{0}c for 30sec and final extension at 72ºC for 7 minutes for 1 cycleand hold 4 ºC until machine off.

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 ACGATG 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 MgSO4 (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.

3.4.3.3. *Agarose gel electrophoresis of PCR products*

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

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

3.4.4. *On-farm Experimental Study and Determination of Antibody Titres*

3.4.3.1. *Ethical statement*

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.

3.4.3.2. Experimental animal and management

One hundred and eighty day old chicks from unvaccinated Lohman Brown parent stock were obtained from Debre Zeit Agricultural Research Center (DZARC) for determination of optimal time vaccination. The chicks were placed into separate rearing cages at Dembi site, Bishoftu and provided with standard feed and management. The parent stock (from day-old) was vaccinated Live attenuated Gumboro vaccine but not vaccinated the inactivated vaccine against IBD at pullet age. Progenies hatched from this parent stock were used for all the experiments. Two types of poultry feed (Starter and Grower) were bought from Alema koudjis feed processing company, bishoftu and Starter feed was given in the first month and Grower feed was given in the second month. Feed and water were provided *ad libitum* with equal number of drinkers and feeders in each group until the end of the experiment. The spacing in each group was 10 chicks/m3. All experiments were performed in animal facilities following international ethical guidelines on animal welfare.

3.4.3.3.The IBD vaccines used for the study

Three commercially available live attenuated infectious bursal disease vaccines (IBDV vaccines) were used. Namely: Intermediate: CEVAC^{R} GumboL contains LIBDV strain, Intermediate Bursal Disease Vaccine, freeze dried form and Batch No. IBD 04/14 Hungary; CEVAC^{R} IBDL Winterfield 2512 G-61 strain, Intermediate plus Bursal Disease Vaccine; freeze dried form, Batch No.3506N, with SPF embryonated hen eggs origin, manufactured by Lohmann Animal Health GmbH, Germany, and Bursitis virus strain LC75 Bursal Disease Vaccine, freeze dried form, Batch No. D037411 Manufactured by U.S.A. LOHMANN ANIMAL HEALTH INT, LTD, with cell culture origin which is actively produced at National Veterinary Institute (NVI) and used in Ethiopia.

3.4.3.4. Determination of Antibody Titres

The titre of antibody in the serum samples collected during this was assayed using enzyme linked immunosorbent assay (ELISA) kit (ID Screen®, IBD Indirect) supplied by ID.Vet, 310, rue Louis Pasteur – France. All the reagents were allowed to come to room temperature before use and homogenized by inversion and vortex. The samples were pre-diluted 1: 500 in Dilution Buffer and 10µL of the pre-diluted samples were added to 90 µL of Dilution Buffer in the ELISA microplate. The plate was covered and incubated for 30 minutes. The wells were emptied and washed 3 times with 300 µL of Wash Solution. One hundred µL of 1x Conjugate was added to each well and incubated for 30minutes. The wells were emptied and washed 3 times with 300 µL of Wash Solution to each well. One hundred µL Substrate Solution was added to each well and the plates incubated for 15 minutes. One hundred µL of Stop Solution was added to each well. The results of the reactions were read at 450nm using ELISA reader. The presence or absence of antibody to IBDV was determined by relating the A (450 nm) value of the unknown to the positive control mean. The positive control had previously been standardized and represented significant antibody levels to IBD in chicken serum. The relative level of antibody in the unknown was determined by calculating the sample to positive (S/P) ratio. The equation for calculation provided in the ELISA kit was used in calculating the antibody titre as follows:

a) Positive Control Mean (NCX)= $$\frac{\text{Well A1(450nm)} + \text{Well A2(450nm)}}{2} = OD_{PCX}$$

b) Negative Control Mean (NCX)= $$\frac{\text{Well A3(450nm)} + \text{Well A4(450nm)}}{2} = OD_{NCX}$$

Interpretation of the results

For each sample the S/P ratio was calculated as described by the manufacture of the kit as:

$$\mathbf{S/P = \frac{OD\ sample - OD\ NC}{OD\ PC - OD\ NC}}$$

Determination of antibody titre from the S/P ratio is as follows

$$\mathbf{Log_{10}\ (titre) = 0.97 * log_{10}(S/P) + 3.449}$$

$$\mathbf{Titre = 10^{\ log10(titre)}}$$

Finally the results of status of infection with IBD are determined as given the table below based on the recommendations of the manufacture.

