VERO CELL ADAPTATION, IMMUNOGENICITY AND EFFICACY TEST OF INFECTIOUS BURSAL DISEASE VIRUS (LC-75) VACCINE STRAIN FOR VERO CELL BASED VACCINE DEVELOPMENT IN ETHIOPIA

MSc. Thesis

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JUNE 2018

BISHOFTU, ETHIOPIA

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

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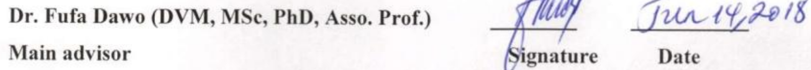

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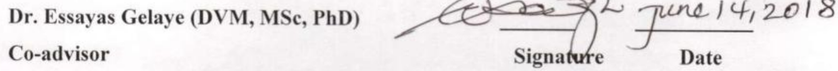

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First, I declare that this thesis is my effortful work that all sources of materials used for this have been duly acknowledged. This thesis has been submitted in partial fulfilment of the requirement for an advanced (MSc) degree at Addis Ababa University, college of veterinary medicine and agriculture and it can then be deposited at the university, college of Veterinary Medicine and agriculture library for borrowing according to the rule of the library. On the other hand, I solely declare that this thesis is not submitted to any other body anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AC-ELISA	Antigen captured enzyme linked immunosorbent assay
AGID	Agar gel immune diffusion
AGP	Agaros gel precipitation
AGPT	Agaros gel precipitation test
AIDS	Acquired immune deficiency Syndrome
ANOVA	Analysis of variances
BF	Bursa of Fabricious
cDNA	Complementary deoxyribonucleic acid
CEB	Chicken embyo bursa
CFC	Chicken fibro blast cell
CPE	Cytopathic effect
CSA	Central statistical agency
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethyl diamide triacetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
HVT	Herpes Virus Turkey
IBD	Infectious bursal disease
IBDICX	Infectious bursal disease immune complex
IBDV	Infectious bursal disease virus
ICX	Immune complex
IPNV	Infectious pancreatic necrosis virus
MAB	Maternal antibody
MDA	Maternal derived antibody
MDV	Marek's disease virus
MEM	Minimum essential media
NAHDIC	National animal health diagnostic and investigation center
NAM	National metrology agency
NVI	National veterinary institute
OD	Optical density

OIE	Office international des epizootics
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription Polymerase chain reaction
SP	Sample positive ratio
SPF	Specific pathogen free
TCID	Tissue culture infective dose
TPB	Tryptose phosphate broth
USA	United State of America
VN	Virus neutralization
VNT	Virus neutralization test
vv	Very virulent
vvIBDV	Very virulent infectious bursal disease

ABSTRACT

Infectious bursal disease (IBD) is an acute and contagious viral disease of young chicks caused by Infectious bursal disease virus (IBDV). The disease is seriously affecting the poultry production. Vaccination is the major controlling method of the disease. Vaccine production using chicken fibroblast primary cell is time consuming, laborious, and uneconomical. The present study was carried out to adapt the LC-75 designated vaccine strain of infectious bursal disease virus using Vero cell culture, to evaluate immunogenicity and efficacy of the adapted virus to develop Vero cell based vaccine. Accordingly, Vero-cells at passage 20 with confluent monolayer was infected with 0.5 ml of LC-75 vaccine strain virus by adsorption method and recorded as passage 1. This process has been done up to 10 successive passages by following the same methods of virus infection onto Vero cells. The virus showed good cytopathic effects starting from passage 3. The infectivity titre of adapted virus was conducted and the result showed a linear increment of the titre along the passage number. The positive results of reverse transcriptase polymerase chain reaction targeting the VP2 gene revealed amplification of the 645 base pair. The VP2 gene nucleotide sequence analysis derived from Vero cell adapted virus and chicken fibroblast cell based prepared vaccine showed the absence of any nucleotide variation. A 0.03ml of Vero cell adapted virus from passage 5 and passage 10 was inoculate into 14 day chicks through eye drop to evaluate the level of antibody produced. Detection of the antibody level was conducted by indirect enzyme linked immune sorbent assay technique. The statistical result revealed that the absence of any significant antibody titre difference between the three groups of experimental chicks. Efficacy test was conducted by challenging non-vaccinated chicks (control group), one group vaccinated with passage 5 and third group vaccinated with passage 10 using Vero cell adapted very virulent infectious bursal disease virus circulating in the country. The Vero cell adapted virus at passage 5 and passage 10 protected the chicken 100%; whereas 60% morbidity and 25% mortality was observed in the control group. Therefore, IBDV LC-75 vaccine strain is well adapted on Vero cell, and immunogenic and efficacious to protect against the circulating virus isolates. Thus, IBD vaccine production using Vero cells is recommended.

Key words: *IBD vaccine virus, chicken, ELISA, immunogenic, vaccine, Vero cell adaptation*

1. INTRODUCTION

Infectious Bursal Disease (IBD) is a highly contagious acute viral infection, affecting three to six weeks chickens (Eterradossi and Saif, 2008). IBD is caused by infectious bursal disease Virus (IBDV) that belongs to serotype-1. It is an important immunosuppressive virus of chickens and may exacerbate previous infections with other infectious agents, and may reduce the capacity of the bird to respond to vaccination, as the virus damages the humoral and cellular immune responses of chickens (Sharma *et al.,* 2000). Rautenschlein *et al.* (2003) reported that IBDV strains with different virulence may differ in their ability to replicate *in vivo*, to induce humoral immunity, and to cause immune-suppression (Rautenschlein *et al.,* 2003). However, Ingrao *et al.* (2013) reported that the relative effectiveness of these strains to stimulate cell-mediated responses, and the exact causes of clinical disease and death are still poorly understood (Ingrao *et al.*, 2013).

IBD is characterized mainly by the swollen and hyperemic bursa of fabricious (BF) during acute stage (3 to 4 days post infection) and then severe atrophy of the organ (Teshager, 2015). The virus has predilection for lymphoid tissue special target organ the bursa and also can be isolated from the thymus, spleen and bone marrow. Besides the loss due to mortality and morbidity, immune-suppression is a very important problem associated with IBD infection (Jones, 2008). The IBDV destroys lymphocytes and macrophages as a result cripples the immune system with marked immunosuppressive effect leading to vaccination failures and concurrent infections (Saif *et al*., 2008).

Since the first discovery of classical IBDV strains in the United States of America (USA) over 50 years ago (Hex *et al*., 2012), the virus has spread throughout the world and complex evolution of the virus has taken place (Rai *et al*., 2017). Very virulent IBDV is endemic in parts of southern Asia, Indonesia, South America, Middle East and Africa and spread to all over the world. All major poultry producing regions report the coexistence of two or more strains of variable pathogenicity of IBDV (Pitesky *et al.,* 2013; OIE, 2016).

Chicken production under back yard system has been practiced in Ethiopia (Tsegaye and Mersha, 2014) that can be used as the source of egg and meat consumption and for generating income. According to central statistics agency (CSA) 2015/16 report, there are

about 60,505,327 poultry in Ethiopia (CSA, 2015/16). Local chicken constitute about 99% of total poultry population in the small scale rural farm, however losses due to chicken mortality that occurs in different age group is very high (61%) (Zeleke *et al.,* 2005). Among the cause of poultry death in Ethiopia, IBD is one of the diseases which are challenging the production rate and the product quality of poultry. IBD first reported in Ethiopia in 2002 at privately owned commercial poultry farm in which 45-50% mortality rate (Zeleke *et al*., 2002) reported. Subsequently, IBD has become a priority problem in commercial and backyard poultry production system despite regular vaccination practices (in some cases) using attenuated IBDV D78 vaccine and improved biosecurity measures (Zeleke *et al*., 2005). On top of this, most control strategies designed in the country do not take into consideration the local chickens, and this may lead to failure of most strategies (Tadelle and Ogle, 2001). Starting from 2004, highly pathogenic (hotter) strains are emerging via mutation and genetic re-assortment (Mardassi *et al.,* 2004). Therefore, vaccination is considered as an important means of protecting birds during their first weeks of life (Eterradossi and Saif, 2008).

The strategy to control IBD in chicks is to hyper-immunize breeders with inactivated vaccines (Camilotti *et al.,* 2016). Although passive immunity promotes good protection of chickens during the first weeks of life, permanent protection against IBD requires the administration of live vaccines (OIE, 2016). It is important to highlight that live vaccines have been developed and are categorized as mild, intermediate and hot according to their degree of virulence. Mild vaccines are safe for specific pathogen free (SPF) chickens, but are not very effective in the presence of high levels of maternal antibodies or against very virulent strains of IBDV. Intermediate and hot vaccines are much more effective, but may induce moderate to severe lesions in the BF (Van Den Berg *et al*., 1991).

Ethiopia has been producing live IBDV vaccine strain of LC-75, by using chicken embryo fibro blast cell (CFC) (Mekuriaw *et al*., 2017) at National Veterinary Institute. Producing vaccine based on chicken embryonated fibro blast cell needs high labor power, time consuming and specific pathogen free eggs with a minimum production. But for the growth of virus including IBDV, different cell lines such as vero-cells are suitable, easily manageable and reproducible with a minimum cost. The present study was aimed to answer, how to produce IBDV vaccine in easy way regarding to time, man power and cost of material in the production system. Therefore, the main objective of the study was

- To develop Vero cell line based IBDV vaccine from vaccine strain (LC-75).

The specific objectives are:

- To adapt IBDV in Vero cell line for vaccine development
- To evaluate immunogenicity and efficacy test of Vero cell adapted IBDV (LC-75) vaccine strain for Vero cell based vaccine production.

2. LITRATURE REVIEW

2.1. History of infectious bursal disease

IBD is an acute and highly contagious disease of young chicken which is caused by IBDV. The causal agent was first isolated in Gumboro, Delawer in (USA), and the disease was originally known as Gumboro disease (Hiram and Vergil, 1999).

In Ethiopia the disease was first reported in a farm at Bishoftu in 2002 with high mortality of 49.89% in the affected 20-25 day-old broiler and layer chicken (Zeleke *et al*., 2002). The disease has since observed in different locations in Ethiopia including smallholders and all investigated commercial farms and multiplication centers occurring at an average outbreak rate of 3-4 farms per year. The disease was encountered commonly in backyard poultry production systems as well (Chanie *et al*., 2009). Seven years ago, there was Gumboro disease surveillance/investigation conducted by the National Animal Health diagnostic and investigation center (NAHDIC) in different Regions and the overall prevalence rates at the time of this NAHDIC surveillance was found to be about 77.48 % from the 706 samples collected and analyzed. Recently, the samples were collected from different regions of the country and serological test result has displayed as about 80% positive (Mekuriaw *et al.,* 2017).

The IBDV replicates in immature B-lymphocyte in BF leading to reduced immunologic responsive of chicken (Dwight *et al*., 2004). The disease is characterized by sudden onset short course and extensive destruction of lymphocyte particularly in BF and other lymphoid organs. It considered as acquired immune deficiency syndrome (AIDS) of chicken since it severely affected chicken immune system (Kaufer, and Weissi, 2005).

2.2. Description of the disease

The etiological agent of the disease is IBDV that belongs to the family *Birnaviridae* of the genus *Avibirnavirus*. The family name *Birnavirus* was proposed to describe viruses with 2 segments of double stranded RNA (Shamaila, 2005; OIE, 2008; Delmas, 2011). This family includes six divergent genetic clusters, four of which are recognized as the genera: *Aqua birnavirus whose* type species is infectious pancreatic necrosis virus (IPNV), which infects fish, mollusks and crustaceans; *Avibirna virus* whose type species is

IBDV, which infects birds; *Blosnavirus* (BSNV) Blotched Snakehead and *Entomo-birna virus*. The virus is very hardy, can survive in a wide variety of environmental conditions, and is difficult to effectively decontaminate (WSU, 2014).

