Thesis Ref, No._____

MOLECULAR CHARACTERIZATION, ADAPTATION AND ATTENUATION OF LOCALLY ISOLATED AND VACCINAL STRAIN OF INFECTIOUS BURSAL DISEASE VIRUS FOR DEVELOPMENT OF VERO CELL BASED INFECTIOUS BURSAL DISEASE VACCINE IN ETHIOPIA

MSc. THESIS

BY:

GETAW DERESSE TADESSE

Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Veterinary Microbiology, Immunology and Veterinary public health

MSc program in Veterinary Microbiology

JUNE, 2017

BISHOFTU, ETHIOPIA

MOLECULAR CHARACTERIZATION, ADAPTATION AND ATTENUATION OF LOCALLY ISOLATED AND VACCINAL STRAIN OF INFECTIOUS BURSAL DISEASE VIRUS FOR DEVELOPMENT OF VERO CELL BASED INFECTIOUS BURSAL DISEASE VACCINE IN ETHIOPIA

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

BY

GETAW DERESSE TADESSE

JUNE, 2017

BISHOFTU, ETHIOPIA

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

As members of the Examining Board of the final Msc open defense, we certify that we have read and evaluate the thesis prepared by: Getaw Deresse Tadesse entitled "Molecular Characterization, Adaptation and Attenuation of Locally Isolated and Vaccinal Strain of infectious bursal disease virus for development of vero cell based infectious bursal disease Vaccine in Ethiopia" and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

Dr. Kibeb Legesse	_____	_____
Chairman	Signature	Date
Dr. Belayneh Getachew	_____	_____
External Examiner	Signature	Date
Dr. Fufa Dawo	_____	_____
Internal Examiner	Signature	Date

Advisors

Dr. Asmelash Tassew (DVM, MSc, Assist. Prof.)	_____	_____
Main advisor	Signature	Date
Dr. Essayas Gelaye (DVM, MSc, PhD)	_____	_____
Co-advisor	Signature	Date
Dr. Molalign Bitew (DVM, MSc, PhD)	_____	_____
Co-advisor	Signature	Date
Dr. Gezahegn Mamo (DVM, MSc, PhD, Asso.prof.)	_____	_____
Department Chairperson	Signature	Date

STATEMENT OF AUTHOR

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 fulfillment 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.

Brief quotations from this thesis are allowable without special permission provided if an accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the college when in his or her judgment.

In all other instances, however permission must be obtained from the author.

Name: Getaw Deresse Tadesse Signature: _____

Addis Ababa University, College of Veterinary Medicine and Agriculture, Bishoftu
Date of submission: June 16 /2017

ACKNOWLEDGEMENTS

First and foremost, I thank my God, the lord of all creations who granted me his blessings throughout my life and gave me endurance to do and complete this research paper.

Next I would like to express my deepest gratitude and appreciation to my major advisor, Dr. Asmelash Tassew for providing his constructive and immeasurable comments, advice, encouragement and desired written materials those help me as guidance throughout my work. I thank him for unlimited support me starting from setting my bench mark work up to the end of my research.

I would also like to express my deepest gratitude for National Veterinary Institute for granting me an excellent opportunity to work in laboratories of the institute, timely supply of laboratory inputs and covering all the cost that need for the research.

I would like to give special thank to my coadvisor Dr. Essayas Gelaye, Head of research and development directorate at National Veterinary Institute, for facilitating all necessary tasks of the research to be performed with recommended time. Especially, molecular identification and sequence analysis of the samples were conducted by him with Ato Alebachew Belay who is professional of molecular biology in the institute.
I would like to express my gratitude to my coadvisor Dr. Molalign Bitew for his constructive instructions, proper guidance and motivation throughout my study period.

I am grateful to my colleagues and the staff of the Virology Research Laboratory; Dr. Teferi Degefa, Mrs. Hawa Mohammed and all other staff members for their patience and valuable assistance during each activity of the laboratory work of the research.

I want to express my thanks to Ato Bogale Gossaye and Ato Kassaye Adamu who cooperated with me to help the editting of my paper.

Last but not the least, I am indeed grateful to my family for their understanding the nature of my hard work, and their great help in accomplishing my study.
Finally my special thanks go to my wife for her patience, continuous encouragement, and care and supporting during this study.

TABLE OF CONTENTS

Table of Contents **Page**

LIST OF TABLE

LIST OF FIGURES

Page

LIST OF ANNEXES

Page

LIST OF ABBREVIATIONS

AGIDT	Agar gel immunodiffusion test
BF	Bursa of Fabricus
BP	Base pair
CAM	Chorioallantoic membrane
cDNA	Complementary deoxyribose nucleic acid
CEB	Chicken embryo bursa
CEK	Chicken embryo kidney
CFC	Chicken fibroblast cell
CPE	Cytopathic effect
DEPC	Diethylpyrocarbonat
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethyl diamide triacetic acid
EID50	Embryo infectious dose
ELISA	Enzyme linked Immunosorbent assay
FCS	Fetal calf serum
GMEM	Glasgow Minimum Essential Media
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
KM	Kilo meter
MDAs	maternally derived antibodies
Ml	Milliliter
MOA	Ministry of agriculture
NAHDIC	National animal health diagnostic and investigation center
NVI	National Veterinary Institute
OIE	Office International des Epizooties
ORF	Open reading frame
P	Passage
PBS	Phosphate Buffer saline
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
Pi	Post infection
RE	Restriction endonuclease

RNA	Ribose nucleic acid
RT	Reverse transcriptase
SPF	Specific pathogen free
SOP	Standard operative procedure
TCID50	Tissue culture infective dose 50
TPB	Tryptose Phosphate Buffer
USA	United States of America
VNT	Virus neutralization test
VP	Viral protein
VvIBDV	Very virulent infectious bursal disease virus
WHO	World Health Organization
WSU	Washington state university

ABSTRACT

Infectious Bursal Disease (IBD) or Gumboro Disease is one of the most common diseases of commercial poultry in Ethiopia.Vaccination is the principle method to control the disease. Currently, NVI is producing IBD vaccine by using standard strains of IBDV on primary chicken embryo blast in Ethiopia. But the production of live attenuated IBD vaccine from locally isolated IBDV stain provides high protection for chickens against field IBDV strains. IBD vaccine production on primary chicken embryo blast is also very laborious, time consuming and economically costy. However, production of live attenuated IBD vaccine using Vero cell can solve these issues. The present study was initiated to propagate, adapt, and attenuate both locally isolated and commercially available IBDV strains in the vero cell lines for development of an effective Vero cell-based IBD vaccine in Ethiopia. Locally isolated and LC-75 vaccinal strain IBDVs were successfully adapted and attenuated in Vero-cell line in NVI and molecularily characterized. The IBDV virus strains were attenuated by further serial passages and pathogenicity test at specific passages were conducted in breeder chickens free from antibodies against IBDV to evaluate the loss of virulence in the virus during passaging on Vero cell line. Vero cell could be used as a model to study the growth of kinetic of the IBDV isolate and the Vero adapted attenuated virus should be further studied for possible adoption as a candidate for an attenuated IBD vaccine development.

Key words: *Chicken, Immunosuppression, Infectious bursal disease, Infectious Bursal Disease Virus, Isolation, Molecular characterization, Vaccination, Vero cell.*

1. INTRODUCTION

Livestock resource is a major portion of global resources. Especially, it plays an important role in the agricultural economy of Africa. Poultry has a lot of contribution and it occupies a unique position in terms of its contribution to the provision of high quality food (protein) in the form of meat and eggs as well as reliable source of petty cash to rural small holder farming families in Africa. Both poultry meat and eggs enrich and contribute to a well-balanced diet of young children in sub Saharan Africa (Tadelle *et al.*, 2003). However, the contribution of poultry production to the small holder farmers and the country economy is still restricted by various factors like low inputs of feeding, poor management, infectious diseases and lack of appropriate selection and breeding practice (Alemu, 1995; Tadelle and Ogle, 2001; Halima *et al*., 2007).In Ethiopia, poultry industry is one of the most productive sectors, which acts as a source of major protein supplements for growing population of the country. However, outbreaks of various diseases are a major constraint in further growth of this industry.

Infectious bursal disease (IBD) is one of the killer diseases of chickens. Infectious bursal disease (IBD), an immunosuppressive viral disease, causes significant losses to the poultry industry either by causing high mortality in an acute disease or as a consequence of immunosuppresion (Van den Berg, 2000). The most 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 (Van Den Berge, 2004).

Infectious bursal disease virus (IBDV) attacks chicken's bursa of fabricius, killing lymphoid cells results in suppression of birds immune system and increase the susceptibility to other avian diseases such as infectious bronchitis, Marek's disease and especially Newcastle disease (Apilak ,2006). The disease is caused by a double stranded RNA virus belonging to the genus *Avibirnavirus* under the family *Birnaviridae* (Dobos *et al.,* 1979). IBDV first described in the USA in 1962 (Cosgrove, 1962). In Ethiopia, the disease was first reported in a farm at Debre-Zeit in 2002 (Zeleke *et al*., 2002) with high mortality of 49.89% in the affected 20-25 day-old broiler and layer chicken (Zeleke *et al*., 2005). There are two distnct serotypes of IBDV (McFerran *et al.*, 1980). Virus strains belonging to the serotype 1 are pathogenic causing disease in young chickens of usually 3 to 6 weeks of age (Lukert and Saif,

2003; Muller *et al.*, 2003) and comprise several pathotypes, such as, classical virulent, antigenic variant and very virulent strains (Lukert and Saif, 1997). Serotype 2 viruses are apathogenic for chickens.

IBDV can infect and grow on various primary cell culture of avian origin and certain cell line of mammalian origin. Commonly used cell lines to replicate IBDV are chicken embryo fibroblast (Sofei *et al.*,1996*)*,chicken embryo kidney, Vero (Peilin *et al*.,1997), baby hamster kidney (El-Ebriary *et al.*,1997), ovine kidney (Kibenge and Mckenna,1992), chicken embryo bursa (Luker and Davis,1974), normal chicken lymphocytes, B-cell lymphblastoid rabbit kidney*(*Rinaldi *et al*.,1972),baby grivet monkey kidney and M4-104 cells (Jackwood *et al.,*1987). In addition to the above cell lines, IBDV can also infect chicken embryo. The uses of continuous cell lines of mammalian origin have several advantages over the use of primary cell culture of avian origin. Continuous cell lines are easier to handle and maintain and free from vertically transmitted extraneous viruses of avian origin (Hassan *et al*., 1996; Jackwood *et al.*, 1987). Among continuous cell lines, Vero-cell line has been used for the growth and replication of IBD virus (Kibenge *et al.*, 1988; Peilin *et al*., 1997) and (Ahasan *et al*., 2002).