Table 2. Interpretation of results of ELISA

S/P ratio	ELISA antibody titre	IBD immune status
S/P ≤ 0.3	Titre ≤ 875	Negative
S/P > 0.3	**Titre > 875**	**Positive**

Serum samples with S/P ratios of less than or equal to 0.3 were considered negative. S/P ratios greater than 0.3 (titres greater than 875) were considered positive and indicated either vaccination or exposure to IBD virus.

3.5. Data Management and Statistical Analysis

Data collected in this study were stored in Microsoft excel and the mean ±s.d of the antibody titres among the experimental groups was computed and compared using STATA version 13.

4. RESULTS

4.1. Clinical and Postmortem findings

During the study period, 26 flock showed very high morbidity (90%) with severe depression in most chickens lasting for 5–7 days. Mortality was risen (80%) sharply for 2 days then declined rapidly over the next 2–3 days. The incubation period was very short, and clinical signs of the disease were seen within 2-3 days after exposure. Signs of infection in a flock was the tendency for some birds to pick at their own vents, soiled vent feathers, whitish or watery diarrhea, anorexia, depression, ruffled feathers, trembling, severe prostration, and finally, death. Affected birds became dehydrated and, in terminal stages of the disease, had a subnormal temperature.

Gross Lesions: Birds that succumb to the infection were dehydrated with darkened discoloration of pectoral muscles. Hemorrhages were present in the thigh and pectoral muscles, increased mucus in the intestine and renal changes were prominent in birds that die or are in advanced stages of the disease. Such lesions are most probably a consequence of severe dehydration. The cloacal bursa appears to be the primary target organ of the virus. The infected bursa often shows necrotic foci and at times petechial or ecchymotic hemorrhages on the mucosal surface. Extensive hemorrhage throughout the entire bursa has been observed. The spleen was slightly enlarged and had small gray foci uniformly dispersed on the surface. Hemorrhages are observed in the mucosa at the juncture of the proventriculus and gizzard.

Figure 3. Postmortem examinations

4.2. Results of Molecular Diagnosis

A total of 11 samples collected from clinical cases of infectious bursal diease in chickens (5 samples from Bishoftu, 3 samples from Adama and 3 samples from Addis Ababa) were analyzed with RT-PCR. Of these 8 samples (72.73%; CI: 39.03 – 93.98) were positive for infectious bursal disease virus RNA (Figure 5). The RNA of IBDV was detected in 4 of 5 (80%) of samples from Bishoftu and 2 of 3 (66.67%) of samples from each of Adama and Addis Ababa (Table 3).

4.3. Results of Experimental study

The results of ELISA tests for MDA performed on the blood of day old chicks (Table 4) from unvaccinated parent flock revealed that antibody titre was on average 2329.045± 898.9366. Ninety percent of the chicks had an S/P ratio greater than the protective level (>0.3). For the unvaccinated chicks the titre was highest on day 1 and it decreased continuously from day 1 to day 21. The MDA falls sharply after day 7. The proportion of chicks in the unvaccinated group with S/P ratio greater than the protective level continuously fall from 0.90 on day 1 to 0.0 on day 14. In group A (those chicks vaccinated with GumboL and IBDL) on day 7 and 14 the antibody titre dropped from 1374.283±884.9499 on day 7 (first vaccination) when 70 % of the chicks had protective antibody level to 344.632± 480.8263 on day 14 (booster vaccination) (Table 5). At this age none of the chicks had protective antibody level and none of the chicks had protective antibody level. Two weeks after the first vaccination (day 21) the antibody titre remains low (1064.61± 748.1621) with only 30 % of the chicks having antibody titre greater than or equal to the protective level. About two weeks after the booster vaccination (day 28 of age) 100 % the chicks had antibody titre greater than the protective level and the mean anti body titre was 3168.555± 1124.5183. In Group B (those chicks vaccinated with NVI LC75) on day 14 and day 21 the antibody titre was 282.5035± 188.6627 on the day of first vaccination. At this time none of the chicks had protective antibody level. The antibody remains low (123.2321 ± 212.0105) when the chicks receive booster vaccination on day 21. About two weeks after the booster vaccination (day 40 of age) 100 % the chicks had antibody titre greater than the protective level and the mean anti body titre was 4349.256±1097.636.