IBD virus strains are classified as serotype 1 and serotype 2 differentiated by a virus neutralization test (Muller *et al*., 2003). There is no cross protection between these serotypes. Serotype 1 - pathogenic IBDV affecting poultry (classic, hyper or very virulent, variant IBDV strains and all IBDV vaccine strains) and serotype 2 – is apathogenic IBDV (Van den berg *et al*., 2004).

2.2.1. Morphology of infectious bursal disease

Infectious bursal disease Viruses of the family *Birnaviridae* is non-enveloped, single-shelled particles with a diameter of about 65 nm, and they have a dsRNA genome that is made of two linear segments. Genomes are linear and segmented, around 2.8-3.6kb in length (virus taxonomy, 2012). The virus comprises two segments: A and B. Segment A is poly-cistronic and encodes two major structural proteins (VP2 and VP3); one protease (VP4) responsible for cleavage of the viral poly-protein; and one nonstructural protein (VP5), which is expressed transiently at the end of the virus life cycle and is believed to be responsible for breaking down the membrane of the infected cell (Lombardo *et al.,* 2000). Segment B is mono-cistronic and encodes protein VP1, which is none other than the viral RNA polymerase releasing the viral particles. Protein VP2 contributes to the antigenicity (Letzel *et al*., 2007), tropism and pathogenicity of the virus (Van Loon *et al.,* 2002). The epitopes responsible for the induction of neutralizing and protective antibodies are located on the VP2 protein (Abdel *et al*., 2001).

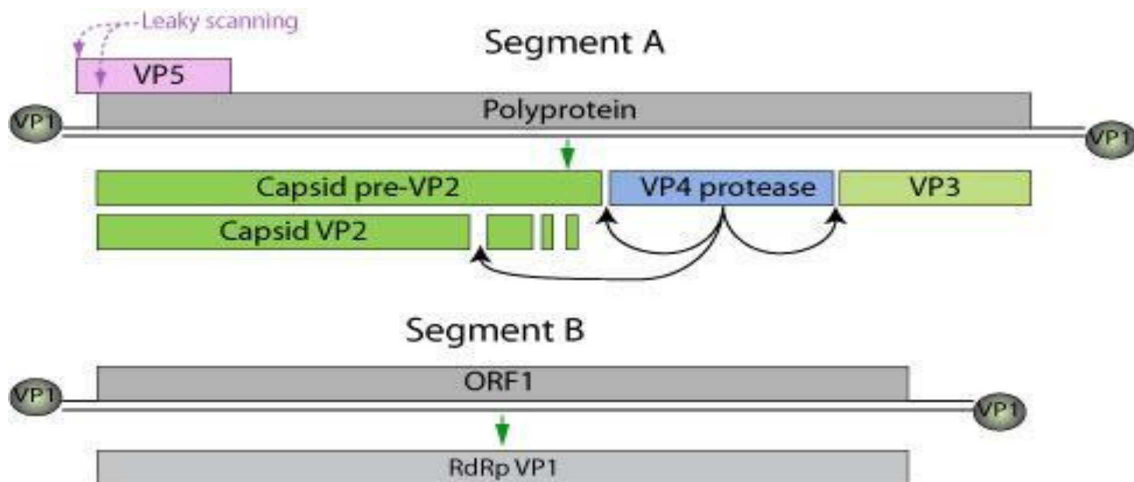


Figure 1 Structure of the segmented linear dsRNA genome of IBD virus.

2 segments (A, B) encode for 5-6 proteins. VP1 is found in a free form and covalently attached at the 5' genomic RNA end (VPg). Segments size is about 2.8-3.6 kb. Genome total size is about 6kb. *Source:* Viral zone, 2009.

2.2.2. Physico-chemical properties of the virus

The virus is non-enveloped and quite resistant to physical and chemical agents. Due to the stability and hardiness of the virus, it persists in poultry premises even after thorough cleaning and disinfection (Shamaila, 2005). IBDV is non-infective above 42 °C and unstable above 72 °C. However, the change in pH does not significantly contribute to the IBDV stability (Rani and Kumar, 2015). The virus is inactivated at a pH of 12.0 but not at pH 2.0 (Lukert and Saif, 2003). The virus is unaffected by the exposure for 1 hour at 0.5% to 30% phenol and 0.125% trimersal. Its infectivity is markedly reduced when exposed to 0.5% formalin for 6 hours (Dwight *et al.,* 2004).

2.3. Epidemiology

Infectious bursal disease occurs worldwide in major poultry production area and 80% of member countries of OIE report the occurrence of acute clinical case or very virulent infectious bursal disease virus (vvIBDV) (Eterradossi *et al*., 2000). Since 1986, Europe has experienced the emergency of very virulent (vv) strain of IBDV, which can cause up to 70% flock mortality in laying pullet (Van Den Berge *et al*., 2004). However, vv IBDV can

establish infection in face of level of moderate vaccine that were previously protect against classical strain, meanwhile. Very virulent IBDV infections also have been observed in Africa, Asia and recently in South America (Enterradossi *et al.,* 1999). Australia, New Zealand, Canada and the USA were unaffected with vvIBDV (Proffitt *et al*., 1999). It is estimated that vvIBDVs are present in 95% of the Office International des Epizooties member countries (Van den Berge, 2000).

2.3.1. Host range

The natural hosts of IBDV are the domestic fowl including chickens and turkeys, but the most severe clinical signs and lesions and the highest mortality rate have been observed in white leghorns (Eterradossi and Saif, 2008). Other wild bird like healthy ducks, guinea fowl, quail and pheasants have been found to be naturally infected by serotype 1 IBDV (Campbell, 2001). There is no evidence that IBD virus can infect other animals, including humans (Sanchez *et al*., 2005).

2.3.2. Susceptibility

The age of maximum susceptibility is between three and six weeks, 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. Clinical cases may be observed up to the age of fifteen to twenty weeks (Lay *et al*., 1979). Light strains of laying stock are more susceptible to disease than the heavy broiler strains (Hassan and Saif, 1996).

2.3.3. Transmission of IBD

Only horizontal transmission has been described, with healthy subjects being infected by the oral or respiratory pathway. Infected chickens excrete the virus in feces as early as 48hr after infection, and may transmit the disease by contact over a sixteen-day period (OIE, 2012). Because of the resistant nature of the IBD virus, it is easily transmitted mechanically among the farms by people, equipment, vehicles and insects may also act as vectors (OIE, 2008).

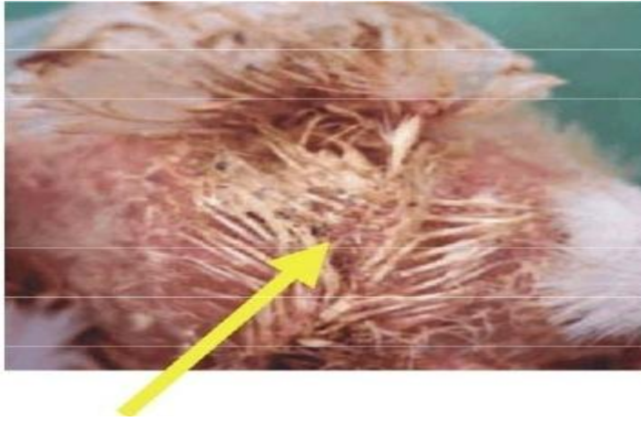


Figure 2 The feathers around the vent stained with faeces containing plenty of urates.

(Jordan *et al.,* 2002)

Subclinical IBD occurs when chickens are exposed to IBDV during the first two weeks post hatch and have sufficient maternal antibody at time of infection to prevent clinical disease but not viral replication in the bursa. Subclinical IBD characterized by bursal atrophy, immune-suppression and resultant increased susceptibility to secondary infections (such as *E. coli*).

2.3.4. Morbidity and mortality

Infectious bursal disease is extremely contagious. In infected flocks, morbidity is high, with up to 100% serological conversion, after infection, whilst mortality is variable (OIE, 2000). Until 1987, the field strains isolated was of low virulence and caused only 1% to 2% of specific mortality. However, since 1987 an increase in specific mortality has been described in different parts of the world. In the USA, new strains responsible for up to 5% of specific mortality were described (Rosenberger and Claud, 1986). At the same time, in Europe and subsequently in Japan, high mortality rates of 50% to 60% in laying hens and 25 % to 30% in broilers were observed. These hyper virulent field strains caused up to 100% mortality in specific-pathogen-free (SPF) chickens (Nunoya *et al.,* 1992).

2.4. Clinical sign

Infectious bursal disease virus has short incubation period of 3-4 days and the infection generally last 5-7 days. One of the earliest sign of IBDV infection is the tendency for bird to engage in vent picking (Ley *et al.,* 1983). Clinical sign are described as acute onset of depression, trembling, white and watery diarrhea, anorexia, prostration, ruffled feather, and

vent feather solids with urates. In severe cases, bird became dehydrated and in terminal stages subnormal temperature and death (Zelleke *et al.,* 2005). The clinical disease has a sudden onset and mortality rate in the flock increase rapidly (Saif and Barnes, 2003).

2.4.1. Gross pathological lesion of IBD

The tissue distribution and severity of lesions is dependent on the subtype and pathogenicity of the virus (Tsukamoto *et al.,* 1995).The lesions observed in bird that are common to IBDV infection include dehydration, hemorrhage in breast and leg musculature, darkened discoloration of pectoral muscles, occasional hemorrhage in thigh muscle and pectoral muscle, increasing mucus in the intestine and renal changes (Teshager, 2015). As the bursa is primary target organ of the virus, it is important to understand the sequence of change while examining bird at post-mortem. Among clinically active cases, 76.67% of BF was found as swollen, edematous and petechial hemorrhage in serosa and mucosal part (Teshager, 2015).

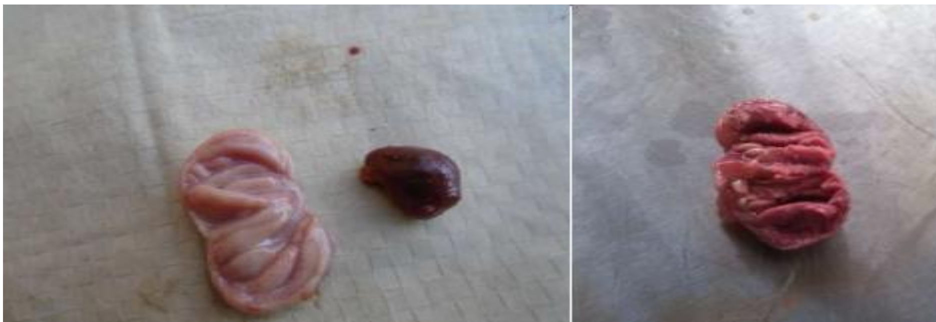


Figure 3 Gross pathological changes in infected bursa of fabricious.
(Teshager, 2015)

Teshager, 2015 had also described that petechial hemorrhage and swollen of thymus gland, spleen, caudal part of kidney and ureter was turgid.