The Vero cell line is one of the most satisfactory based on its stability and well-documented performance in quality and quantity of viral yield. Vero cells are considered as a substrate for the production of viral vaccines. Vero cells were first separated from a normal adult African green monkey kidney cell line by Yasumura of Chiba University, Japan in 1962. This cell line presents several advantages over primary and diploid cell substrates. Vero cells are easily available, grow fast and require no rigor culture conditions which can support various virus proliferations; Vero cells have been extensively characterized to evaluate their oncogenic potential. Several studies have shown that this cell line is free from oncogenic property and is not presenting any threat to human health when used as a substrate for biological production authorized by the World Health Organization (WHO); Vero cells can be used in microcarrier and suspension cultures for large scale production in bioreactors. Moreover, virus titer achieved is higher than that reached using other types of cell substrates. These properties greatly facilitate the transfer of vaccine-producing capability to developing countries, which is an important goal of WHO.This cell line is being used worldwide to grow and propagate a number of viruses of avian and non-

avian origin (Tian and Keping, 2009). In other hands, the traditional isolation method for IBD virus using the chorioallantoic membrane of 9 to 11-day-old chicken embryos is no longer reliable (Hitcher, 1970), as some variant strains of virus cause no embryo mortality (Rosenberger and Cloud, 1986) and most field isolates cannot be readily adapted to grow in primary chicken cell cultures (Lee and Lukert, 1986; McFerran *et al*., 1980). These primary cell cultures produce low yield of virus, even contaminated with some extraneous virus of avian origin and have limited growth properties (Lukert *et al*., 1975).

Ethiopia is producing IBD vaccine by using primary chicken embryo fibroblast and standard strains of IBDV at present time. The production of vaccine from locally isolated IBDV absolutely give high protection against field challenge of IBDV. Producing IBD vaccine using primary chicken embryo fibroblast is also very laborious, time consuming and economically costy. But Vero cells are easily available and grow fast for vaccine production.

Therefore, the objectives of this study were:

- To assess the capacity of the virus to propagate on Vero cell line effectively
- To adapt and attenuate the local isolate and vaccinal strain of IBDV in vero cell for development of vaccine
- To determine the molecular characteristic of the adapted and attenuated IBDV strains
- To determine pathogenecity of the live attenuated IBDV in antibody free chicks against IBD.

2. LITERATURE REVIEW

2.1. Description of the disease

Infectious bursal disease is a viral infection, affecting the immune system of poultry. It is a highly infectious disease that affects young chickens, turkeys and ducks. The disease is caused by the infectious bursal disease virus (IBDV), which is a member of the *Birnavirus* genus. The virus destroys the lymph cells within the cloacal bursa (of fabricus) where B lymphocytes mature and differentiate tonsils and spleen. The target cell of the virus is the B lymphocyte in an immature stage, and the infection, when not fatal, causes an immunosuppression, in most cases temporary, the degree of which is often difficult to determine. The disease normally affects chickens up to six weeks and the onset can be sudden **(**Jonathan and John, 2013).

In all poultry producing regions of the world, IBDV continues to be a major constraint for poultry farmers. The consequences of immunosuppression associated with IBDV are vaccination failure and susceptibility of chickens to opportunistic pathogens. It was also shown that IBDV-infected birds may become a good propagator for other viral pathogens. For example, low pathogenic duck adapted avian influenza virus becomes more virulent when serially passaged in IBDV-infected chickens. Moreover, highly virulent IBDV can cause high mortality in unprotected flocks (Tamiru *et al*., 2016).

IBDV causes a highly immunosuppressive disease in chickens and is a pathogen of major economic importance to the poultry industry worldwide. IBD is an acute, highly contagious viral infection in chickens manifested by inflammation and subsequent atrophy of the bursa of Fabricius, various degrees of nephroso-nephritis and immune suppression. Clinically the disease is seen only in chickens older than three weeks. The feathers around the vent are usually stained with faeces containing plenty of urates. This form can result in high mortality. Infection prior to three weeks of age results in immuno suppression and bursal atrophy due to destruction of undifferentiated lymphocytes. It is economically important to the poultry industry worldwide due to increased susceptibility to other diseases and negative interference with effective vaccination (Gary and Richard, 2003).

2.2. History of IBD

IBD was first recognized as a distinct clinical entity in 1957 (Cosgrove, 1962). A. S. 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 diarrhea, 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 (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 (Winterfield *et al*., 1962), Hitchner (Hitchner, 1970) proposed that the disease be termed "infectious bursal disease" due to its pathognomonic bursa lesions.

The first experiment to isolation the etiologic agent was impeded by a lack of specific-pathogen-free (SPF) eggs and by deficiencies in viral and serologic techniques. By 1967, the highly infectious nature of the agent was recognized. Dependable methods were developed to isolate the virus in embryonated eggs and to adapt it to tissue culture. The agent was characterized as a virus belonging to a new taxonomic group in 1976 (Lukert and Saif, 2003).

The immunosuppressive property of IBD virus was first recognized in 1970 and was confirmed in structured trials in 1976. An early method of control involved planned infection of chickens (Lukert and Saif, 2003). Between 1960 and 1964, the disease affected most regions of the USA, and reached Europe in the years 1962 to 1971 (Lukert and Saif, 2003). From 1966 to 1974, the disease was identified in the Middle East, southern and western Africa, India, the Far East and Australia.

IBD was first reported in a farm at Debre-Zeit in 2002 (Zeleke *et al*., 2002) with high mortality of 49.89% in the affected 20-25 day-old broiler and layer chicken (Zeleke *et al*., 2005).The disease has since spread to 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. There was Gumboro disease surveillance/investigation conducted by the 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 (MOA, 2011).

2.3. Etiology

IBD is caused by a virus called infectious bursal disease virus (IBDV), which belongs to the *Birnaviridae* family. The virus is very hardy, can survive in a wide variety of environmental conditions, and is difficult to effectively decontaminate (WSU, 2014).

2.3.1. Taxonomy of Avibirnavirus

The family *birnaviridae* has three genera. The genus *Avibirnavirus* whose type species is infectious bursal disease virus, which infects birds and this genera have two serotypes (serotype 1 and serotype 2) which they differentiated by virus neutralization test**.** *Aquabirnavirus* included infectious pancreatic necrosis virus, which infects fish. *Entomobirnavirus* is whose type species Drosophila X virus, which infects insects.

The *Avibirnavirus* viral genomes is nonenveloped with icosahedral symmetry and a diameter varying from 55—65 nanometer, consisting of two segments of double stranded RNA (dsRNA), hence the name *birnaviruses*.

2.3.2. *Morphology and structure of Avibirnavirus*

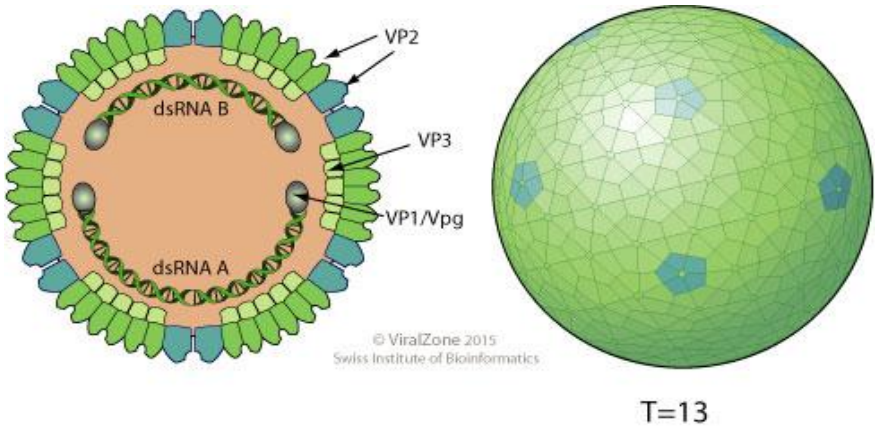


Figure 1: Morphology of the IBDV

Source: http://viralzone.expasy.org/all_by_species/572.html

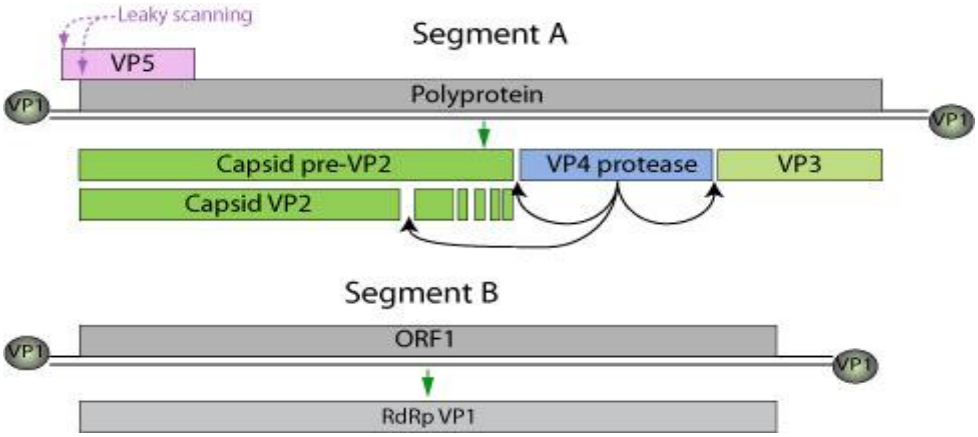


Figure 2: The structure of the segmented linear dsRNA genome

Source: http://viralzone.expasy.org/allbyspecies/572.html

Viruses in *Avibirnavirus* are non-enveloped, with icosahedral and Single-shelled geometries, and T=13 symmetry. Genomes are linear and segmented, around 2.9-3kb in length. Five viral proteins designated VP1, VP2, VP3, VP4, and VP5 are recognized **(**Hirai and Shimakura, 1974; Dobos, 1979; Müller *et al.*, 1979 and Ozel and Gelderblom, 1985).

VP2 and VP3 are the major structural proteins, forming the outer and inner capsid of the virus, respectively. The epitopes located on the VP2 protein are capable of eliciting the host's protective neutralizing antibodies against IBD, they are serotype determinant. But as the hyper-variable region of VP2 is subject to mutations, it often leads to changes in virus antigenicity and pathogenicity.

The genome of IBDV and other Birnaviruses consist of double-stranded RNA (two segments), which are packaged in a nonenveloped icosahedral shell 60 nm in diameter. The largest part, A is 3,261 nucleotides long and it encodes an 110-kDa precursor protein in a large single open reading frame (ORF), which is cleaved by autoproteolysis to yield mature VP2, VP3, and VP4 proteins . The major structural proteins of the virion are VP2 and VP3 while VP4 is a minor protein share in the processing of the precursor protein single-shelled. The major protective antigen of IBDV is the VP2 protein that contains specific epitopes responsible for inducing neutralizing antibody responses. Variations in antibody recognition may occur due to changes in an amino acid within the variable region of VP2, as well as, antigenecity, immunogenicity, virulence, and tissue tropism of IBDV strains. The smallest segment, B is 2,827 nucleotides long and it encodes VP1, a 97-kDa protein having RNA-dependent RNA polymerase activity. IBDV has two serotypes; however, only serotype I viruses are naturally pathogenic to chickens. Serotype I strains are classified as very virulent IBDV strains, classic or variant and differ in their virulent, antigenic, and pathogenic properties. A second serotype (serotype II) is isolated from a fowl, turkeys, and ducks. It is apathogenic (does not cause any clinical disease) and was originally isolated from turkeys. The two serotypes can differentiate by cross-neutralization assay and ELISA using monoclonal antibodies (Nawzad *et al*., 2016).