5. DISCUSSION

Infectious bursal disease is one of the important diseases of poultry with huge loss in young flock. It continues to be a major problem of chickens in poultry rearing areas. It has been documented in Ethiopia before one decade and considered to be endemic in many parts of the country. This confirmed by the results of molecular investigation made in this study. The gene of infectious bursal disease virus was detected in samples collected from all sites. This shows that the disease is widespread at least in central Ethiopia. In this study 72.27% of the samples gave positive signals for IBDV. This is quite in agreement with previous reports made on infectious bursal disease in Ethiopia (Aregitu, 2015) and the results of Zahoor *et al.* (2010) from Pakistan. However, the proportion of samples yielding positive results in this study is higher than the reports of Ramzy *et al.* (2015) from Egypt. It is clear that infectious bursal disease is one of the standing problems of poultry farmers.

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 gelelectrophoresis 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.

The level of MDA is high for the first week for chicks derived from unvaccinated immune stock, but it decreases rapidly after day 7. The assay of the antibody collected on the second day of hatching showed that 90% of the chicks have protective antibody level and 10% of them were susceptible. That means in chicks hatched from unvaccinated parent flock the optimal time of vaccination is when they are day old. The World Organization for Animal Health recommends that chicks need to be vaccinated at day old with intermediate IBD vaccine and booster dose to be given when 10% of the chicks become susceptible (OIE, 2016). After two weeks of age low levels of antibodies were present in the blood of the chicks but the proportion of chicks with protective antibody level fell sharply to zero on day 14. That means when the booster vaccination was given 85% of the chicks were susceptible. Thus, in group of chickens vaccinated on day 7 and 14 (Group A), the antibody fell initially and two weeks after booster vaccination was given it jumped sharply concurrent with rise in the proportion of chicks with protective antibody level to 100%. This implies that administration of GumboL, which is intermediate type of IBD vaccine on day 7, followed by vaccination with IBDL (intermediate plus) on day 14 provided sufficient level of antibody but the schedule, has to be reconsidered. There has been contrasting results regarding the time of vaccination in chicks that hatch from unvaccinated parent flocks. One study showed that an intermediate vaccine given at 7 days of age did not provide protection to vaccinated chicks in the face of virulent challenge with field strain. However, when given at 14 days of age, the same vaccine fully protected the chicken (Hair-Bejo et al., 2004). In contrast vaccination of chicks at 7 days of with intermediate plus vaccine elicited protective antibody level against virulent challenge in Ghana (Otsyina et al., 2009). Although we did not carry out artificial challenge with virulent infectious bursal disease virus, absence of clinical cases throughout the study period in endemic areas where several outbreaks caused significant loss in chickens suggests that the vaccination schedule works well.