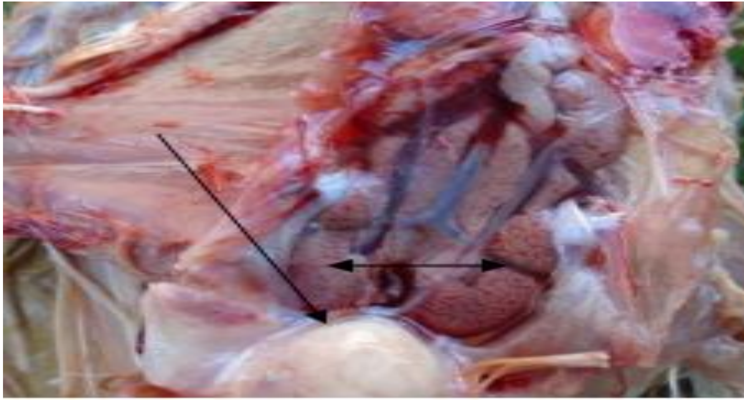


Figure 4 Swollen kidney and bursa of fabricious

(Teshager, 2015)

By day 2 or 3 post-infection, the bursa had a gelatinous yellowish transudate covering the serosa surface. Longitudinal striations became prominent and the color changed from white to creamy. The transudate disappeared as the bursa returned to its normal size and the organs turned gray during the period of atrophy (Lukert *et al.,* 2003). By the 4 day, bursa usually is double its normal weight and size and then after begins to decrease in size. From 8 day onward, it is about one-third its normal weights. The bursa usually show necrotic foci (area of dead tissue) and cheesy mass is found within its lumen from fallen cell of tissue. At time small large hemorrhage on its inner surface (mucosal surface) is also seen. Sometimes widespread hemorrhage throughout the entire bursa are present in such case, bird may pass blood in their drooping (Herdt *et al*., 2005; Teshager, 2015).

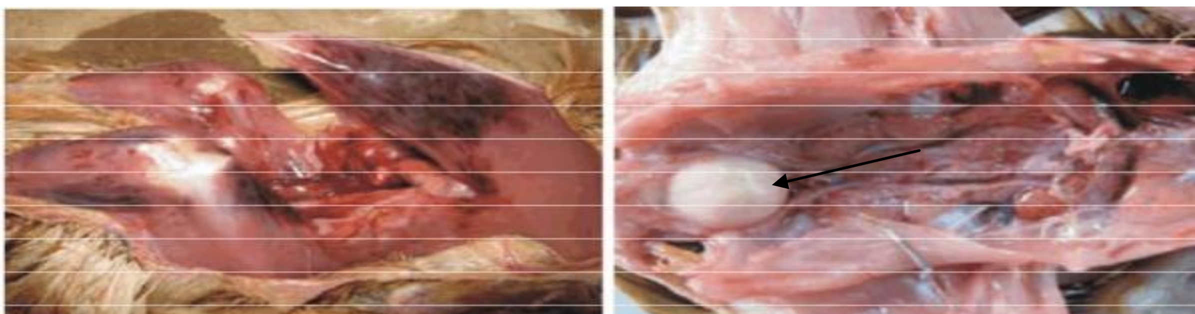


Figure 5 Hemorrhages in different organs and inflamed bursa

(Jordan *et al.,* 2002)

2.5. Pathogenesis of IBD

Pathogenesis is defined as the method used by the virus to cause injury to the host with mortality, disease or immuno-suppression as a consequence (Van den Berg *et al.,* 2000). IBDV usually infects young chickens between 3-6 weeks of age and causes a clinical disease, while sub-clinically infecting older birds. The outcome of IBDV infection is dependent on the strain and amount of the infecting virus, the age and breed of the birds, route of inoculation and presence or absence of neutralizing antibodies (Muller *et al.,* 2003). The virus affects lymphoid tissue causing destruction of B lymphocyte cell within the BF. The bursa is infected via the blood stream and by many cell in this organ contain antigen. A viremia follows when the virus infects other organ including spleen, the harderian gland and the thymus lymphocyte and their precursor appear to viral antigen can be found in the bursa up to 14 days post infection (Jordan *et al.,* 2002). In some birds the kidneys appear swollen and may contain urate deposit and cell debris which is probably result of blockage of ureters by severely swollen bursa. Bursa depletion as the result of virulent IBD virus infection in early life can result in impaired immune responses to antigen and the response to IBD virus itself. The consequence of immune-suppression is lowered resistance to disease and suboptimal response to vaccine given during this time (Sharma *et al.,* 2000).

2.6. Economic significance of infectious bursal disease

The economic loss due to IBD could be due to direct mortality of chicken during acute lethal course or by the immune suppression. In fully susceptible flock, the clinical signs appear suddenly with high morbidity rate usually approaching 100% follows the loss in body condition and loss of productivity (Paul *et al.,* 2004). Most the economic devastation associated with IBD is due to its immunosuppressive effect that leads to poor vaccination response, secondary bacterial, viral, protozoan infection and poor performance and poor economic return. It has high morbidity and mortality rate, which can lead to, loses of economy for the owner of the poultry farm or house hold individuals as well as for country (Zeleke *et al*., 2005).

2.7. Diagnosis of infectious bursal disease

Diagnosis involves consideration of flock history, clinical sign and post mortem lesion. Pathological change observed at the BF is characteristic and histophatological investigations combined with the demonstration of viral antigen by immune-histochemistry confirm an IBDV infection. Viral antigen can be demonstrated by agar gel precipitin assay (AGPA) or by antigen capture enzyme linked immune-sorbent assay (AC-ELISA) (Dwight *et al.,* 2004)

2.7.1. Isolation of infectious bursal disease virus using cell cultures

Classical strains of IBDV can be isolated in chicken embryo bursa (CEB) and chicken embryo kidney (CEK) (Ahasan *et al*., 2002). The virus however did not replicate in kidney cell until serial passage in chicken fibro blast cell (CFC) cells. After serial passage done on CFC, the virus produces a cytopathic effect (CPE) in kidney cells in about 3-5 days (Schat and Purchase, 1989). IBDV grows in CFC and produces CPE characterized with an appearance of round retractile cells in about 3-5 days (Sivanadan *et al.*, 1986).

2.7.2. Serological tests of infectious bursal disease virus

Serological tests generally used for the detection of IBDV are ELISA, virus neutralization (VN) and agar gel precipitation (AGP). The antigen capture enzyme linked immune sorbent assay (AC-ELISA) is the most commonly used test for the detection of antibodies to IBDV. It is economical, simple and quick and tests a large number of samples at the same time and is adaptive to automation to computer software (Lukert and Saif, 2003). The AC-ELISA allows the quantification of antibodies to IBDV and is therefore used for monitoring the immune status of the chicken flocks (Marquardt *et al.,* 1980) to check response to vaccination, natural field exposure and decay of maternal antibody titer (Lasher and Shane, 1994). However, ELISA cannot differentiate between the antibodies specific to the two serotypes (OIE, 2016). Therefore, while using ELISA for monitoring the chicken flocks for antibodies to IBDV, careful consideration should be given to the fact that the serotype 2 viruses are widespread in commercial chickens and could result in erroneous impression of antibody levels of the flock (Abdel-Alim and Saif, 2001).

Virus neutralization tests are carried out in cell culture. The test is more laborious and expensive than the agar gel immune diffusion (AGID) test, but is more sensitive for

detecting antibody. This sensitivity is not required for routine diagnostic purposes, but may be useful for evaluating vaccine responses or for differentiating between IBDV serotype 1 and serotype 2 (Ismail and Saif, 1996). Serum neutralization presents the disadvantages that specialized equipment and five days incubation is required. The technique is much more sensitive than AGID and correlates better with the level of protection of the subjects tested. Another serological method used to detect antibodies to IBDV is the AGP test. This test has been adapted to the quantitative format (Cullen and Wyeth, 1975). It is rapid but insensitive. It does not detect serotype differences and measures primarily group-specific soluble antigens (Lukert and Saif, 2003).

2.7.3. Identification by molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation. The most frequently used molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reaction (RT-PCR) (Wu *et al*., 1992; Lin *et al*., 1993). This method can detect the genome of viruses that do not replicate in cell culture, because it is not necessary to grow the virus before amplification. RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription (RT) of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotidic primers that are short sequences complementary to the virus-specific nucleotidic sequence (HeX *et al.,* 2012). Different areas of the genome will be amplified depending on the location from which the primers have been selected (Le Nouen *et al*., 2006).

2.7.4. Pathogenicity testing

Studies to compare the pathogenicity of IBDV strains must be carried out in secure bio-containment facilities to avoid the dissemination of the studied virus (OIE, 2016). SPF chickens must be used to avoid interference by contaminating agents. The main variables when comparing the results of pathogenicity trials are the breed, age and immune status of the challenged chickens, the dose and route of inoculation of the challenge virus, and the possible presence of contaminating agents in the inoculums. Light layer breeds have been reported to be more susceptible than heavy broilers (Van den Berg *et al.,* 1991). Differences in susceptibility may also occur between different SPF chicken lines. The highest susceptibility to acute IBD occurs in chickens between 3 and 6 weeks of age

(Lukert and Saif, 1997). High dose of challenge virus is necessary so that all inoculated chickens become infected at once without requiring bird-to-bird transmission of the inoculated virus. Finally, the presence in the inoculum of contaminating agents, such as adenovirus or chicken infectious anemia virus, may modify the severity of IBD and signs observed after challenge (Camilotti *et al.,* 2016). The terms variant, classical and very virulent have been used to describe IBDV strains that exhibit a different pathogenicity.

2.7.5. Antigenicity testing

Antigenic relatedness among IBDV strains may be assayed in cross VN tests, which correlate best with cross protection. Such tests have to be performed in SPF embryonated eggs when the studied viruses do not grow in CEF e.g. (vvIBDV). Differences in cross VN test results among serotype 1 IBDV strains have led to the definition of serotype 1 ‘subtypes’ (Jackwood and Saif, 1987).

Another approach to the study of genetic relatedness is the use of mouse MAbs that bind to IBDV neutralizing epitopes. Several panels of MAbs exist world-wide for use in AC-ELISA (Eterradossi *et al*., 1999; Snyder *et al*., 1992). Some of the MAbs have been included in commercially available kits, but no unified MAb panel as yet been proposed. All neutralizing epitopes of IBDV characterized to date have been mapped into a major immunogenic domain in the middle third (amino acid positions 200 to 340) of the VP2 capsid protein (Vakharia *et al*., 1994). This region is termed VP2 variable domain‟ because most amino acid changes observed among IBDV strains are clustered in it. Within VP2, four amino acid stretches are of critical importance to antigenicity and are referred to as VP2 hydrophilic peaks. These are amino acid positions 210 to 225 (major peak A), 249 to 252 (minor peak 1), 281 to 292 (minor peak 2) and 313 to 324 (major peak B) (Van den Berg *et al*., 1996)

2.8. Prevention and controlling mechanism of the disease

An effective IBD prevention and control program must involve an effective bio-security and effective vaccination program.

2.8.1. Using effective Bio-security

The prevention methods of IBDV includes several precautions such as practicing "all-in/all-out" farming methods; cleaning and disinfecting premises; and having a period of rest between depopulation and restocking (Lukert and Saif, 1997). Before cleaning, all insects and pests (for example rats and mice) need to be eliminated. After removing and decomposing the old bedding and dung, all farm equipment's are disassembled and relocated into a cleaning room outside the farm buildings. This is followed by washing with hot water (60^0C) and detergent at a pressure of 80 to 150 bars. Before introducing the new chicks, second disinfection of the full premises is warranted. The feed that remained from the previous flocks must never be reused (Van den Berg *et al.,* 2000).