2.3.3. Physico-chemical properties of the virus

IBDV is very stable. IBDV resisted treatment with ether and chloroform, is inactivated at pH 12 but unaffected by pH 2, and is still viable after 5 hours at 56°C. Only the iodine complex had any deleterious effects. Certainly, the hardy nature of this virus is one reason for its persistent survival in poultry houses even when thorough cleaning and disinfection procedures are followed. One of the most interesting features of IBDV is its ability to remain infectious for a very long period of time and its resistance to commonly used disinfectants. Infectious bursal disease is usually a disease of three to

six week old chickens. But an early subclinical infection before three weeks of age is also observed (Lukert and Saif, 1997). Therefore without proper disinfection the virus can survive on the premises for more than 4 months. The virus is extremely resistant to disinfection and pH changes between pH 2-12. The virus remains viable following incubation for 5 hours at 56 °C and 30 min at 60 °C. The infectivity can be reduced by treatment with 0.5% formalin for 6 hours or 1% formalin for 1 hour.

2.4. Distribution of IBD

IBD is emerged in 1957 as a clinical entity responsible for acute morbidity and mortality in broiler chickens. The first report of a specific disease affecting the bursa of Fabricius in chickens was made by Cosgrove in 1962. The first outbreak due to the classical IBDV were observed in the area of Gumboro and was initially described as avian nephrosis, in Delaware (United States of America) which is the origin of the name, although the terms 'IBD' or 'infectious bursitis' are more accurate descriptions. It was characterized by flock morbidity of 10-25% and mortality averaging 5% (Cosgrove, 1962). Between 1960 and 1964, the disease affected most regions of the USA, and reached Europe in the years 1962 to 1971 (Lukert and Saif, 2003). The condition spread rapidly and was recognized throughout the U.S broiler and commercial egg production areas by 1965. From 1966 to 1974, the disease was identified in the Middle East, southern and western Africa, India, the Far East and Australia.

IBD is currently an international problem: 95% of the 65 countries that responded to a survey conducted by the Office International des Epizooties (OIE) in 1995 declared cases of infection, including New Zealand which had been free of disease until 1993. These findings led to the adoption of a specific resolution of the International Committee of the OIE during the 63rd General Session in May 1995 (Islam, 2005).

2.5. Transmission of IBD

The route of infection is usually oral by contaminated feed, water, servicemen and trucks, but may be via the conjunctiva or respiratory tract, with an incubation period of 2-3 days. The disease is highly contagious. Mealworms and litter mites may harbour the virus for 8 weeks, and affected birds excrete large amounts of virus for about 2 weeks post infection.

IBD has been an economically significant, widely distributed condition affecting flocks of chickens. The causal virus is transmitted laterally by direct and indirect contact between infected and susceptible flocks (Lasher and Shane, 1994), but not transmitted vertically by transovarian rout (Lukert and Saif, 2003). Indirect transmission of virus most probably occurs on fomites (feed, clothing and litter) or through air, whereas no evidence of egg transmission of the virus and no carrier state have been detected in chickens (Saif, 1998).

Infected chickens shed IBDV at one day after infection and can transmit the virus for at least 14 days but not exceeding 16 days (Lasher and Shane, 1994). The virus can remain viable for up to 60 days in poultry house litter, in addition, rodent, wild birds and insects including mites may be playing an important role in transmission of IBDV. Beside, the lesser meal worm was recognized as a carrier and the virus has been isolated from mosquitoes and evidence of infection in rats has been reported but there is no indication that either species is reservoir for virus (Saif, 1998).

2.6. Pathogenesis of IBD

Pathogenesis is the process through which the virus causing injury to the host leading to mortality, disease or immunosupression. The different pathogenesis of IBDV has different degree of pathogenicity. The natural infection is usually via the oral route accompanied by the gut associated lymphoid cells (Becht, 1980).

Following oral inoculation of IBDV in susceptible birds, the virus replicates primarily in the macrophage and lymphoid cells of the gut-associated lymphoid tissue during 4-6 hours post inoculation and lead to primary viremia. Then the virus travel to the liver via portal vein and localized in the bursa of fabricius as the target organ via blood stream where IBDV replication will occur at 13 hours post inoculation (Muller *et al.*, 1979). After massive replication in the follicle of the bursa of fabricius, the virus will be released into the blood as secondary viremia. This will be followed by virus replication and destruction to another organ such as cecal tonsil, spleen, bone marrow, gut associated lymphoid tissue and also replication in bursa of fabricus (Becht, 1980; Muller *et al.*, 1979). Consequently, clinical sign and mortality occur within 48 to 72 hours. The cause of death in clinical IBDV is mainly due to circulatory failure as a

result of severe hemorrhages. Severe dehydration owing to diarrhea and reduce water intake could also lead to circulatory failure and death.

Haemorrhage in IBDV infected chicken can be due to impairment of the clotting mechanism due to destruction of thrombocyte (Skeeles *et al*., 1980) and depletion of haemolytic components. In addition haemorrhages can also be the result of formation of immune complexes culminating to an arthus reaction.

Microscopic lesion particularly in the bursa of fabricius is similar to an Arthus reaction, which is caused by deposition of antigen antibody complement complexes which in turn induces production of chemotactic factors, haemorrhage and leukocytes infiltration **(**Skeeles *et al*., 1980). Two week old chicks showed less circulating complement than 8 weeks old chicks and did not show the arthus reaction. In addition, IBDV infected chickens showed prolonged clotting time, which has consequently induced hemorrhagic lesions in the birds (Skeeles *et al*., 1980).

The target organ of IBDV is the bursa of Fabricius at its maximum development, where B lymphocytes mature in avian species. Bursectomy can prevent illness in chicks infected with virulent virus. Actively dividing, surface immunoglobulin- M-bearing B cells are lyses by infection but cells of the macrophage lineage can be infected in a persistent and productive manner and play a crucial role in the dissemination of the virus. The severity of the disease is directly related to the number of susceptible cells present in the bursa of fabricius; therefore, the highest age susceptibility is between 3 and 6 weeks, when the bursa is at its maximum development. This age susceptibility is extended in the case of vvIBDV infection. Necropsies performed on birds that die during the acute phase (2–4 days following infection) reveal hypertrophied, hyperemic and odematous bursas. After oral infection or inhalation, the virus replicates primarily in the lymphocytes and macrophages of the gut-associated tissues (Muller *et al.*, 1979).

2.7. Clinical signs and gross pathological lesions of IBD

IBD is an acute, highly infectious and contagious disease of only young chickens (mostly 3-6 in age) and characterized clinically by self-vent picking, profuse watery yellowish white diarrhea, and tremor of the whole body and spicking curve of mortality and pathologically by inflammatory enlargement of BF followed by atrophy,

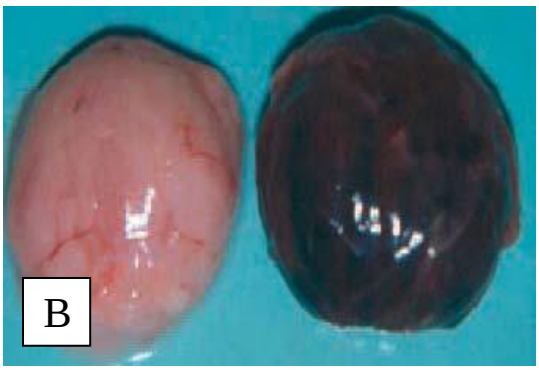
edematous to hemorrhagic BF and peripheral yellowish infarction. The incubation period of IBD range 2-4 days. The infection of susceptible broiler or layer pullet flocks is characterized by acute onset of depression. Chickens are averse to move and peck at their vents (Cosgrove, 1962). In acute outbreaks, the chick appear sleepy and have a reduce food intake. Terminally, birds may show sterna recumbence with coarse tremor and most of the birds have ruffled feathers, droopy appearance and may be seen pecking at the vent. Morbidity and mortality begins 3 days pi, peaks and recedes in a period of 5-7 days. White or watery diarrhea, sold vent feathers and vent pecking are seen. The feathers are ruffled, the birds have an unsteady gait and may become prostrate and trembling prior to death (Lukert and Saif, 2003).

The short duration of clinical signs and mortality pattern are considered to be of diagnostic significance in IBD (Lasher and Shane, 1994). Affected flocks showed depression for 5-7 days during which mortality rise rapidly for the first two days then declines sharply as clinical normality returns.

There is usually 100% morbidity, but the mortality varies depending on the virus strains. Clinical signs alone are not sufficient to make a diagnostic, but when combined with gross lesion; it is possible to arrive at preliminary diagnosis. Changes in lymphoid organs are typical of the disease. The bursa of fabricus which is the main target of the virus undergoes major changes beginning at 3days post infection. It increases in size reaching twice the normal size by 4 days followed by atrophy, and reaching one third of its original weight by 8 days (Saif, 1998).

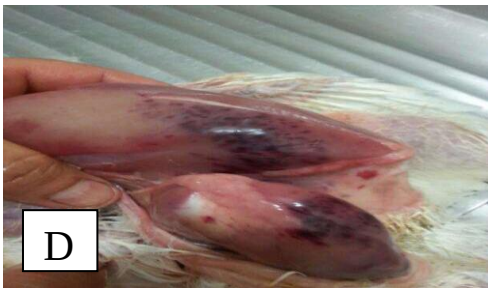
By day 2 or 3 post-infection, the bursa usually has a gelatinous yellowish transudate covering the serosal surface. Also longitudinal striations became prominent and the color changed from white to creamy. The transudate disappeared as the bursa returned to its normal size and the organs turned gray during the period of atrophy (Lukert and Saif, 2003). Infected birds are dehydrated; Petechial hemorrhages with darkened discoloration are present in the thigh and pectoral muscles and this hemorrhage (also reported from the mucosa at the juncture of the proventriculus-gizzard and on the serosal surface and bursa.

A). IBD Infected birds which are depressed
Source: Gumboro disease special (2014)

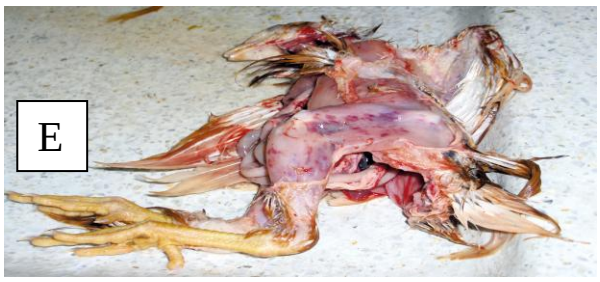


B). Severe haemorrhagic inflammation
Source: Http//vetmed.uodiyala.edu.iq

C). Enlargement of the spleen
With Gumboro
Source: Rabee (2014)



D). Petechial haemorrhages
Source: Rabee (2014)



E). Post mortem of a very virulent case of IBD with damage.
Source: Gumboro disease special, 2014

Figure 3: Different gross pathological signs of IBDV

The tissue distribution and severity of the lesions is dependent on the subtype and pathogenicity of the virus. Pathogenic changes in the spleen and thymus were less prominent than those of the bursa (Hitchner, 1970).The spleen might be slightly enlarged and usually had small gray uniformly dispersed on the surface. Lesions in these organs are noticed at the same time as the changes occurred in the bursa. These lesions resolved within 1 or 2 days of appearance.