Data from this study revealed that the change in antibody to IBDV is variable. This may be explained by the influence on the half-life of MDA of the vaccine type and its time of application (Alam et al., 2002. When offspring of different parent flocks are raised together, this may result in different levels of MDA and compartmentalisation of the herd into individuals with low or high susceptibility to virulent IBDV (Tsukamoto et al., 1995). Early vaccine failed to stimulate the immune system in the chicks because maternal antibody reacts with live vaccine virus and becomes neutralised or interferes with MDA (Zhuo et al., 1998). Several studies under laboratory conditions have indicated that high MDA at the time of IBDV vaccination might interfere with the vaccine response, neutralises the vaccine virus and delays or even prevents the induction of humoral immunity (Hair-Bejo et al., 2004; Jung 2006; Morães et al., 2005). This means that the vaccination in the first days may not offer the chick any protection against disease. Nevertheless, an increase in titre was observed when vaccination was performed at 14 days (Knezevic et al., 1999), as also observed by Kumar, Singh and Prasad (2000) using a quantitative agar gel precipitation test. As shown in Tables 2 and 3 the MDA to IBDV was high before vaccination (day 7) but decreased below the protective level after first vaccination. This shows that the MDA neutralized the vaccine and when booster vaccine was given the antibody level remained low. Three weeks after the first vaccination (two weeks after booster vaccination) the antibody level rose to above protective level in 100% of the chicks. That is, booster vaccination is must since the first vaccine is neutralized to elicit protective antibody level.

In the group of chickens vaccinated at 14 and 21 days of age using NVI LC75 (intermediate plus) type of vaccine (Group B) the antibody level was nearly nil during the first vaccination. It remained remarkably low during the booster vaccination (on day 21). It is not clear why the antibody remain low since the vaccine was given when the MDA has wane. Similar to the chicks in group A chicks in group B mounted antibody two weeks after the first vaccination but still 45 % of the chicks were susceptible. After two weeks of the booster vaccination the antibody level reached protective level in 100% of the chicks. Previously Mahasan and Rahman (2004) recommended vaccinating chickens at an age of 2 weeks with intermediate strains of IBD and boosting them with the 'hot' vaccine at an age of 3 weeks. Suzuki et al. (2009) reported estimated optimal vaccination timings against IBDV of each flock at the three sampling time points between 16 and 24 days of age. In this study we intermediate plus at both ages and this may be the reason for the failure lower protective antibody titre observed but this need to be elucidated in the future. The appropriate time to implement active vaccination without risk of vaccine failure or incidence of infection seems to vary according the vaccine used.).

In this study, the MDA of control group was decreased from the 1^{st} to the 28^{th} day by half every week. The antibody titer of group B was decreased in a way similar to that of the control group, but a significant ($p<0.05$) difference was present between them at 14^{th} day and thereafter, whereas in group A it was decreased from the 1^{st}-14^{th} day and then increased at 21^{st} 28^{th} of age. Although single dose at the 7^{th} day old vaccination could induce slight increase of IBD antibody in comparison to that of the control, vaccination at the 14^{th} day of age induced high and protective level of IBD antibody titer after one week. These may be due to the ability of vaccine at each time of vaccination to neutralize different levels of MDA. single dose vaccination at the 7^{th} day in chickens with high MDA was ineffective and could not used in chicks so that booster dose should be given at 14^{th} day. It was concluded that in chickens with low MDA, the 1st vaccination should be given between 7^{th} and 14^{th} days and repeat after one week. Early single vaccine failed to stimulate the immune system in the chicks because maternal antibody reacts with live vaccine virus and becomes neutralised or interferes with MDA (Zhuo *et al.* 1998). Several studies under laboratory conditions have indicated that high MDA at the time of IBDV vaccination might interfere with the vaccine response, neutralises the vaccine virus and delays or even prevents the induction of humoral immunity (Hair-Bejo *et al.* 2004; Jung 2006; Morães *et al.* 2005). This means that the vaccination in the first days failed to offer the chick any protection against disease. Nevertheless, an increase in titre was observed when booster vaccination was performed at 14 days (Knezevic *et al.* 1999), as also observed by Kumar, Singh and Prasad (2000) using a quantitative agar gel precipitation test. Under field conditions, however, the decay pattern of IBDV-specific MDA proved to be more complex, as it depends largely on initial antibody levels, which may vary between batches and also within a batch, making it difficult to predict the optimal time for vaccination (De Wit 1998).