2.8.2. Genetic Selection for Resistance

The susceptibility of the host to various poultry pathogens depends mainly on its genetic makeup (Bumstead *et al.,* 1993). Resistance to IBDV infection could be breed-dependent, and crosses between resistant and susceptible lines had indicated the resistance is a dominant hereditary phenotype (Van den Berg *et al*., 2000). Light breeds of chickens may have higher mortality rates than the heavier breeds, but inoculating IBDV in other avian species failed to cause the disease (McFerran, 1993). Unfortunately, the genes that confer the resistance against IBDV are yet identified and it is not a common practice to selectively breed the resistance lines (Bumstead *et al.,* 1993).

2.8.3. Vaccination of infectious bursal diseases

Infectious bursal disease virus is stable in nature and resistant to many physical and chemical disinfectants and then despite a properly cleaning and disinfection procedures, IBDV infections continue to occur and spread widely (Van den Berg, 2000). Vaccination of chicken is the principal method used for the control of IBD (Jyothsna, 2017).The success of vaccination depends on the choice of vaccine strain, vaccination schedule, and the strains of the field isolate. In the field, outbreaks of IBD have been controlled by vaccination practices (Fussell, 1998).Therefore vaccination is inevitable under high infection pressure and mandatory to protect chickens against infection during the first weeks of age (Eterradossi and Saif, 2008).

Four major types of vaccines are available for the control of IBD, these are: i) live attenuated vaccines; ii) immune-complex vaccines; iii) live recombinant vectored vaccines expressing IBDV antigens; and iv) inactivated oil-emulsion adjuvanted vaccines (OIE, 2016).

Live attenuated vaccines

Live vaccines are produced from classical and variant IBDV strains by passaging these viruses in tissue cultures or embryonated chicken eggs (Jackwood and Sommer-Wagner, 2011). Therefore live IBD vaccines are produced from fully or partially attenuated strains of virus, known as mild, intermediate, or intermediate plus (hot plus). Conventional live attenuated IBDV vaccines are suitable for mass vaccination and when applied in drinking water can induce robust immunity, usually at 3- 8 weeks of age that can induce strong humoral and cellular immunity (Alkie and Rautenschlein, 2016). Mild vaccines are safe for specific pathogen free (SPF) chickens, but are not very effective in the presence of high levels of maternal antibodies or against very virulent strains of IBDV (Camilotti *et al.,* 2016). Mild vaccine cannot cause immune-suppression as it not causes bursal damage, but gives weak antibody response (Shamaila, 2005).

The intermediate type IBD vaccines are most commonly used. These vaccines can stimulate the broiler to produce antibodies earlier than the mild-type vaccines, without significant damage to the BF as may occur with the virulent type vaccines. But it causes slightly immune-suppression (slight bursal damage), and gives good immune response (Van den Berg, 2000).

Intermediate plus gives high level of antibodies and advanced protection as compared with that of mild and intermediate vaccine.The use of hot vaccines, although providing protection, is not safe as they carry higher inherent risk of reversion to virulence and may result in immune-suppression in chickens. Therefore, in an effort to develop appropriate vaccination program for the farm, interference of MDA have become a critical factor in choosing the right vaccines (Van den Berg, 2000). The potential for reversion to virulence, residual immunosuppressive effects, as well as their role as genetic sources for the generation of re-assortment new viruses are major safety concerns of their extensive field applications (Rautenschlein *et al.,* 2005; Rautenschlein *et al.,* 2007).

Inactivated oil-emulsion (adjuvant vaccines)

Inactivated IBD vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural exposure to field virus during rearing (Müller *et al.*, 2012). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. For the vaccination to be effective, inactivated vaccines may be used in programmes combining inactivated and live vaccines, in young valuable birds with high MDA levels reared in areas with high risk of exposure to virulent IBDV (OIE, 2016). Passive immunity protects chicks against early immunosuppressive infections for one to three weeks and this protection might be extended to four or five weeks by boosting the immunity in breeders with oil adjuvant vaccines (Eterradossi and Saif, 2008).

Immune-complex vaccines

Infectious bursal disease 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. The viruses are released from the ICX when the levels of maternal antibody (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 (Ivan *et al.,* 2005).

Live recombinant vectored infectious bursal disease vaccines

Vector vaccines are genetically engineered vaccines in which a gene from one organism the donor is inserted into the genome of another organism the vector to elicit a protective immune response against both organisms. Among others, fowl poxvirus (Heine and Boyle, 1993), Newcastle disease virus (Huang *et al*., 2004), herpes virus of turkey (HVT) (Darteil *et al.,* 1995), Marek's disease virus (Tsukamoto *et al.,* 1999), avian adenovirus (Francois *et al.,* 2004) and T4 bacteriophage (Cao *et al.,* 2005) have been used as vector viruses for expressing VP2, the only antigen inducing protective immunity to IBDV. Meanwhile, several HVT plus IBDV-VP2 vector vaccines have been developed for application in ovo or by the subcutaneous route in 1-day-old chickens. Some have been licensed in various

countries, and data on field efficacy trials have been reported (Bublot *et al.,* 2007; Le Gros *et al.,* 2009).

2.9. Infectious bursal disease virus vaccine Failure

Failure of vaccination against infectious bursal disease is associated mainly with early vaccination in flocks of unknown immune status and with the evolution of viruses circulating in the field, leading to antigenic drift and a sharp rise in pathogenicity (Boudaoud *et al*., 2016), inappropriate storage of vaccine, inappropriate vaccination techniques, the use of expiry vaccine and using of un recommended dose (OIE, 2012)

2.10. The status of infectious bursal disease in Ethiopia

Infectious bursal disease is one of the newly emerging disease threats to chicken in different corners of Ethiopia as described by Zeleke *et al. (*2005) that the disease has been speculated to be introduced concurrent with the increased number of commercial state and private poultry farms flourishing in the country and causing reduction of both the number and productivity in the sector. The disease was first reported in a farm at Debre-Zeit in 2002 (Zeleke *et al*., 2005) with high mortality of 49.89% in the affected 20-25 day-old broiler and layer chicken.

The first study on the incidence of IBD in Ethiopian village poultry was in the Amhara region which had received "improved" chicks from a commercial farm (Mazengia *et al*., 2009), and it has been suggested that this was the cause of the introduction of the disease to village poultry. Serological studies have since demonstrated infection in indigenous birds in several areas of the country (Chaka *et al*., 2012), with the proportion of positive samples ranging from 75% to 96%. Gumboro disease surveillance/investigation was also conducted by the NAHDIC in different regions of the country and overall prevalence rates at the time of that NAHDIC surveillance was found to be about 77.48 % from the 706 samples collected and analyzed (Bewket *et al.*, 2012).

3. MATERIAL AND METHODS

3.1. Study location

The study was carried out in the National Veterinary Institute (NVI) at Bishoftu, Ethiopia from November 2017 to May 2018. Bishoftu is located 47km south east of Addis Ababa in Oromia regional state at 9°N and 40°E (Fig 6), at an altitude of 1850 meters above sea level in central high land of Ethiopia (National Metrology Agency, Addis Ababa, Ethiopia (NMA, 2010). NVI is a government organization established at 1964 under the ministry of agriculture to produce different veterinary vaccines. This company has different international accreditation certificates in vaccine production and diseases diagnosis. Those certificates include: ISO/QMS 9001: 2008 by an international accrediting company ALCUMUS/ISOQAR and it is dedicated for development, manufacture, sales and distribution of veterinary vaccines as its primary mandate and certified ISO/IEC 17025:2005 in research and development laboratory for serological tests (NVI public communication office).

Figure 6 Map of the study area

(Source: http://www.maplandia.com/ethiopia/oromiya/east-shewa/bishoftu/

3.2. Study materials

3.2.1. Media and solutions

The medias used for Vero cell replication was growth medium and maintenance medium which was prepared from Dulbecco's modified Eagle's medium (DMEM) with 10% and 2% sterile foetal calf serum (FCS), respectively. Other media and solutions such as Trypsin versine solution, Phosphate buffered saline (PBS) and tryptose phosphate broth (TPB) where used. The media was prepared according to the manufacturer's instruction. The history of the media is described on (Appendix 1).

3.2.2. Source of virus (LC-75), Vero cell line and challenge virus

Working seed of IBDV vaccine strain (LC-75) Lot number 5/17 with log $_{10}5.4$ titer/ml was obtained from NVI quality assurance department, vaccine seed preparation section. African green monkey kidney cells (Vero-cell line) passage 20 with confluent monolayer was also obtained from NVI vaccine quality assurance department of cell line managing unit. The challenge virus code MB/263/17 with titre $\log_{10}5.3$ $TCID_{50}$/ml was obtained from virology laboratory of NVI research and development department

3.2.3. Experimental chickens and management

Eighty, 1 day old chickens were purchased from Ethiopian Agriculture Research Institute center, Bishoftu, Ethiopia. These chicks were floor reared, fed on balanced commercial poultry ration, and kept under strict hygienic measures throughout the experiment, till they became 14 days of age. The chicks were maintained throughout the duration of the experiment in separate animal experiment isolation unit as described on OIE (2016).

3.3. Study design

Experimental study design was applied for Vero cell adaptation, immunogenicity and efficacy test of Vero cell adapted LC-75 vaccine strain of IBDV. Monolayer of Vero cells were used for adaptation of IBDV vaccine strain (LC-75). The infected cells were monitored for virus adaptation as manifested by CPE developed. The yield of the virus was determined by virus titration and subjected to identity test using reverse transcription polymerase chain reaction (RT-PCR) at the NVI molecular biology laboratory by targeting viral protein 2 (VP2) of the IBDV. The PCR positive product was sent to LGC Genomics,

Germany for sequencing. Immunogenicity and efficacy tests were conducted on IBD antibody free chicks obtained from the Ethiopian Institute of Agricultural Research, Bishoftu. The chicks were initially screened for presence of maternal antibody by the use of AC-ELISA (ID-Vet product) test before starting the experiment. All chicks with low maternal antibody (S/P ratio <0.3) grouped into four groups. Each group contains 20 chickens. Group 1 designed for inoculation of Vero cell adapted IBDV passage 5; Group 2 with passage 10; Group 3 inoculation of Gumboro vaccine produced based on chicken fibroblast cell (CFC) batch Gum 1/18 for comparison of immunogenicity test of Vero cell adapted IBDV and Group 4 was retained as non-vaccinated (negative) control. Serum samples were collected from each group at day 7, day 14, day 21, and day 28 post-inoculation and subjected to indirect ELISA for antibody (AB) detection and titration. All chicks inoculated and non-inoculated group were challenged with challenge virus. Challenged groups were kept for 14 days under strict follow up and all observed findings were recorded.

3.4. Study Methods

3.4.1. Sub culturing of adherent monolayers of Vero cells

The 75cm^2 cell culture flask containing confluent monolayer Vero cells were examined under inverted microscope (Olympus CK2, Japan) to observe the formation of complete monolayer. Confirmed confluent monolayer Vero cells were transferred to class-II safety cabinet (Lab care, England) and the present media was removed and washed three times by pre warmed PBS with pH 7.4 to remove dead cells. Confluent monolayer was brought into suspension using pre-warmed 0.25% trypsin/EDTA solution. The viability of cells was checked with trypan blue stain by using of cell counter machine. The viable cells were counted and reseeded into new flasks containing pre-warmed 10% DMEM at concentration of 3x10^{4}cells/cm^2. Based on the concentration of the cell obtained, 90ml DMEM with sterile 10% FCS was added and a cell suspended in the flask was splited into two into three 25cm^2 tissue culture flasks and incubated at 37°C in the presence of 5% of CO2 in CO2 incubator. The cells were examined twice daily under the inverted microscope for the formation of complete monolayer (Hussain and Rasool, 2005). In the same way, sub-culturing was performed when needed.