The vvIBD infections are characterized by severe clinical signs, high mortality and a sharp death curve followed by rapid recovery. The vvIBD strains have the same clinical signs and incubation period of 4 days as classical viruses but the acute phase is exacerbated (Van den Berg, 2000). The vvIBD strains cause more sever lesions in the cecal tonsil, thymus, spleen and bone marrow. It has been shown that the pathogenicity of the field strains of IBD correlate with lesion production in non bursal lymphoid organs. The result also suggests the pathogenicity of IBDV may be associated with virus antigen distribution in non-bursal lymphoid organs. Chickens affected by the variant IBDV are characterized by sever bursal atrophy and immunosupression (Lukert and Saif, 1997) without showing inflammation induced symptoms associated with the infection of IBDV.

The destruction of immature B lymphocytes in the bursa creates an immunosuppression, which is more severe in younger birds. In addition to the impact on production and role in the development of secondary infections, this affects the immune response of the chicken to subsequent vaccinations which are essential in all types of intensive poultry production (Jonathan and John, 2013). Attenuated strains have been adapted to chick embryo fibroblast cells or other cell line. These strains do not cause disease in chickens, and therefore some of them are being used as live vaccines.

2.8. Morbidity and mortality rate

In fully susceptible flock, the clinical signs appear suddenly with high morbidity rate usually approaching 100%. Mortality rate ranges from 20-30% usually peaks (maximum) on the 3rd day post infection, recedes (gradually decrease) in a period of 5-7 days post infection and no deaths are observed after 8-9 days post infection (spiking death or mortality curve) (Wafaa, 2006).

The economic impact of IBD is difficult to assess due to the multi-factorial nature of the losses involved. In addition to direct losses related to specific mortality (which in turn depends on the dose and virulence of the strain, the age and breed of the animals and the presence or absence of passive immunity), indirect losses also occur, due to acquired immunodeficiency or potential interactions between IBDV and other viruses,

bacteria or parasites. Further losses may occur as a result of growth retardation or the rejection of carcasses showing signs of haemorrhages (Van den Berg, 2000).

2.9. Diagnosis of IBDV

2.9.1. Immunological tests of IBDV

The disease can be diagnosed by clinical signs and postmortem changes. However in practice, laboratory diagnosis of IBD depends on detection of specific antibodies to the virus, using immunological methods.

Immunoflurescent

It is dentification of the virus by direct immunoflurescent staining or direct examination by electron microscopy (McFerran *et al.,* 1980). A direct immunoflourescent smear of bursa of fabricius and direct electron microscopy examination are more sensitive method than virus isolation and direct electron microscopy (Allan *et al*., 1994).

Agar gel immunodiffusion test (AGID)

AGID is the most useful for detection of specific antibodies in serum or urinal antigen in bursal tissue and the test can be used to measure antibody levels (Cullen and Wyeth, 1975). AGIDT is economic to use and simple to perform and the result could be obtained within 28 hours. Agar gel immunodiffusion is the simplest, but least sensitive technique. Variability in results may be due to the investigator, as well as the nature of the viral strain used as an antigen (Weisman and Hitchner, 1978).

Virus neutralization test (VNT)

VNT is more laborious and expensive than AGID, but is more sensitive in detecting antibodies, so it may be useful for evaluating vaccines response or differentiate between the two serotypes (Isamil and Saif, 1996). Serum neutralization presents the disadvantages that specialized equipment and five days incubation are required. The technique is much more sensitive than AGID and correlates better with the level of protection of the subjects tested (Rooney and Freund, 1988).

Enzyme linked Immunosorbant assay (ELISA)

ELISA was firstly developed for detection of IBDV antibodies (Marquard *et al.,* 1980). The ELISA had been used for detection of IBDV antigens directly from infected tissues (Synder *et al.,* 1988). It was for serosurveyes of chicken flock (Synder *et at.,* 1986). The ELISA is the most rapid test and its results are easily entered into computer software. The ELISA is the most rapid and sensitive method, and presents the fewest variations due to the viral strain used as an antigen (Rooney and Freund, 1988).

2.9.2. Virological diagnosis

Embryonated Eggs

Hitchiner (1970) demonstrated that the chorioallantoic membrane (CAM) of 9-11 days old embryos was the most sensitive rout for isolation of IBDV which could subsequently be adapted to allantoic sac and yolk sac rout of inoculation. Hitchiner (1970) observed that the most mortality of embryos occurred between the 3rd and 5th day PI. The recent very virulent strains are difficult to cultivate in embryonated eggs (Bumstead *et al*., 1993).

Isolation of IBDV using cell culture

The old strains or classical strains of IBDV can be isolated in chicken embryo bursa (CEB) and chicken embryo kidney (CEK) (Lukert & Davis, 1974). The virus however didn't replicate in kidney cell until serial passage in CEB cells. The virus produces a cytopathic effect (CPE) in kidney cells in about 3-5 days (Lukert & Davis, 1974). IBDV grows in chicken embryo fibroblast and produces CPE characterized with an appearance of round retractile cells in about 3-5 days (Sivanadan *et al.,* 1986). IBDV isolation in cell culture is not a routine use as a diagnostic test because the virus is difficult to culture. Some field strains failed to grow on cell cultures (Bumstead *et al*., 1993). Wild–type IBDV strains particularly very virulent strain do not grow in tissue culture. Comparison of genome sequence of wild-type and tissue culture adapted IBDV strains pointed to several mutations that might be responsible for in vitro growth of IBDV in tissue culture (Islam, 2002).

2.9.3. Molecular detection of IBDV

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) (Lin *et al*., 1993; Wu *et al*., 1992). 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. Different areas of the genome will be amplified depending on the location from which the primers have been selected. The amplification of the middle third of the gene encoding the outer capsid protein VP2 (Eterradossi *et al*., 1998) or the partial amplification of the 5‟ extremity of the VP1 gene in IBDV segment B are examples of this technique ((Le Nouen *et al*., 2006).

The reverse transcriptase polymerase chain reaction restriction endonuclease (RT \ PCR – RE)

RT \ PCR – RE assay can be used to diagnose IBD in chickens and IBDV strains (Jackwood and Nilsen, 1997). Currently, RT-PCR is a molecular tool frequently applied for IBD diagnosis. RT-PCR in combination with restriction enzyme analysis allows the rapid identification of vvIBDV (Lin *et al.,* 1993; Zierenberg *et al.,* 2001). RT-PCR allows the detection of viral RNA in homogenates of infected organs or embryos, as well as in cell cultures, irrespective of the viability of the virus present. The choice of amplified genomic zones depends on the objective. When the only objective is to detect multiple strains of the virus, primers are selected in the highly preserved zones (Stram *et al*., 1994; Tham *et al*., 1995).

Conventional PCR

Conventional RT-PCR, amplifying the VP2 hypervariable region, in combination with DNA sequencing of the PCR product, can differentiate classic, variant, and vvIBDV

strains because variant and vvlBDV have characteristic nucleotide and amino acid substitutions (Peter, 2007).

Real time PCR

Real-time RT-PCR, targeting different regions of the IBDV genome, including VP1, VP2, and VP4 genes, in conjunction with melting-curve analysis is being investigated as a promising tool for molecular diagnosis of IBDV infection. These methods potentially allow for more rapid, sensitive, and specific detection and differentiation of IBDV classic, very virulent, and variant subtypes (Peter, 2007).

2.10. Economic importance of IBD

Immunosuppressive viral diseases are a great concern for the poultry industry for several years. Indeed the reemergence of IBDV in variant or highly virulent forms have been the cause of significant economic losses (Rautenschlein *et al.,* 2001). IBDV has an economic impact not only due to the direct losses it provokes, but also to the indirect losses as a consequence to immunosuppression or due to interaction it might have with other factors.

The direct losses are due to specific mortality, depending on the virulence and the dose of the inoculums, the age and the breed of the bird, and the presence or absence of passive immunity. Moreover, IBDV is also responsible for indirect losses due to acquired immunodifficiency, impaired growth and condemnation of carcasses (Shane *et al*., 1994). Furthermore the increase use of antibiotics and chemicals to fight against opportunistic (secondary) infections is a major concern of human health, if we consider the risks linked to the presence or residues in meat products, the release of residues into environment and increased antibiotic resistance (Marian, 2001). It can be stated that there is a significant variation in body weight in Gumboro affected broilers due to the existing and imposed vaccination program under farm condition and thus the imposed vaccination program should be recommended for use in farm condition to attain better body weight (Paul *et al*., 2004). The economic importance of this disease is fundamentally based on these two aspects: on one hand, the high mortality rate caused by some IBDV strains in 3-week old chickens, and even older, and on the other hand, the second clinical manifestation of the disease consisting of a prolonged

immunosuppression of the birds infected at early ages (Naqi *et al*., 2001). The main sequelae associated to said immunosuppression are: dermatitis gangrenosa, anemia-hepatitis syndrome with inclusion bodies, E. coli infections and failures in the efficacy of other vaccinations, such as the vaccinations against the Newcastle disease and infectious bronchitis (Naqi *et al*., 2001).

2.11. Treatment of IBD

There is no specific treatment. Antibiotics, sulfonamides, and nitrofurans have little or no effect. Vitamin-electrolyte therapy is helpful. High levels of tetracyclines are contraindicated because they tie up calcium, thereby producing rickets. Surviving chicks remain unthrifty and more susceptible to secondary infections because of immunosuppression. Another similar idea say that no known chemotherapy treatments of IBDV, but antibiotic are given to avoid or to prevent secondary infections which may occur due to immunosuppression which become a result of IBDV infection. Electrolytes and minerals are given as supportive treatment to keep the acid-base balance in equilibrium (Pettit *et al*., 1983).

2.12. Prevention and control measures for IBD

Protection and Prevention are the two objectives assigned to a Gumboro vaccination program aiming for a real Control of the disease.

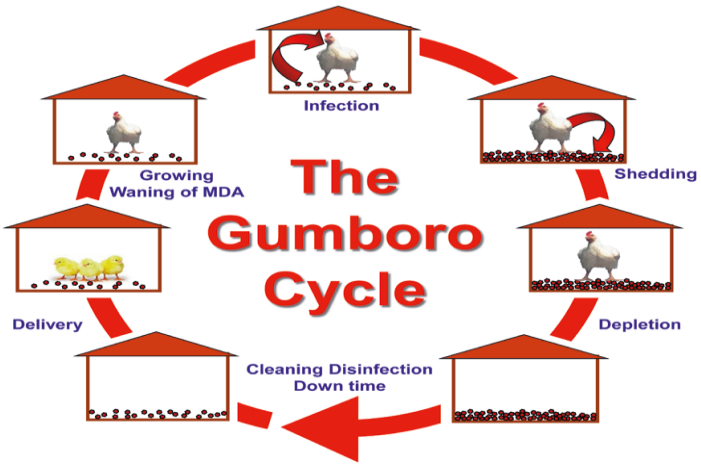


Figure 4: Gumboro transmission cycle

Source: Gumboro disease special (2014)

Only a sound vaccination and Gumboro prevention scheme can break the disease cycle and stop the buildup of virus pressure (Yannick *et al.*, 2014).