In the presentt study the half-life of MDA to IBDV is between 5 and 7 days. Similarly, other studies reported that the rate of decline was by about half every 5 days (Alam *et al.* 2002; Shrestha *et al.* 2003) and between 4 and 5 days (Sheku 2013). Others have reported that the half-life MDA to IBD in chicks was 3.46 days (Saijo & Higashihara 1998) and decreased every 4 days (Gardin 1994). Others In newly hatched layer-type chicks, MDA exhibits a linear or curvilinear decline with a mean half-life of 5 to 6 days (Müller *et al.* 2012). Fahey, Crooks and Fraser (1987) reported a half-life of 6.7 days for IBDV-specific MDA. It is generally thought that the half-life of MDA in broiler lines is much shorter, approximately 3 days (Block *et al.* 2007). Data from this study revealed that the decrease of MDA to IBDV is variable during the growing period. This divergence may be explained by the influence on the half-life of MDA of the vaccine type, its time of application in hens (Alam *et al.* 2002) and probably the immune status of the hen (Kouwenhoven & Van den Bos 1992).

Despite the time of vaccination, the present study agree with Sahar, Ali Mahasan and Rahman (2004) who recommended vaccinating chickens at an age of 2 weeks with intermediate strains of IBD and boosting them with the ‘hot’ vaccine at an age of 3 weeks in a closed system. Suzuki *et al.* (2009) reported estimated optimal vaccination timings against

IBDV of each flock at the three sampling time points between 16 and 24 days of age. Similarly, Block *et al.* (2007) indicated that the optimal vaccination time was between 17 and 23 days post-hatch based on the Deventer formula, whilst Lone *et al.* (2012) suggested that broiler chicks vaccinated at days 8, 15 and 23 with live attenuated vaccine or live attenuated vaccine followed by inactivated vaccine at days 8 and 21 could be adequately protected against the virulent form of IBDV. Furthermore, Al-Mufarrej (2013) observed that chickens vaccinated at days 10 or 18 showed better immune response to IBDV vaccination. In practice however, if a (sub-clinical) infection has occurred during the life of a hen, the antibody titre of the hen will rise, particularly in hens that have not been re-vaccinated with a killed vaccine. The progeny of these hens will need to be vaccinated later than normally expected. On the other hand, one doesn't want to wait too long before vaccinating as this will leave the flock unprotected against early challenge.

The present study clearly shows that a high level of maternally derived antibody at day 1 in the chicks hatched from unvaccinated parent flock. This may be due to subclinical infection in the parent flock. The MDA in the chicks interferes with the vaccine, resulting in no immune response after the 1st vaccination in both groups. However, re-vaccination induces an immune response, particularly when carried out at days 14 and 21. Therefore, two vaccinations should be recommended to achieve good protection against infection by bursal disease virus in a flock.

6. CONCLUSION AND RECOMMENDATIONS

Infectious Bursal Disease (IBD) is an acute and highly contagious disease affecting young chickens from 3-6 weeks of age. The disease causes immunosuppression in chickens and rendering them vulnerable to a variety of other infections. Early subclinical infections are the most important form of the disease because of economic losses. They cause severe, long-lasting immunosuppression due to destruction of immature lymphocytes in the bursa of Fabricius, thymus, and spleen. The more frequent reason for outbreaks in vaccinated flocks is incorrect application of the vaccine. The present study clearly shows that infectious bursal disease is causing problem in poultry farms. Results of gross and molecular diagnosis of BF and evaluation of Ab titers demonstrated that IBD was indeed a subclinical disease problem on the poultry farms. It is revealed that the vaccination schedules used in central Ethiopia need to be revised as the maternally derived antibody interferes with the vaccines. It seems that two vaccinations starting at 7^{th} day old are necessary for chicks hatched from unvaccinated parent flock. Clear time of vaccination could not be determined in this study. Therefore, the following recommendations are forwarded:

- Further evaluation of optimal time of vaccine delivery in vaccinated parent flock is needed
- Large scale evaluation of different commercially available vaccine need to be carried out
- Appropriately staffed and equipped diagnostic laboratories should be established for timely diagnosis of poultry diseases and accurate titration of level of maternal antibodies.
- Efforts should be initiated to bring the prevalence of IBD as low as possible by adoption of authentic vaccination schedules in many intensive poultry farms.
- Better hygienic and strict bio-security measures should be applied for successfully overcoming the predisposing factors which act as conducive media for the emergence of outbreaks of IBD.
- Molecular epidemiology of IBDV virus should be studied with a planned interval to assess the antigenic diversity of the IBDV virus.