3.4.2. Inoculation of infectious bursal disease Virus into Vero cells

Healthy and confluent mono-layers of Vero cells at 36 hours after sub-culturing were used for virus infection by adsorption method. The working area under laminar air flow cabinet was disinfected with 70% ethanol alcohol. PBS and maintenance media were warmed at 37°C water bath. The growth medium in the flask of $25cm^2$ with confluent monolayer was removed and cell monolayer surface was washed three times with pre-warmed PBS. Then the Vero cells were infected by adding 0.5 ml of IBDV and spreading uniformly over the mono-layers' and incubated at 37°C for 1 hour with intermittent rotation to allow adsorption. After 1 hour incubation 10ml 2% DMEM was added into an infected flask and placed into 37°C in the presence of 5% of CO2 in CO2 incubator. One flask of fresh cells with confluent mono layers was kept as control under similar condition. The infected cells were observed twice a day under inverted microscope for CPE formation from inoculation date up to six days. At six days of post inoculation virus infected cells were harvested and labelled as passage 1(P1) and stored at -20°C for over night. The P1 virus was freeze thawed three times and inoculated again to fresh monolayer of Vero cells using the same technique and observed for CPEs twice a day up to six day post infections. At six days post infection the virus was also harvested and labeled as P2 and stored at -20°C for overnight. Similarly, P3 virus was obtained through third infection and CPEs were observed twice a day up to 6 days post-inoculation. This process was repeatedly done up to passage 10(P10). At each passage the harvested flasks were labeled and stored at -20°C for different tests until the experiment work had finished (Hussain and Rasool, 2005).

3.4.3. Titration of IBDV (LC-75) adapted on Vero cells

The viral suspension to be titrated was diluted in sterile tubes 10x from 10^{-1} to 10^{-10} (0.5ml viral suspension in 4.5ml of DMEM without serum) of passage 3, passage 5, passage 7, passage 9 and passage 10. The 100 µl viral dilutions were dispensed into 96 micro plate wells containing 100 µl Vero cells in a way to have ten replicates for each dilution in 100µl volumes of cells. Column 11 was left empty and column 12 was inoculated only cell for controls. Finally, the plate wells were sealed by micro plate sealer and incubated at 37°C in the presence of 5% CO_2 incubator for 8 days. The inoculated plates were observed under inverted microscope twice daily starting from inoculation date up to day eight. The titres for each virus passages were determined according to the following formula (Spearman, 1908).

$$Log10= ((xo - (d/2) + d (\sum ri/ni))$$

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

d=Log 10 of the dilution factor that is the difference between the log dilution intervals

ri =number of positive test monolayer's out of ni.

$\sum$ (ri/ni) = $\sum$ (P) sum proportion of the tests beginning at the lowest dilution showing 100% positive result. The summation was started at dilution Xo (Annex 2titration procedure).

3.4.4. Molecular identification of Vero cell adapted IBDV (LC-75)

To confirm that the Vero cell adapted virus was IBDV, the homogenates of infected Vero cells from passages 5, 6, 7, 8, 9, and the initial IBDV vaccine working seed separately and also Vero cell control collected by freeze thawing three times were subjected to molecular detection test. Therefore, viral RNA was extracted from mentioned sample suspension by the use of RNA extraction kit and RT-PCR was carried out for detection and amplification of adapted IBDV Vp2 gene. This includes RNA extraction, master mix preparation, PCR amplification, and RNA detection on agarose gel electrophoresis and sequencing techniques.

RNA extraction and cDNA synthesis

RNA of the IBDV was extracted from 350µl of harvested virus using Qiagen RNeasy® Mini Kit (Qiagen, Germany) following the procedure as described on manufacturer instruction. An extracted RNA was eluted by using RNase free water and used as template directly for RT-PCR. According to the Kit procedure, two steps RT-PCR cDNA synthesis was conducted. A 10 µl volume reaction mix was prepared first from 3 µl RNase free water, 1µl Oligo(dT), 1 µl 10 Mm dNTP mix and mix by vortex and then 5 µl template RNA was added and incubate at 65ºC for 5 minutes and placed 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 for 50 minutes. The reactions was terminated at 85ºC for 5 minutes and chill on ice and then collect by brief centrifugation and added 1

µl RNase to each tube and incubate for 20 minutes at 37ºC and finally the obtained cDNA was immediately used for PCR.

Master Mix preparation

A total of 20µl master mix was prepared by using 3µl of RNase free water, 2µl of forward primer, 2µl of reverse primer, 10µl of IQ super mix and 3µl of Template (cDNA). A set of primers used for the RT-PCR reaction were used for the sequencing PCR products by amplification of 645bp fragment IBDV on VP2 Gene. The following primers were used for RT-PCR reaction according to master mix preparation protocol and PCR reaction procedure. The sequences of the primers used for RT-PCR are:
Forward primer (5pm/µl) is
5'-TGTAAAACGACGGCCAGTGCATGCGGTATGTGACGCTTGGTCAC-3'and
Reverse primer (5pm/µl) is
5'-CAGGAAACAGCTATGACCGAATTCGATCCTGTTGCCACTCTTTC-3'

Polymerase chain reaction (touchdown PCR) for IBDV

The prepared master mix was loaded into touchdown PCR machine (applied Bio-system, code 2720 thermal cycler) for exponential amplification of premixed IBDV RNA. 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, elongation at 72ºC for 30sec and again followed by 20 cycles of 2nd denaturation at 95ºC for 30sec, annealing at 56 ºC for 30sec, extension at 72ºC for 30sec and final extension at 72ºC for 7 minutes for 1 cycle and hold 4ºC until machine off.

Agarose gel electrophoresis of PCR product

The PCR amplified products (amplicons) were analyzed by gel electrophoresis. A 10-µl sample of the PCR reaction mixture was electrophoresed and separated on a 1.5% agarose gel in TAE buffer. After staining with gel red (0.5µg/ml), the electrophoresis was run for 1:20 hour at 120V and PCR products were visualized by viewing the gel with an ultraviolet light and photographed (Visi-Doc-It system, UVP, UK) and compared with standard DNA size markers.

Sequencing and Sequence analysis

Amplicons of PCR positive were extracted using QIAGEN Gel extraction kit; with the concentration of extracted DNA determined by using spectrophotometer using micro volume a Thermo Scientific Spectrophotometer (Nano Drop 2000c, USA). The concentration of the quantified purified PCR product was adjusted following the requirements set by sequencing company. The quantified DNA, and also IBDV VP2 gene specific forward and reverse sequencing primers were added in to the separated labeled eppendorf tubes. The purified PCR products were mixed with the sequencing primers and submitted for sequencing to the commercially sequencing LGC Genomics (Berlin, Germany). The raw sequence data were edited and fragments were assembled using Vector NTI Advance™ 11.5 software (Invitrogen, Carlsbad, CA, USA). For each isolate, the fragments produced were sequenced with the forward and reverse primers were edited and assembled together and the clean gene sequence was extracted. Multiple sequence alignments were performed using the ClustalW algorithm implemented in BioEdit software package to compare the gene of the Vero cell adapted virus at different passages and the reference strain.

3.5. Immunogenicity test

Eighty chickens with minimum maternal derived antibody level (less than positive range) at 14 day old were grouped into four groups for immunogenicity test (Group 1, 2, 3 and 4) of 20 chickens each group according to OIE (2016). All groups except group 4 were infected with 0.03ml Vero cell adapted IBDV from passage 5, passage 10 and CFC based prepared live Gumboro vaccine batch (Gum 1/18), respectively and Group 4 were remain unvaccinated to serve as negative control. The three groups (1, 2 and 3) were infected via ocular route at 14 days old. All vaccinated groups were boosted by the same dose and the same site of inoculation from passage 5, passage 10 and Gum 1/18 on 14 day post inoculation. Blood samples for serum separation were collected from each chicken at days 7, 14, 21 and 28 post infections. The collected blood sample was left at room temperature in slant position for 10 hours and the serum was collected in 1.5ml cryo vial, labeled and stored at -20ºC until the tested or conducting further experiment.

3.5.1. Indirect ELISA

The indirect ELISA test was done by using 96 wells micro plate coated with purified IBDV antigen, positive control, negative control, concentrated conjugate(10x), dilution buffer 3, dilution buffer 14, wash concentrate (20x), substrate solution and stop solution (0.5M). The entire ELISA test was done at NVI research and development department serology laboratory as per manufacturer instruction (ID.VET innovative diagnostic, France). Finally the test result was read by using micro titer plate reader at 450nm absorbance having the first blank on air. The reader, connected to the computer loaded with Excel packages, was used to automate the reading of optical density (OD) value. The percentage positivity for test samples in relation to the negative and the positive controls was calculated as per the formula given by the kit manufacture.

$$\text{SP value} = \frac{\text{ODsample} - OD_{NC}}{OD_{PC} - OD_{NC}}$$

Where: SP samples to positive ratio

OD sample- optical density of the test sera,

OD NC- optical density of negative control,

ODPC -optical density of positive control

3.5.2. ELISA validity test

SP value > 0.3 and OD of negative control $\leq$ 0.25 is considered as valid. But, if S/P value is less than 0.3 and the mean OD values of the positive control is $\leq$ 0.250 the entire result of the kit is considered as invalid according to manufacturer instruction.

3.5.3. ELISA test result interpretation

S/P ratio $\leq$0.3 or antibody titer $\leq$ 875 is negative

S/P ratio > 0.3 or antibody titer > 875 is positive

3.6. Efficacy test

Efficacy test was done by inoculating challenge virus into chicken groups vaccinated before by Vero cell adapted LC-75 IBDV strain from (passage 5 and passage 10) and Gum

1/18 and non- vaccinated group. All challenged groups were followed for 10 days after inoculation of the challenge virus. Chicken groups vaccinated and unvaccinated or control group were challenged with the same dose of challenge virus at 21 days post vaccination by 0.2 ml/bird of vvIBDV containing titer of $10^{3.5}$ EID50/ml, via eye drop route as described by El-Bagoury *et al*. (2015). The challenged birds were observed 10 days for morbidity and mortality result observed and recorded during the challenge period (OIE, 2016).

$$\text{Protection \%} = \frac{\text{Number of survived birds}}{\text{Total number of challenged birds}} \times 100$$

3.7. Ethical consideration

Ethical clearance for this study was obtained from Addis Ababa University College of veterinary medicine and agriculture minutes of animal research ethics and review committee (Appendix 6).

3.8. Data management and statistical analysis

All the data that were collected was entered to Micro Soft (MS) Excel spread sheet program to create data base and it was analyzed by using STATA version 12 and subjected to a one-way analysis of variance (ANOVA). Statements of statistical significance were based on $p < 0.05$

4. RESULTS

4.1. Adaptation of IBDV LC-75 on Vero cells

At the first passage the infected cells were remained intact on the surface of tissue culture flask and did not show any CPEs formation. During second passage minor CPEs were observed starting from day four post-inoculation. But starting from passage three up to passage ten, good CPEs were happened at day three post infection by observed typical aggregation, rounding and clumping of large number of cells and detachment of cells with few cells floating in media under inverted microscope. Negative control monolayers did not show any changes throughout the observation period except lowering of pH of the medium as indicated by yellowish colour (Fig.6A, 6B, 6C and 6D).