The very high resistance of IBDV to physical and chemical agents (Benton *et al.,* 1967) accounts for persistence of the virus in the outside environment, particularly on contaminated farms, despite disinfection. Eradication in the affected countries therefore seems unrealistic. Prevention of IBD necessitates hygiene measures and medical prophylaxis. No vaccine can solve the problem if major sanitary precautions are not taken. These precautions include 'all-in or all-out' farming methods, cleaning and disinfection of premises and observance of a 'down time' (a period of rest between depopulation and restocking) (Maris, 1986).

An effective IBD prevention and control program must involve an effective breeder vaccination program, an effective biosecurity program, and an effective broiler vaccination program (Gary and Richard, 2003).

Immunization of breeders is an important part of the IBD control program. Antibodies produced by the hen are passed through the egg to the broiler chick. IBD virus infections within the first week do not entail any noticeable clinical signs or mortality. However, they cause severe immunosuppression if chicks are not protected by passive immunity. The immunosuppressive effect of sub-clinical IBD decreases in intensity or vanishes as birds grow older. Therefore, in order to prevent losses arising from early challenges, it is a common practice in the poultry industry to hyperimmunise breeders (through a combination of live and inactivated vaccines) with a view to maximizing, in terms of quantity and quality, the passive immunity transferred to their progeny.

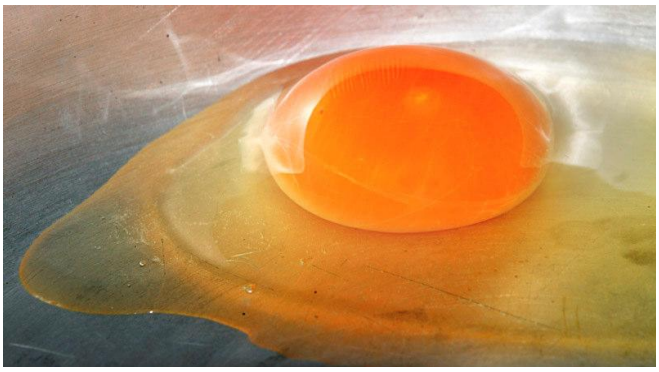


Figure 5: Egg yolk

Source: Passive immunity: part 2 (2008)

Most IBD immunoglobulins (Ig) are transferred from the egg yolk to the offspring via embryonic circulation before hatching. However, part of it is transferred after hatching through the resorption of the yolk of the egg. As a consequence, the level of maternally derived antibodies (MDAs) remains approximately stable in the first four days after hatching, regardless of their natural metabolisation. Several publications have described the percentage of IBD antibodies transferred from dams to their progeny (60-80%). However, it is not possible to assess accurately the quantity of MDAs transferred from hens to chicks without resorting to a test at day-old.

Serological monitoring via ELISA or VN tests is usually carried out in order to determine the level of maternal antibodies in young birds and to determine whether flocks are adequately protected. The duration of the protection provided by MDAs may vary depending on the level of MDAs transferred and the breed of the chicken. Broadly speaking, the half-life of MDAs in broilers varies between 3 to 3.5 days. However, regarding long living birds like breeders and layers MDAs' half-life lasts 4.5 days and 5.5 days respectively (Roberto, 2008).

The development and enforcement of a comprehensive biosecurity program is the most important factor in limiting losses due to IBD. Phenol and formaldehyde compounds have been shown to be effective for disinfection of contaminated premises (Gary and Richard, 2003).

Efforts at biosecurity (cleaning, disinfecting, traffic control) must be continually practices, as improvement is gradual and often only seen after 3 or 4 flocks. The important factors to consider in the control of IBD are the prevention of broiler losses through an effective IBD breeder vaccination program (maternal titers) and decreasing exposure through a comprehensive biosecurity program. Relying on broiler vaccination has met with only limited success when not coordinated with effective breeder vaccination and biosecurity programs. Biosecurity requires the adoption of a set of attitudes and behaviors by people, to reduce risk in all activities involving poultry production and marketing. Basic management practices such as limited controlled site access, separate footwear and equipment for each site/house, and footbaths at the entrance to sites/houses all minimize the risk of introducing the virus. Due to the resistance of IBDV an infection on a site easily leads to an endemic situation. Hygienic

measures are aimed at minimizing infection pressure. Priority is to remove contaminated litter from site as soon as possible.A structured approach is required to prevent back tracking of the virus. Dry clean is the removal and disposal of all organic material from the site (In case of earthen floors this should include removing the top 4-5 cm of soil). Wet clean is the cleaning poultry house using water at high pressure (35 – 55 Bar) to ensure removal of all organic material. It is advisable to add detergents to assist cleaning process. Disinfection is the application of suitable disinfectant to reduce infectivity of any remaining virus particles. Applying disinfectants at the correct concentration with a suitable contact time is critical. Generally products containing formaldehyde, iodophores, chlorine-releasing agents or quaternary ammonium compounds are suitable. The downtime between successive broiler flocks must be maximized (a minimum of 10 days is recommended between successive flocks). Control of IBDV on multi-age sites is extremely challenging and requires strict control of the movement of personnel and equipment between houses. Due to high resistance of IBDV to environmental exposure hygienic measures alone are ineffective and vaccination is thus essential (Lukert and Saif, 1997).

A third factor to consider in the IBD prevention and control program is vaccination of the broilers to prevent clinical IBD. Three categories of vaccines, based on their pathogenicity, have been described: 1) mild, 2) intermediate, and 3) virulent. 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. The timing of broiler vaccination depends on the level of maternal antibody present in the chicks. High levels of maternal antibody at the time of vaccination will neutralize the vaccine virus. Thus, only a limited active immune response results and chickens will be susceptible to disease as maternal titers decrease. If low levels of maternal IBD titers are present in the chicks, vaccination may not be effective on farms contaminated with virulent field virus. Approximately 10 to 12 days are required after vaccination for chickens to develop minimal protective titers. During this "lag time," chickens are susceptible to IBD. In addition, virulent IBD viruses are able to break through higher maternal titers than milder vaccine viruses (Gary and Richard, 2003).

Live vaccine is generally derived from the serial passage in embrionated eggs or tissue culture. The degree of attenuation of the vaccines strain can be classified as mild, intermediate, and hot, depending on the its ability to cause varying degree of histological lesions. Although serotype I vaccine strains cause no mortality, it is still causing different degree of bursal lesions that range from mild to moderate or even severe (Van den Berg, 2000).

2.12.1. Mild vaccine

This vaccine cannot cause immuno-suppression as it not causes bursal damage, but gives weak antibody response (Van den Berg, 2000).

2.12.2. Intermediate vaccine

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 immuno-suppression (slight bursal damage), and gives good immune response (Van den Berg, 2000).

2.12.3. Intermediate plus (hot vaccine)

It gives high level of antibodies and causes immuno-suppression due to bursal damage. It is used in case of improper immunized flocks and in epidemic areas and high maternal immunity (Van den Berg, 2000). Inactivated oil-adjuvant vaccine: for breeders and layers only at 16-18 weeks old. In endemic areas it is necessary to monitor the titer of maternal antibodies and in case of presence of high level of maternal antibodies use intermediate plus live vaccine as other vaccines can be neutralized by maternal antibodies. The living vaccines used in chickens only at age 10-14 days of age using eye drop or drinking water method and revaccinate at 3-4 weeks of age (Wafaa, 2006).

2.13. The status of IBD in Ethiopia

Ethiopian poultry production has a long traditional practice which is mainly used as an immediate cash income for the rural communities although careless production system is practiced. Especially, women are more involved in keeping backyard chickens for

egg collection and selling adult chickens so that this extensive breeding practice has a significant role in the livelihood of the farmers although managed poorly (Zeleke *et al.*, 2005).

The majority of Ethiopian poultry is indigenous birds kept in small backyard flocks, belonging to rural smallholders, and is particularly important to landless in society, and also to women. Disease is reported by smallholders to be a major constraint to production, but most outbreaks are attributed to Newcastle disease without any investigation of the pathogens responsible. IBDV was first reported in Ethiopia in 2002 in a commercial flock, but the very virulent (vvIBDV) strain type has since been identified in all production systems. This strain is associated with high mortality in birds between 3 and 6 weeks of age (Bettridge *et al., 2013).*

IBD 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 (Zeleke *et al.*, 2002). IBD has become a problem during the past few years and it is assumed that it was introduced to the country through the importation of infected poultry, poultry products or poultry equipment. IBD is a disease of economic importance, especially to the government operated breeding and rearing centers and commercial poultry sector. The breeding and rearing centers and the commercial farms in Ethiopia vaccinate against IBD (Solomon, 2007).

As mentioned in the above, in Ethiopia, there was no recorded occurrence of IBD case up to 2002 (OIE, 2003) and it was reported in Ethiopia for the first time in 2002 – before that, the country was thought to be free of the disease. The disease was first reported in a farm at Debre-Zeit in 2002 (Zeleke *et al*., 2002) with high mortality of 49.89% in the affected 20-25 day-old broiler and layer chicken (Zeleke *et al*., 2005). A report for the same area in 2004-2005 identified lesions suggestive of IBD on one farm (Chanie *et al*., 2009). The first study on the incidence of IBD in Ethiopian village poultry was in two areas 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; Jenbrie *et al*., 2012; Kassa and Molla, 2012; Zeryehun and Fekadu, 2012), with the proportion of positive samples ranging from 75 to 96%. However, at least two of these studies have purposely selected areas where there is regular distribution of chicks from the government multiplication centers to village producers. Two papers on the molecular characterization of virus isolates have identified very virulent IBD strains from commercial and indigenous birds (Negash *et al*., 2012).

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 analyze (Bewket *et al.*, 2012).

Table 1: Reported prevalence of IBD in Ethiopia

Study area	Prevalence	Authors
Central Ethiopia	82.2%	Zeryehun and Fekadu, 2012
Selected sites of Ethiopia	83.1%	Jenberie *et al*., 2012
Gondar and west Gojjam	73.5%	Kassa and Molla, 2012
Bahir Dar	29.4%	Mazengia *et al*., 2010
Farta	21.7%	Mazengia *et al*., 2010
Andassa poultry farm	100%	Woldemariam and Wossene, 2007
Debre Zeit	93.3%	Zeleke *et al.*, 2005

Source: Teshager (2015)

These reports indicate that the disease is present in many parts of the country and is disseminating at a faster rate in recent years. 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, there is not any endeavor towards the implementation of cost effective control strategies of IBD. But there is recommendation from the Federal Ministry of Agriculture and Rural Development that regional states should implement vaccination against IBD to combat the loss of poultry at this stage (Mazengia *et al.*, 2010).