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8. ANNEXS

Annex. I: Formulation of growth and maintenance Cell Culture Medium

Base cell culture medium preparation

Ingredients	Amounts
GMEM (MEM)(powdered)	12.5 g
$NaHCO_3$	2.75 g
H2O (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 +4oC

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

Diluted completely and Sterilized by autoclave 121 oc for 15 minutes

Annex. 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
$NaHCO_3$	0.35 g
Purified H_2O	1 liter

Adjust pH to 7.4 with $NaHCO_3$ 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 (37oC) trypsin solution (0.25%) and put on stir plate at slow speed into 37 oC incubator for 10-15 minutes.

 Pour off supernatant into centrifuge tube with calf serum.

 Add 50 ml Trypsin solution and stir slowly in 37 oC incubator for 8 minutes. This may be

repeated 1 more time for a total of 2 trypsinization.

 Centrifuge 10 min. at 1500 rpm. Note the amount of Pelleted cells obtained.

 Pour off Trypsin solution and resuspend cells complete cell culture medium (10% calf serum and tryptose phosphate broth contained GMEM or MEM. The cells may be counted or diluted 1:200and dispensed with in tissue culture flask and incubate at Co2

connected 37 oC 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 cm2)or 2 ml for the larger size

 Rock each plate gently to distribute inoculums evenly over the cell monolayer.

 Incubate inoculated cultures in 37°c incubator for 45 minutes to 1 hour to allow virus to adsorb.

 Rock tray once or twice during incubation if possible.

 Add 20 ml maintenance medium to each small T C flask (25 cm2).

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 -80o 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 +4oc until successful results are confirmed. Cover the plates with an adhesive cover and incubate at +37oc 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 +37oc 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 +37oc 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: 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 elude the RNA from the membrane in to the recovery tube

k). Label (the virus name and day of extraction) and preserved at -20oC 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 56oc 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

Annex. IX : Master mix preparation

Ser. no	Type of reagent	For one reaction	Total reaction	Remark
1	RNase free water	3µ		
2	Primer-IBD3-Fow-5pm/µl- 5'TGTAAAACGACGGCCAGTGCATGCGGTATG TGACGCTTGGTCAC-3'	2 µ		
3	PrimerIBD3-REV-5pm/ µl 5'CAGGAAACAGCTATGACCGAATTCGATCCT GTTGCCACTCTTTC-3'	2 µ		
4	IQ super mix	10 µ		
5	Add Template (cDNA)	3 µ		
	Total volume	20 µ		

Annex. X. Agarose Gel Electrophoresis

 Prepare 60 ml of 1.5% agarose in 1X TBE buffer

 Either heat in microwave for approximately 2min or boiling water until melt it

 Allow to cool to about 45oc and add 2.5µl /red gel

 Pour gel and insert well former (comb). Allow o set on a flat surface for about 15 min.

 Pour puffer 1X TBE in to tank and remove the comb from gel

 Prepare samples in to tubes, multichannel pipette or on paraffin

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 H2O

 Load samples in to the wells formed in the gel. It is often use full to load the molecular weight markers in both the first and last lanes

 Electrophoresis at 120 volt for 50 minutes

 View the photograph the gel on an UV-trans illuminator. Use UV safety spectacle.

Annex. XI: Run Touchdown PCR

	Temperature	Time	Cycle	Remark
Initial Denaturation	95^{o}c	5 mints	1-Cycle	
1^{st} Denaturation	95^{o}c	30sec	15 cycles	
Annealing	60^{o}c	30sec		
Elongation	72^{o}c	30sec		
2^{nd}Denaturation	95^{o}c	30 Sec	20 Cycles	
Annealing	56^{o}c	30 Sec		
Elongation	72^{o}c	30Sec		
Final Elongation	72^{o}c	7mints	1-Cycle	
Put at	4^{o}c	Until machine off		