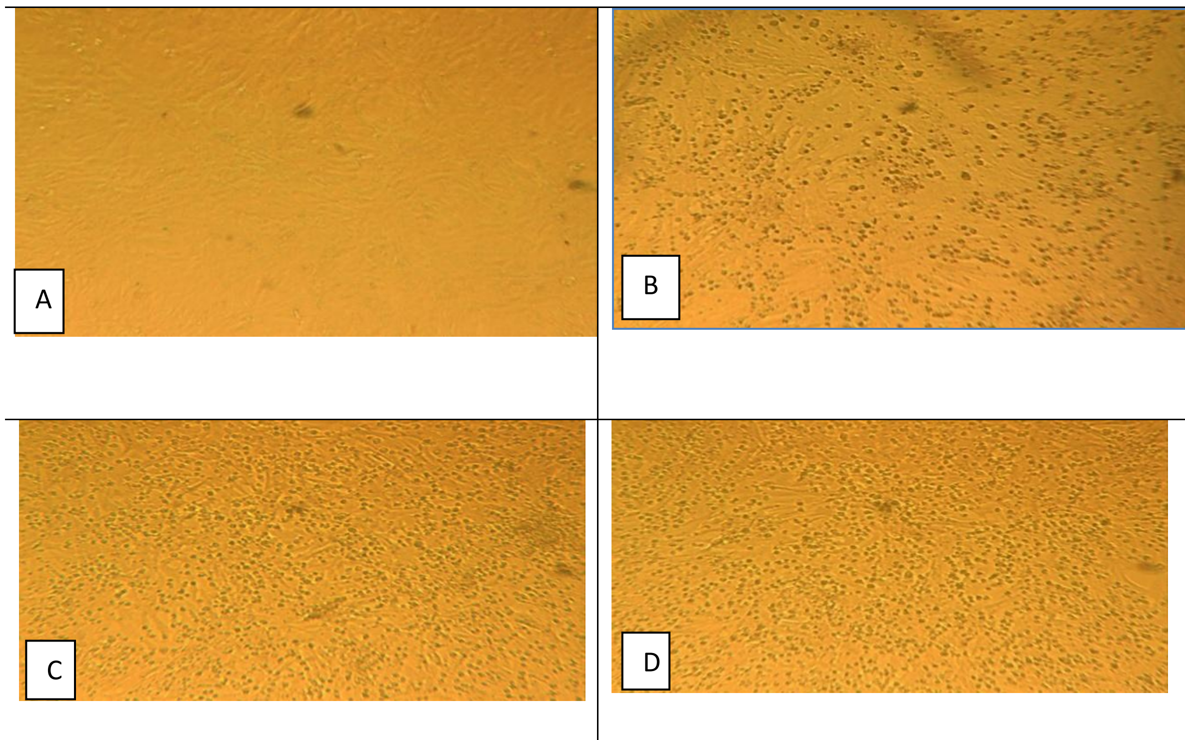


Figure 7 Cythopatic effect (CPE) observed on Vero cell at different passages.

A, Vero cell control; B, CPE, P-5; C, CPE, P-7; D, CPE, P-10

4.2. Titration of the IBDV

The infectivity of adapted IBDV to Vero cells was determined by calculating 50% end point, as described by Spearman (1908). The total infectious titers of Vero cell adapted at

passage 3, passage 5 passage 7 passage 9 and passage 10 were $log_{10}5.4$ $TCID_{50}/ml$, log_{10} 5.60 $TCID_{50}/ml$, log_{10} 5.9 $TCID_{50}/ml$, log_{10} 6 $TCID_{50}/ml$, and $log_{10}6.2$ $TCID_{50}/ml$, respectively (Figure 8).

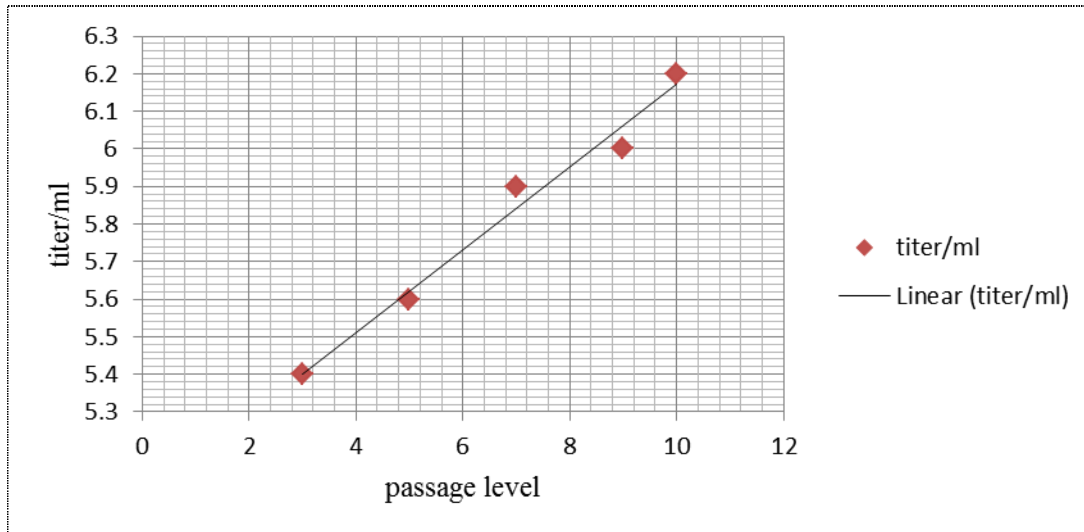


Figure 8 Vero cell adapted virus titration result on different passages.

4.3. Molecular identification result

The amplified products were detected on agarose gel electrophoresis resulted in generation of an amplicon band at the correct expected size of the VP2 gene (645bp). PCR amplicons obtained from virus infected Vero cell homogenates were compared with positive control amplicons and the base pair estimation was used by molecular marker (Figure 9).

Figure 9 Agarose gel electrophoresis picture showing 645bp fragment of the VP2 gene IBDV

M- Molecular Ladder started 100bp (Fermentas); 1. Vero cell control; 2. Original culture; 3.Vero cell culture passage 5; 4. Vero cell culture passage 6; 5. Vero cell culture passage 7; 6. Vero cell culture passage 8; 7. Vero cell culture passage 9; 8. Negative control; 9. RNA free water; 10. Known positive control

4.3.1. Sequence analysis result

The nucleotide amino acid sequence of the VP2 HVR was determined for IBDV vaccine strain (LC 75) prepared on chicken fibroblast cell, IBDV vaccine train (LC 75) adapted on Vero cell at passage 5 and IBDV vaccine train (LC 75) adapted on Vero cell at passage 10 from cDNA transcripts. The nucleotides (700 nt of VP2 gene) as well as amino acid alignment result had analyzed and recorded (Figure 10 and 11).

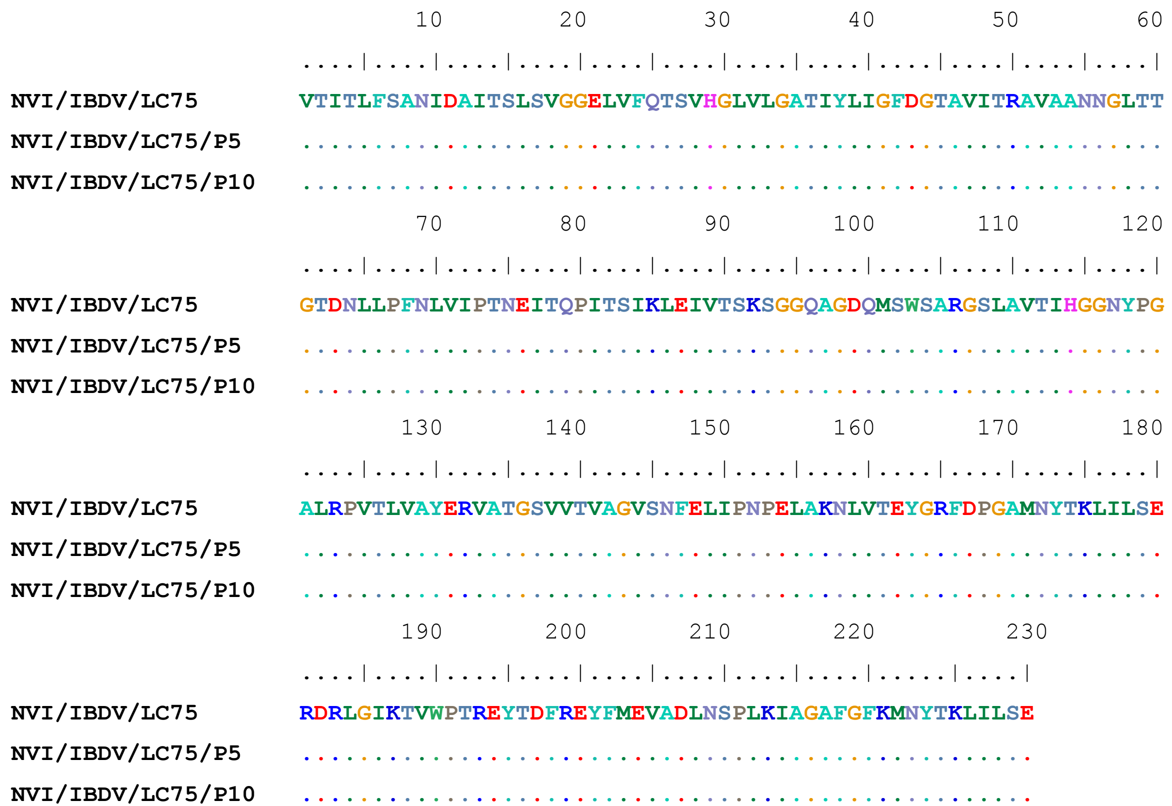
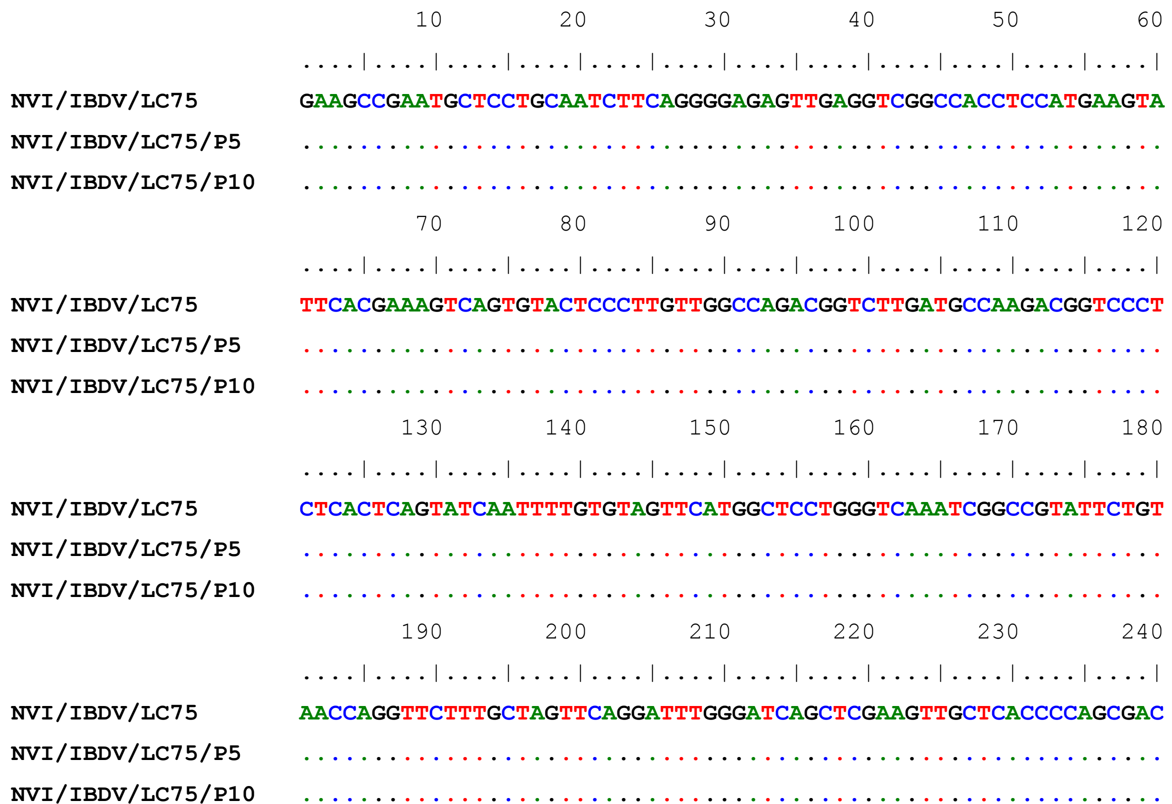