3. MATERIALS AND METHODS

3.1. Study area

The study was carried out in National Veterinary Institute at Bishoftu, Ethiopia from October, 2016 to May, 2017. The geographyical location of Bishoftu town is at $8^0$44‘40‘‘N latitude and $38^0$59‘9‘‘E longitude and covers about 5,444 hectares of area. It is found in Oromia Region, East Shewa zone of Ada‘a *Woreda.* Bishoftu town is found at about 47kms to the southeast of Addis Ababa and situated between Dukem and Mojo towns along the old Addis Ababa-Djibouti railway **(**Gezahegn, 2009).

Figure 6: Map of Bishoftu town

Source: http://www.worldatlas.com/af/et/or/where-is-bishoftu.html

3.2. Study Design

Experimental study design was applied for isolation, adaptation, attenuation of IBDV in vero cell. Unvaccinated and IBD free chickens were selected based on random sampling techniques for IBDV pathogenecity tests.

3.3. Study animals

Thirty five chickens free of antibody against IBDV in various experimental groups were maintained through the duration of the experiments in separate isolation units under suitable condition for pathogenecity tests.

All of the tests on chickens were carried out in way of humane according to ethial clearance provided by Addis Ababa University College of Veterinary Medicine and Agriculture guidelines.

3.4. Study Methods

3.4.1. Isolation, adaptation and attenuation of IBDV on cell culture

An appropriate media containing essencial supplements with suitable conditions was used to propagate, adapt and serial passaging of the IBDV on Vero cell line.

3.4.2. Cells and media

African green monkey kidney (Vero) was used to grow the IBDV. The frozen cells were resuscitated to prepare monolayer, which further sub-cultured to confluent monolayer were grown in Glasgow Minimum Essential Medium (GMEM) supplemented with TPB and 2-10% calf serum. The cells were grown at 37°C in a humidified incubator set at 5% CO_2. Cells were subcultured after they formed a monolayer on the flask. The cells were detached by treating them with trypsin-EDTA after the old growth medium covering the confluent monolayer removed and washed with sterilized PBS. After pippeting the cell to be detached from the flask and to make the single cells, the fresh medium was added to the cells and dispensed into new flasks.

3.4.3. Isolation and propagation of IBDV on cell culture

Isolation and propagation of IBDV in cell culture was successfully conducted. Two IBD virus strains were used to grow on the vero cell line. The first one was local IBDV isolated from IBD suspected outbreak samples collected from a poultry farm located in Debre Zeit. The processed and homogenized bursa samples were frozen and thawed three times before using as inoculum. The second IBDV strain used under this study was LC-75 vaccine strain. The vaccine was reconstituted with 3ml of base medium before inoculation in to the vero cell.

Both the reconstitute vaccinal strain and local isolate were used to infect the healthy, semi confluent monolayers of vero cells in 25 cm^2 flasks. The growth medium of the flask was removed and the monolayers were washed twice with prewarmed sterilized PBS. For virus isolation, 0.5ml of IBDV inoculum was dispensed over each monolayer, the inoculum was spread uniformly over each monolayer and flasks were incubated at 37 c^o for one hour to the virus to adsorb on the surface of vero cell. 10ml of sterilized prewarmed mainainance medium was added in each flask and incubated them at 37c^o in 5% co_2. The monolayers were examined twice daily under inverted microscope for cytopathic effects (CPEs). Freezing and thawing of the cultures and inoculating the

resulting lysates were onto fresh cultures. This procedure was repeated at least three times consecutively, as described by Peilin *et al.* (1997).

3.4.4. Harvesting of the virus

Infected cells exhibitting advanced CPE were harvested to deep freezer. The virus infected cells and culture medium were frozen and thawed three times. The fluid used as IBDV inoculums inoculated in subsequent cell cultures for further passages.

3.4.5. Adaptation and attenuation of IBD virus on vero cell

During the isolation of viruses, there may emerge variants capable of multiplying more efficiently in the host cells used for this purpose than the original wild type virus. This phenomenon is known as adaptation. Often such variants damage the original host less severely than the wild type virus and are, therefore, said to be less virulent. Viruses are often purposely adapted to alter growth and virulence characteristics. An example is provided by the attenuated vaccine virus strains, which are obtained by repeated passaging of virus virulent for one host in some other host, until virus strains with decreased virulence for the original host have been selected. Following adaptation, the infectivity of virus to the adapted cells is increased but the virulence is decreased (Ahasan *et al*., 2002).

Adaptation of the local strain virus and LC-75 vaccine strain were conducted in Vero cell line through serial passaging.The passage (P1) virus was inoculated again to fresh, healthy and semi confluent monolayers of vero cells using the same technique for CPEs. The virus was harvested by three freeze- thaw cycles, clarified and labled as passage 2 (P2).

In this way IBDV was adapted to vero cells when the CPEs were clear and consistent. The adapted virus was serially passage until it became attenuated (non pathogenic). Under this study, local IBDV isolate and the vaccinal strain LC-75 were adapted on vero cell up to 10 and 7 successive passages respectively as evidenced by stabilization of cytopathic effects.

3.4.6. Titration of the IBDV

Ten-fold serial dilution of IBDV was prepared in basal medium from 10^{-1} to 10^{-10}.

The titer of both the local isolate and the vaccinal strain virus in each serial passage was measured. Each virus passage in the Vero cell were frozen and thawed for three times. 0.5 ml of virus containing fluid (supernatant) was serially passed in five tubes containing 4.5 ml of base medium (GMEM) for each passage separately. 100 µl Vero cell was dispensed into all wells of the first five rows of the microplate leaving 11^{th} column. 100µl of GMEM was added in to the first five wells of twelveth column for control. 100µl serially diluted virus was added to all wells of five rows upto 10^{th} column starting from low concentration to higher one. This was the same procedure for all passages to deterimine the titer of each passage. The plates were incubated at $37c^{o}$ incubator for five days and examined twice daily for CPEs. The titer for each virus passages was determined according to the following formula (Spearman, 1908).

$$\text{Log10} = ((xo - (d/2) + d\,(\text{Ɛri/ni}))$$

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

3.4.7. Molecular identification and characterization of IBDV

The IBDV was identified on Vero cells through its CPEs. The characteristic changes in infected monolayers were carefully examined in each passage. The time for the appearance and intensity of CPEs were also recorded in each passage.

To confirm that the adapted virus on Vero cells was IBDV, the supernatant of passages 1, 3 and 5 from both local isolate and vaccinal strain adapted in vero cells separately were chosen as the sample for RT-PCR. The virus infected cells from the cell culture flasks were repeatedly fozen and thawed three times. After clarification, the supernatant

fluid was collected for RNA extraction. RNA extraction was done by using Qigein RNeasy® mini Kit procedure. RNA was eluted by using RNase free water and stored -20°c. The cDNA was used superscript™ III first- strand synthisis system (Invitrogen, USA), cat.no 18080-051 for RT-PCR. According the Kit procedure, two step 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°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™ (40U/ µl) (1 µl) and Superscript ™ 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°c for 50 minutes. Then the reaction was terminated at 85°c for 5 minutes and chilled on ice and briefly centrifuged. 1 µl RNase H was added and incubated at 37°c for 20 minutes. Finally the prepared cDNA was used immediately for PCR based on the SOP manual used by National Veterinary Institute.

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'TGTAAAACGACGGCCAGTGCATGCGGTATGTGACGCTTGG TCAC-3'and

Reverse primer: 5'CAGGAAACAGCTATGACCGAATTCGATCCTGTTGCCACTC TTTC-3' (OIE, 2016).

3.4.8. 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°c for 30sec ,extension at 72°c for 30sec and final extension at 72ºC for 7 minutes for 1 cycleand hold 4 ºC until machine off (NVI SOP, 2012).

3.4.9. Agarose gel electrophoresis of PCR product

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), 6X loading buffer, Gel red and transilluminator (an ultraviolet lightbox).

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. 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 result of gel picture captured by the camera was saved and printed out for documentation.

3.4.10. Sequencing and Sequence analysis

The positive PCR products were purified using the Wizard SV Gel and PCR clean-up system kit (Promega, Germany). The concentration of the purified PCR product was quantified using the NanoDrop 2000c spectrometer (Thermo Scientific, USA). The concentration of each purified product was adjusted and prepared according to the instruction recommended by the sequencing providing company. 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 Meq gene of the outbreak isolates and the reference strain. For comparative studies, blast was used to collect additional Marek's Disease virus Meq gene sequences from GenBank for inclusion in the data set. For construction of phylogenetic tree, multiple sequence alignments were performed to align the sequences as codons using the Muscle algorithm in MEGA6 (Tamura *et al*., 2013). The Neighbor-Joining algorithm was used with the maximum composite likelihood nucleotide substitution model with the pairwise deletion option was used. For construction of phylogenetic tree, 1000 bootstrap replicate was used.

The primers used both for the PCR and sequencing activities in this study were synthesized by VBC Biotech, Austria and purified by reverse phase high performance liquid chromatography.

3.4.11. Sample collection and Serological test

Day old chickens were housed in isolation units at the experimental house of National Veterinary Institute and reared under suitable conditions. Clean drinking water and commercial feed were provided to the chicks. After 21 days, blood sample was collected from the chickens to assess the immunity against IBD prior to inoculation by the attenuated virus to them. The blood samples were collected from different groups of chickens via wing vein using 5 ml sterile syringe and kept in slanting position to collect serum. The serum samples were stored in -20 c^{o} until used for serological test.

According to the instruction of the kit manufacturer IDvet, the indirect ELISA diagnostic kit was used to detect the anti bodies of the chicken directed against IBDV. Briefly, five hundred fold (1:500) dilutions were used. About 245µl of dilution buffer 14 was added to each wells of microplate. Then 5µl of negative control was added to wells A1 and B1 and 5µl of positive control was added to wells C1 and D1.After that 5 µl of each samples to be tested were added in the remaining wells to make prediluted samples. Then 90 µl of dilution buffer 14 and 10 µl of the pre diluted samples were dispersed in to the appropriate 96-well plate coated with IBDV viral antigen and the plate was covered and incubated in room temperature for 30 minutes.The plate was washed 3 times with 300 µl of the wash solution 1x at the end of incubation period followed by addition of an anti chicken horseradish peroxidase conjugate into each well. The plate was allowed to incubate at room temperature for 30 minutes and washed 3 times again before adding 10µl of the substrate solution to each test well

which was then incubated for 15 minutes at dark place of room temperature. Finally, 100µl of stop solution was added in to each well to stop the reaction and the absorbance were read at 450 nm.

3.4.12. Pathogenicity test

After serological test was conducted, pathogenicity test was carried out in the chickens. Thirty five chickens were divided into two treatment groups; A and B each containing 16 chickens for both local isolate and LC-75 IBD vaccine strains respectively. The remaining 3 chickens were kept as control. Both the first and the second groups were further divided in to four sub groups each containing four chickens. Each of chickens in four sub groups of the first group were inoculated by original bursa suspension, Vero cell adapted and attenuated passages local isolate (P3, P6 and P10 with the titer of $10^{5.3}$, $10^{5.5}$ and $10^{5.7}$ /ml).The second sub group was infected with LC-75 vaccine and its Vero cell adapted passages (P3, P5 and P7 with the titer of $10^{6.2}$, $10^{6.4}$ and $10^{6.4}$/ml respectively). All the chickens were inoculated with 2µl of the virus orally. Each treatment group and the control group were carefully kept in separate room for easy observation of changes. The virulence test was evaluated by observation of the clinical sign of chickens infected with each virus strains.