Figure 10 Aligned amino acids sequences for IBDV LC75 adapted on Vero cells



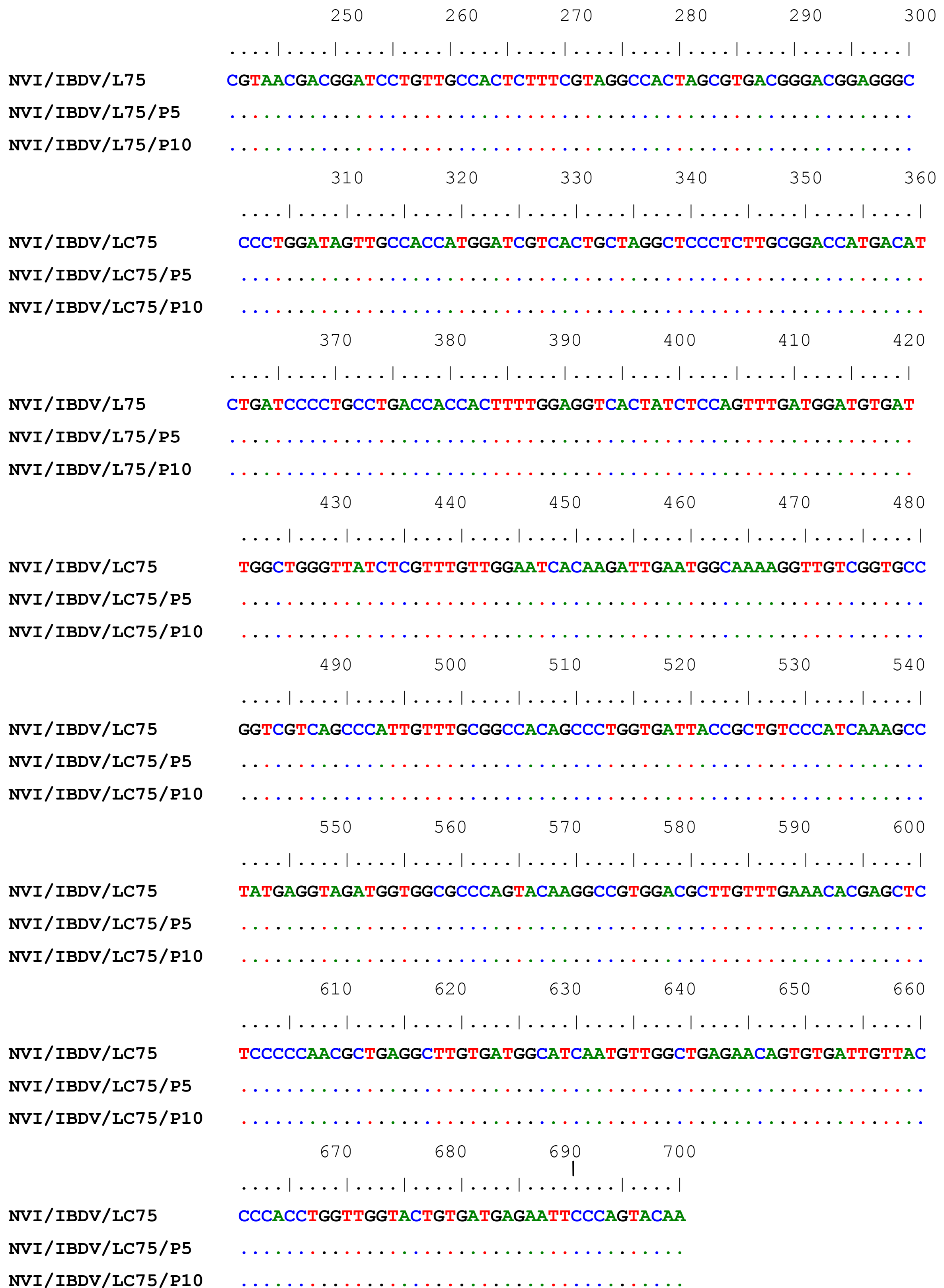


Figure 11 Aligned nucleotides sequences for IBDV LC75 of Vp2 adapted on Vero cells

4.4. Immunogenicity test result

The results of ELISA tests for MDA performed on the serum collected from day 7 old chicks before vaccination revealed that antibody S/P ratio was on average 0.12. At day 21 old (7 days post inoculation) the mean S/P ratio for Group 1; 0.05, Group 2; 0.07, Group 3; 0.094. The mean S/P ratio for the three groups was 0.07 (Figure 12). At day 14 post inoculation S/P ratio for P 5, 1.28; for P 10, 1.53; and for (Gum 1/18), 1.76 . The mean S/P ration for the three groups at 14 day post inoculation was 1.52. At day 21 post infections the S/P ration or antibody titer seems equal with that of day 14 post inoculation (Figure 12). The chickens were boosted at day fourteen post inoculation and then after 7 day boosted S/P ratio were strongly increased from 1.53 to 2.15 or antibody titer (4247.67 - 6307.77) (Figure 12).

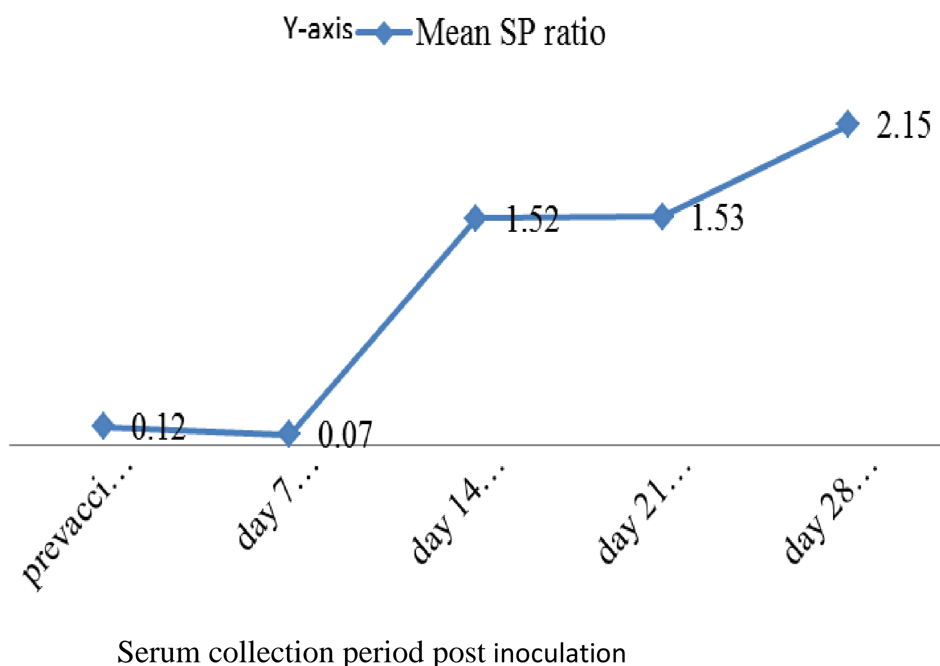


Figure 12: Mean S/P ratio of antibody at pre and post vaccination

4.4.1. Comparison of immunogenicity test result

The comparison of antibody produced against Vero cell adapted IBDV from passage 5, passage 10 and from CFC based prepared vaccine (batch Gum 1/18) was done at 7 days

post vaccination; 14 day post vaccination and 21 day post vaccination. The result was statistically described by the use of one way Analysis of Variance (Table 1, 2 and 3).

Table 1 Statistical comparison of antibody produced at 7 day post-inoculation

Source	**ss**	**df**	**ms**	**f**	**prob > f**
Between groups	0.01	2	0 .004	0.53	0.59
Within groups	0.45	57	0.008		
Total	0.46	59	0.012		

As indicated on the above table, the P-value is 0.59 which is greater than 0.05.

Table 2 Statistical comparison of antibody produced at 14 day post-inoculation

Source	**ss**	**df**	**ms**	**f**	**prob>f**
Between type of infection	0.02	2	0.12	1.49	0.23
Within type of infection	0.44	57	0.01		
Total	0.46	59	0.01		

As indicated on the above table, the P-value is 0.233 which is greater than 0.05.

Table 3 Statistical comparison of antibody produced at 21 day post-inoculation

Source	**ss**	**df**	**ms**	**f**	**prob > f**
Between type of infection	0.21	2	0.11	0.39	0.68
Within type of infection	15.41	57	0.27		
Total	15.62	59	0.27		

As indicated on the above table, the P-value is 0.68which is greater than 0.05.

4.5. Efficacy test results

Starting from day 4 post inoculations of challenge virus, 60% chickens from control groups showed clinical signs such as ruffling feather, in appetence, difficulty in movement, yellowish diarrhea and dehydration, and 25% of the chickens were died starting from day 6 post challenged (Figure 11 and 12). Whereas chicken groups vaccinated with Vero cell adapted LC-75 IBDV strain from passage 5; passage 10 and CFC based prepared vaccine (Gum batch 1/18) was safe with the absence of any clinical signs observed.

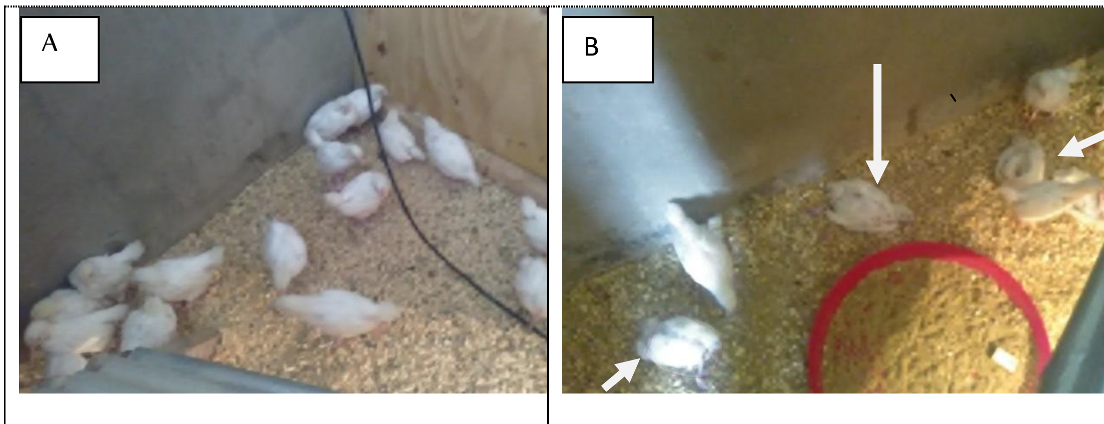


Figure 13 Picture showing the efficacy test result between vaccinated and unvaccinated group

Group A: vaccinated group (no clinical sign, no death); Group B: non-vaccinated group (5/20 death was occurred), white arrows indicate dead chicken.

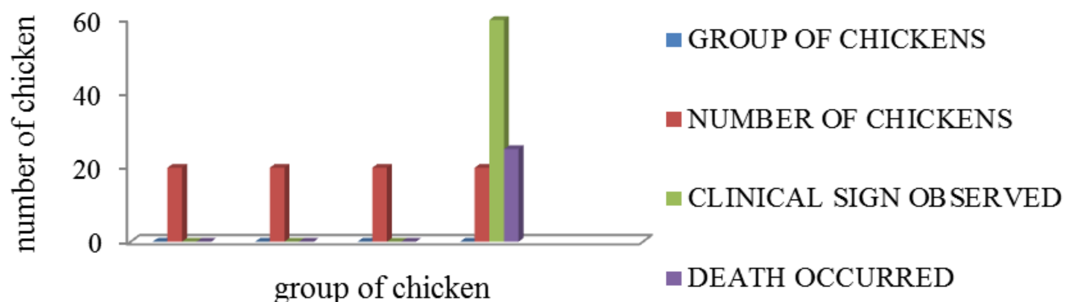


Figure 14 percentages of clinical sign observed and death occurred

5. DISCUSSION

The present study conducted to adapted IBDV (LC-75) strain in Vero cells with ultimate objective of Vero cells based IBDV vaccine production at NVI, Ethiopia. The vaccine strain IBDV (LC-75) was adapted on Vero cells and proved to be immunogenic and efficacious in chickens and is recommended for ultimate vaccine production. Previously it was suggested that the use of Vero cells in growing avian viruses including other viruses has become an increasingly economical, less laborious, and continuous and efficient tool with an advantage of measuring various effects outside the host animal (Ahmed *et al.,* 2015) as compared to the use of chicken fibroblast cells (primary cells).

Normal and confluent monolayer of Vero cells was obtained following 36 hours of sub-culturing in growth medium. Following with infection with IBDV LC-75, the monolayer remained fully intact in passage 1 (P1) up to six days post-infection and then blindly harvested. During second passage, some changes in Vero cell monolayer began to develop after four days of incubation. Monolayer showed rounding of infected cells. Nevertheless, complete CPEs of IBDV on Vero cells were not found in this passage. But starting from passage 3 CPE was observed at day three post infection. This indicated that the virus had adapted to Vero cell culture environment and started replication with good and observable CPEs. In all the cases Vero cell control was the standard to compare the formation of CPEs. Since the virus had shown good CPEs starting from P 3 up to P 10 constantly, IBDV can be adapted very well on Vero cells starting from P 3. Hussain and Rasool (2005) also reported that the passage level and the time at which the CPE formation had started and completed was similar with the present study. Another study conducted by Isabela *et al.* (1999) reported support the present study which revealed at first inoculation of IBDV inoculated on Vero cell no CPE was observed. The result also indicated difference in time of CPEs observed with that of Silva *et al*. (2004).

The total infectious titer of Vero-cell adapted IBDV LC-75 strain starting from passage 3 had indicated that as the passage level increases the titer of the virus is also increases (Figure 8). This confirmed as the virus was adapted on Vero cells starting from passage 3 with a sufficient titer/ml ($\log_{10}$ 5.40TCID/ml). The value of obtained titer/ml was greater than the recommended titer/dose according to the parameters of Code of American Federal Regulation (CFR, 2012) in which IBDV titre must not be less than $\log_{10}$ 3.40 $TCID_{50}$/dose.

So the prepared Vero cell adapted IBDV vaccine strain starting from passage 3 were containing satisfactory TCID/dose. Previously, Hussain and Rasool (2005) also reported similar findings in growth pattern of vvIBDV in Vero cells at passage 3 after 72 hours of infection.

To confirm identity of the Vero cell adapted IBDV LC-75, RT-PCR test was conducted to amplify the VP2 region which is responsible for the pathogenicity of the virus. The PCR result had indicated that homogenates from virus infected Vero-cells were positive but negative in cell control. The positive RT-PCR result had confirmed that the similarity of the PCR products with vvIBDV circulating in different parts of Ethiopia as described by Mekuriaw *et al.* (2017). To arrive on more conclusive evidence, the VP2 gene of the cell adapted IBDV LC-75 was sequenced and no variation of nucleotides between different passages were observed as compared with the CFC based prepared vaccine. This indicated that even though the passage level was done from passage 1 up to passage 10, there was no mutation occurred in the part of gene examined. The present study contradicts with the report given by Getaw (2017) as he reported evidence of nucleotides substitutions of Vero cell adapted IBDV vaccine strain.

Serology test was conducted by the use of AC-ELISA at 7 days post inoculation, all groups of chicken had contained antibody titre less than positive range (titre <875); whereas starting from day 7 up to day 14 post inoculation, all groups of chickens inoculated with both Vero cells adapted virus and Gum 1/18 vaccine had strongly increased antibody titre from 213.18 to 4220.74 or S/P (0.07 to 1.52). Before boosting mean antibody produced at day 14 post inoculations were closely similar with day 21 post inoculation (7 days post boost) (Figure 12). This can show that at day 14 post inoculation antibody level could have reached peak titre. Similar idea was reported by Ahmad *et al.* (2014) that the antibody titre at 14 days of post infection resulted in a gradual increase of antibody values in all inoculated groups. This result also agreed with the description given by Rautenschlein *et al.* (2005) about immunity respond to intermediate plus or “hot” vaccine of IBDV. As reported by Rautenschlein *et al.* (2005) the intermediate plus vaccine induced antibody levels already at 14 days post vaccination. The chickens were also boosted at 14 day post infection. The mean S/P ratio was highly increased starting from day 21 post inoculation. As all groups of chickens were boosted at day 14 post inoculation, antibody production was enhanced and mean S/P ratio or the antibody titre increased. In all types of infections

there was no significant difference of antibody production observed. This was described by using ANOVA statistical analysis since the p-value is greater than 0.05 (Table 1, Table 2 and Table 3). Therefore, Vero cell adapted vaccine strain IBDV is equally immunogenic at passage 5 and passage 10 with that of chicken fibroblast cell based prepared vaccine.

The challenge test was done to evaluate efficacy of Vero cell adapted virus as compared to CFC based prepared vaccine. The control groups responded to challenge virus by high morbidity and mortality rate starting from 3 days post challenge. There was clinical manifestation of anorexia, ruffling of feathers and death but no any clinical sign and death occurred in pre vaccinated chickens. The chicken vaccinated with Vero cell adapted LC-75 IBDV strain at passage 5, passage 10 and the vaccine produced based on CFC were fully protected against the effect of the challenge virus that could be due to absence of nucleotide variation in the VP2 gene of the viruses. Therefore Vero cell adapted virus is efficacious to protect the vvIBDV circulating in Ethiopia as that of CFC based prepared vaccine. The validation result of the Vero cell adapted virus efficacy was similar as described by OIE (2016).

6. CONCLUSION AND RECOMMENDATIONS

The present study confirmed that protective and efficacious IBDV vaccine could be produced using Vero cells just like that of chicken fibroblast cells that needs SPF eggs. The IBDV vaccine strain LC-75 was adapted well on Vero cells as early as passage 3 with a good virus titre/ml. The absence of variations in nucleotide and amino acid of the Vero cell adapted vaccine strain at passage 5, passage 10 and CFC based prepared IBDV vaccine could suggest adaptation of the virus and protection of the vaccine against field strains. The adapted vaccine is equally immunogenic and efficacies with no difference between passages and chicken fibroblast cell based prepared vaccine is more economical to produce IBDV vaccine using Vero cells.

Based on the above conclusions, production of IBDV vaccine using Vero cell line is recommended.

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8. APPENDIXCES

Appendix 1 Table of media used for cell culture

No,	Types of media used	Manufacturer	Lot number	Country
1	DMEM	HIMIDIA	0000244296	India
2	TRYPSIN 1:250	HIMIDIA	0000130542	India
3	FOETAL CALF SERUM	HIMIDIA	0000249659	India
4	TRYPTOSEPHOSPHATE BROTH	HIMIDIA	0000244033	India

Appendix 2 Formulation of growth cell culture medium

Number	Ingredients	Amounts	Function
1	DMEM (powdered)	12.5 g	The main medium
2	NaHCO3	2.75	To give red color and to balance PH
3	Bi distilled water	1000ml	To dissolve and homogenize
4	Dilute and adjust t pH to 7.35 to 7.4 by adding NaHCO3 solution.		
5	Sterilize by filtration with a filter pad 0.22 µl pore size in sterile bottles and store at +4^{0}C		
5	Add 100ml of sterile foetal calf serum and 100ml of tryptose phosphate brose in 800ml base medium and 2ml antibiotic		

Appendix 3 RNA extraction procedure

a. Add one volume of 70% ethanol alcohol to each volume of cell homogenate i .e 350 µl
b. vortex to thoroughly to dispersed any visible precipitate
c. Transfer up to 700 µl of homogenate sample to the spine cartridge with collecting tube
d. Centrifuge 12500rpm for 30 seconds at room temperature and discard the flow through
e. Repeat procedure d until the sample homogenate is completed
f. Add 700 µl wash buffer I to the spine cartridge and centrifuge at 12500rpm for 3min.
g. Place the spine cartridge in to new collection tube and add 500 µl wash buffer II and then centrifuge as above indicated
h. Repeated centrifugation at 13400 rpm for 3 minute to avoid the remaining ruminants and to dry and transfer the spine cartridge in to new collection tube
i. Add 40 µl RNase free water to the centre of the spine cartridge and incubate for 1 min.
j. Centrifuge the spine cartridge at 3400rpm for 3 min at room temperature to elute the RNA from the membrane in to the recovery tube
k. Label (the virus name and day of extraction) and preserved at -20°C for further cDNA synthesis

Appendix 4 cDNA synthesis for one reaction

- Mix RNAse free water 3 µl, 10mM dNTPs mix 1 µl , IBD Specific primer 2 µl for each (IBD3 forward and IBD3 reverse)
- Mix by vortexing and dispense 5 µl mixtures in to PCR tube and add 5 µl of extracted RNA sample and mix by votexing and then incubate for 56°c for 5 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 37°c for 20 min chill on ice

Appendix 5 Agarose gel preparation and sample loading procedures

a. Mix agarose powder with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted (Prepare 60 ml of 1.5% agarose in 1X TBE buffer) in which the comb is inserted
b. Allow to cool and solidify the gel to about 25°c for 20-40 minutes
c. Pour puffer 1X TBE in to tank and remove the comb from gel
d. Prepare samples in to tubes, multichannel pipette or on paraffin

1 µl loading buffer

5 µl PCR product

f. Prepare molecular weight marker

0.5 µl molecular weight marker VI (Bioehringer)

1 µl loading buffer

4.5 µl dH2O

- 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.

Appendix 6 Experimental animal Ethical Clearance

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

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/28/05/10/2018

Name of Applicant: Wakjira Kebede(BSc in VLT, MSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: ***Vero Cell adaptation, immunogenicity and efficacy test of vaccinal strain infectious bursal disease virus for vero cell based vaccine development***

Date of application:	**09/11/2017**
Nature of the project:	**invasive**
Target animal species:	**Chicken**
Number of animals involved:	**80**
Study area:	**National Veterinary Institute, Ethiopia**

Minutes No. and date of review: **VM/ERC/05/10/018, 03/01/2018**

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

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

Dr Getachew Terefe
Chairman

Signature

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Please quote Our Ref. No. When replying

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