3.5. Data management and analysis

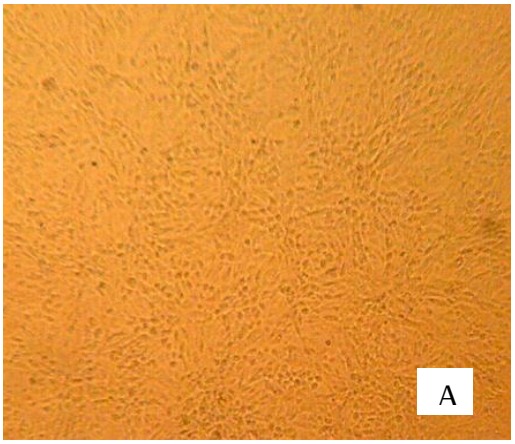
Serological data were encoded in MS-excel sheet and analyzed by using SPSS version 20 including decripive statistics (Percentage) and multiple sequence alignments were performed to align the sequences as codons using the Muscle algorithm in MEGA6.

4. RESULTS

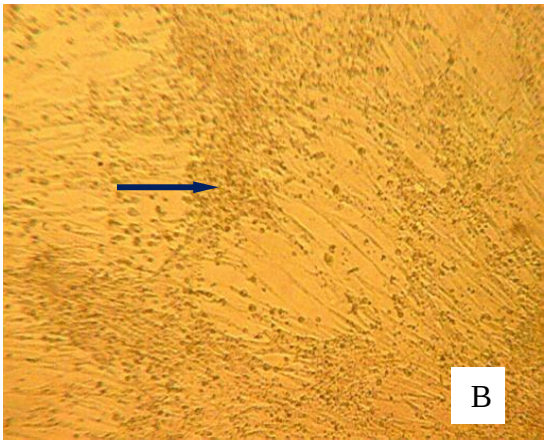
4.1. Isolation and adaptation of IBDV on cell culture

The result of present study showed that both local isolate and the live vaccine of IBDV were completely adapted and attenuated in Vero cell culture. The virus titer was low in Vero in early passages but increased with the passages.

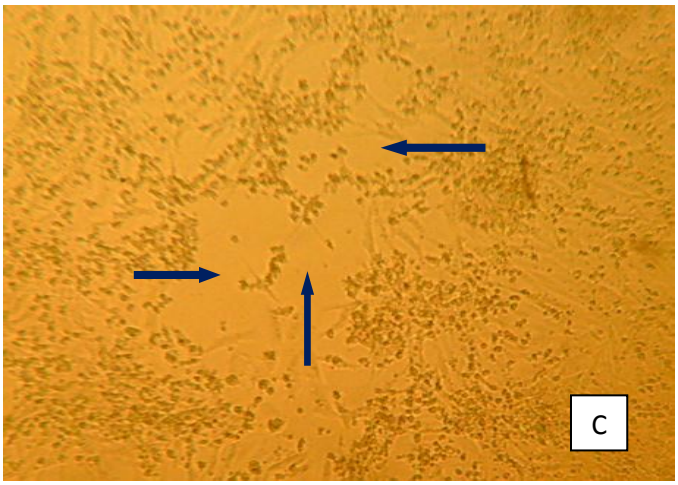
Vero cell monolayers inoculated with the bursal homogenates and reconstitute vaccine at separately using similar conditions resulted in isolation of the virus. Local isolate and LC-75 vaccine strains were adapted to cell culture for several successive passages. Typical aggregation, rounding and granulation of Vero cells were noticed in successive passages from 72 hours upto 144 hours post-infection (Fig 7. B). At 96 hour, rounding and clumping of large number of cells with intense cytoplasmic granulation and detachment of cells with few cells floating in media as well as attached to the monolayer were observed. This virus was serially passaged 10 times in vero cell. Along with the local isolate, IBD vaccine virus (designated as LC-75) was also propagated in Vero cell culture about 7 successive passages. Using live LC-75 IBD Vaccine isolate as inoculum, rounding and clumping of cells, detachment and floating of very few cells were observed at 48 hours PI during first passage itself. Excessive cytoplasmic granulation and detachment of considerable number of cells forming empty spaces were observed at 72 hours PI. More than 80 percent cells were detached from the monolayer and extensive cellular degeneration was observed at 96 hours PI (Fig 7.C). As the number of passage increases in both virus strains, more clear and defined CPE was detected in all passages except passage one in the local isolate that was weak CPE. Negative Control monolayers did not show any changes throughout the observation period except slight lowering of pH of the medium (Fig. 7.A).



A) Uninfected control monolayer



B) CPE by local isolates IBDV on vero Cell at Passage 4 (the arrow shows CPE)



C) CPE of LC-75 vaccine strain on vero cell at Passage 7 at day 6 post infection.The arrows show detachment of cells from the flask with the eventual destruction of the entire monolayer.

Figure 7: Vero cell before and after infection with IBDV

4.2. RT-PCR for LC-75 IBD vaccine and local isolate

Cell culture supernatants and infected monolayers from each passage level of both virus strains were used for extracting viral RNA for RT-PCR to confirm the presence of IBDV in cell culture. Viral RNA samples extracted from cell culture supernatant and infected monolayer of locally isolated (3 samples) and that of IBD vaccine virus (3 samples) at similar passages levels (1, 3 and 5 passages) from each were done and all resulted in generation of an amplicon at the correct expected size of the VP2 gene

(645bp) as ascertained by agarosegel electrophoresis (Fig. 8). On electrophoresis, RT-PCR product of both the local isolate and LC-75 vaccine strain virus revealed similar migration pattern and produced three bands each.The positive control was amplified as similar migration pattern as positive samples. But there was no amplification in lane 8 that contained RNase free water and lane 9 that contained negative controls.

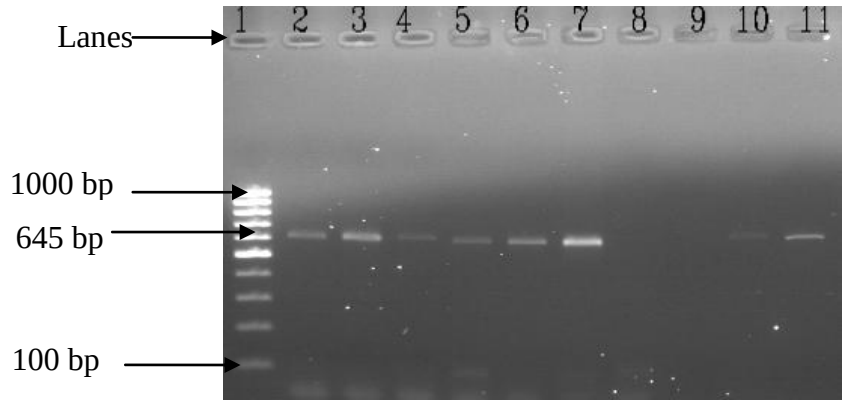


Figure 8**:** Agarose gel electorophpresis (1.5%) of 645-bp fragment of the VP2 gene.

1= 100-1000 bp DNA ladder marker; Lanes 2 to 4 = Local isolate IBDV strain adapted on Vero cell (positive; band at 645 bp); Lanes 5 to 7 = LC-75 IBD vaccine strain adapted on Vero cell (positive; band at 645 bp); Lane 8= Extraction control (RNase free water); and Lane 9=negative control; Lanes 10 and 11 positive controls (Known IBD vaccine, band at 645 bp).

4.3. Nucleotide sequencing for LC-75 vaccine virus on CFC and Vero Cell

A 573 bp fragment of the amplified hypervariable region of VP2 gene of the IBD vaccine virus passaged in vero cells five times was subjected to sequencing and sequence alignment with IBD vaccine prepared using chicken fibroblst cells (CFC). There were only four bases differences between IBD vaccine viruses prepared from CFC and Vero cell adapted virus of this vaccine in NVI with 99.3% identity. But there were nucleotides substitution of Vero cell adapted IBD vaccine virus strain at positions 116 and 286 A with G, at position 346 A with T and at position 462 G with C compared to IBD vaccine prepared using CFC as shown in annex 7. There was no

insertion and deletion of nucleotide (at the 5th passage) on vero cells when compared with the sequences of vaccinal strain prepared using CFC.

The local isolate currently adapted on vero cell was not sequenced and characterized in this study because it was previously studied by other researchers (Shiferaw *et al., 2014)*. However, two IBDV local isolates currently circulating in the country were sequenced and compared with previously characterized isolates.

4.4. Amino acid alignment of VP2 for LC-75 vaccine virus on CFC and Vero Cell

About 191 amino acid residues were used for sequence analysis of the deduced amino acid sequences of vero cell adapted IBD virus (at passage five) correspond to the amino acid of the IBD vaccine virus produced using CFC. Substitution mutations were observed at 3 amino acid residues (Aspartic acid replaced by Glycine at 39th, Threonine replaced by Alanine at 96th and Methionine replaced by Leucine at 116th) amino acid sequence. Asilent point mutation was detected in IBDV adapted on Vero cell at nucleotide position 462 (G → C). The vero cell adapted LC-75 virus showed about 1.6 % variation in amino acid sequence compared to the LC -75 IBDvaccine virus produced on CFC in NVI.

Even though the LC-75 IBD Vaccine was already attenuated, it was important to be sure there was no reversion of its virulence during serial passaging on vero cell line through mutation. In the current study, amino acid 96th was found a novel variation in conserved regions which is responsible for virulence. As explained by Fenaux *et al.* (2004), the notion that just a few sequence changes at specific sites may lead to attenuation has been proven in other viruses. This study offers one novel site for virus attenuation and strengthens the possibility that more than one site is involved in IBDV attenuation.

4.5. Phylogenetic tree for Ethiopian IBD Virus isolates

The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option was compute using MEGA6. The genetic relationship between the IBDV isolates of the current Ethiopian isolates with the previously characterized Ethiopian isolates and other reference isolates retrieved from the GenBank were included in the analysis. The analysis involved data of 26 VP2

coding sequences (nucleotides). The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The current sequenced isolates clustered together with the previously characterized Ethiopian vvIBDV and are indicated in color circle.

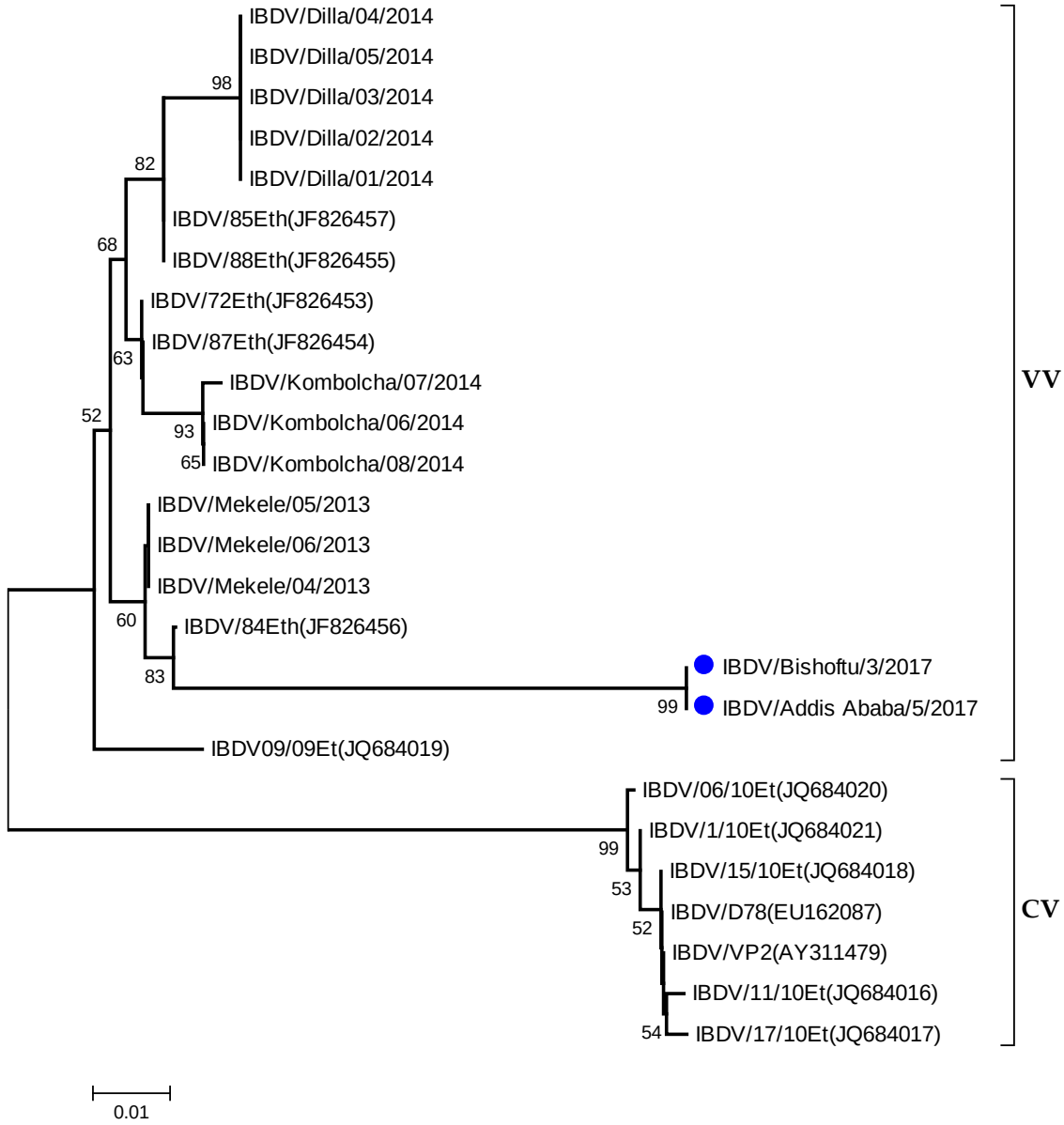


Figure 9: Phylogenetic analysis of the VP2 hypervariable coding sequence of IBDV isolates.

VV-very virulent IBDV, CV-classical virulent IBDV

Phylogenetically, Ethiopian IBDVs were represented two genetic lineages: very virulent (vv) IBDVs or variants of the classical attenuated vaccine strain (D78). From a

total of 27 IBDV isolates presented in figure 9, 19 very virulent and 5 classical variant isolates were Ethiopian IBDV isolates sequenced in the last years (Shiferaw *et al., 2014)*. The remaining two isolates were represented by classical attenuation IBDV (USA.D78, EU162087) and (Egypt, AY311479) which were phylogenetically similar with Ethiopian classical isolates. The IBDV strains indicated by blue color circle in the figure 9 are very virulent strains currently circulating in commercial and breeding poultry farms of Ethiopia and their phylogenetic tree was constructed and incorporated in this study.

4.6. Serological test results

From the total purchased experimental chickens, about 95% of chickens were antibody negative against IBDV during ELISA test prior to inoculation of the virus. Only anti body negative chickens were choiced to serological tests. So, the attenuated virus inoculated to chickens could not neutralized by the maternaly derived antibody of the chickens.

4.7. Pathogenecity test results

Based on the pathogenecity test, both the vaccine and local IBDVs were observed to have lost their pathogenecity after serial passage in Vero cells as evident by no mortality or sign of disease was detected on chickens.

This study was tried to evaluate the pathogenecity of the vaccine and local IBD virus strains which were propagated, adapted and attenuated in Vero cell line with the view of understanding the probable effect of the host sysem on the pathogenecity of the virus at different passages.

5. DISCUSSION

Infectious bursal disease (IBD) also called Gumboro disease in chicken is caused by infectious bursal disease virus that was first reported in Ethiopia in 2002 (Zeleke *et al*., 2002). The causative agent of this disease is a dsRNA virus with a bisegmented genome enclosed within an icosahedral, non-enveloped capsid of 55-65 nm. Vaccination is the only option to control IBDV since it can resist physical and chemical controlling methods.

The use of cell culture in growing avian viruses has become an increasingly economical, less laborious, and continuous and efficient tool with an advantage of measuring various effects out side the host animal (Kibenge *et al*., 1988 and Ahasan *et al., 2002).* As the result of the time consuming nature, the over burden cost implication with the use of specific pathogen free (SPF) eggs or for SPF chick, which are the traditional mothod for propagating IBDV, there had been a call for the use of cell culture.

The present study was attempted to propagate, adapt and attenuate both the local and vaccinal IBDV strains to cell culture of mammalian origion (Vero) with the hope of achieving a positive step towards a cheaper means of IBD vaccine production.

Complete CPE of the LC-75 IBD vaccine virus was observed during the first passage with the higher titer than the locally isolated virus in all passages.The CPE of local field isolate IBDV was started in the first passage on Vero cell but the infectivity was low at this stage. During second passage, CPE was rapid and consistent. After 144 hours of infection, rounding of infected cells and aggregation of rounding cells were observed.

Ahmed (1999) observed CPE of Newcastle disease virus on vero cell line following 36 to 40 hours of infection during 3rd passage. CPE of reovirus was also observed by Islam (1999) following 36 hours of infection during 3rd passage. Peilil *et al.* (1997) complete CPEs of IBDV on vero cell line was stably produced in 65 to 72 hours of inoculation during 4th passage. According to the findings of Ahasan *et al.* (2002) clear and consistant CPEs were observed during 3rd passage. Similarily, Hussain and Rasool (2005) noticed that typical aggregation, rounding and granulation of Vero cells was

noticed in passage 3 (P3) from 72 hours upto 144 hours post-infection. The present study showed slight difference comparing with the above researchers in those passage levels of the virus that time of optimum CPE was observed. This variation might be occurred due to the use of different IBDV strains, the media used to propagate the virus, the infectivity dose of the virus and other factors.

The hyper variable region (HVR) of VP2 is among the most studied parts of IBDV genome. The variable region of VP2 comprises a tight cluster of vulnerable amino acid variation sites, which may be responsible for generation of antigenic variants among the strains (Bayliss *et al*., 1990). The hypervariable region of VP2 is therefore the obvious target for molecular techniques applied for IBDV detection and strain variation studies.

The nucleotide sequence of the VP2 HVR was determined for LC-75 IBD vaccine and Vero cell adapted virus of this vaccine strain from cDNA transcripts. Nucleotide identity between the two was about 99.3% over the region sequenced and were genetically related to to each other with slightly varaion in some of nucleotide. The deduced amino acid sequence of the hypervariable region was determined for vero cell adapted LC-75 IBDV and compared to well characterise LC-75 NVI vaccine on CFC. The vero cell adapted LC-75 virus showed about 1.6 % variation in amino acid sequence compared to the LC -75 IBDvaccine produced on CFC in NVI. The nucleotide identity between the foremer Ethiopian produced IBDV vaccine (D78) and Ethiopian IBDV isolates ranged between 90.2%and 94.6% and the deduced amino acid sequence of the hypervariable region was determined for each of the isolates and compared to classical attenuated IBD vaccine (D78) as explained by Jenberie *et al*. (2014). The main limitation of this study was that the chickens infected by the original local isolate of IBDV were not exibit any clinical sign of the disease.This may be by IBDV strains differences in pathogenicity (OIE, 2016).

6. CONCLUSION AND RECOMMENDATIONS

This study has shown that the Vero cell adapted LC-75 vaccine virus and local isolate strain were successfully propagated and attenuated in Vero cells with higher titers recorded ,hence Vero cell could be used as a model to study the growth of kinetic of the IBDV isolate and the Vero adapted attenuated virus should be further studied for possible adoption as a candidate for an attenuated IBD vaccine development. RT-PCR could be successfully used to confirm the growth of IBDV in cell culture. Three amino acids were mutated in Vero cell adapted virus compared to the vaccine virus after sequencing of segment of VP2 hyper variable region.

Based on the above conclusion the following recommendations were forwarded:

- ✓ The immunogenicity and efficacy tests should be continued for the Vero cell adapted and attenuated IBDV.
- ✓ Further research work should be carried out to the local IBDV strains especially vvIBDV through sequencing in comparison to the vaccine in use in the country.
- ✓ Production of IBD vaccine using Vero cell line and comparing the efficacy with the vaccine currently producing in the country should be done in the future.

7. REFERENCES

Ahamed, T., K. M. Hossain, M. M. Billah, K. M. D. Islam, M. M. Ahasan and M. E. Islam (2004): Adaptation of Newcastle disease virus (NDV) on Vero cell line. *Int. J. Poult. Sci.,* **3**(2): 153-156.

Ahasan, M., Hossain, K.and Islam, M. (2002): Adaptation of infectious bursal disease virus on Vero cell line. *Online J Biol Sci*; **2**(9):633–635.

Alemu, Y. (1995): Poultry production in Ethiopia. *World Poult. Sci. J*. 51:197-201. *Avian Patho,* **28**: 470-476.

Allan, G., McNulty, M., Conner, T., McCracken, R. and McFerran, J. (1984): Rapid diagnosis of infectious bursal disease infection by immunoflourscence on clinical material. *Avian Patho.* **13**(3): 410-427.

Apilak, S. (2006): Potential of using infectious bursal disease virus produced in primary cell and Newcastle disease virus produced in continuous cell line for vaccine production. Master of Science Thesis in biotechnology, Graduate school, Khon Kaen University.Australian Animal Health Laboratory.

Bayliss, C.D.Spies, U., Shaw, K., Peters, R.W., Papageorgiou, A., Muller, H. and Boursnell, M.E. (1990): A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. *J. Gen. Virol.* **71:** 1303-1312

Becht, H. (1980): Infectious bursal disease virus. Current Topics in Microbiology and Immunology, **90**: 107-121.

Benton, W.J., Cover, M.S., Rosenberger, J.K. and Lake, R.S. (1967): Physicochemical Properties of the infectious bursal agent (IBA). *Avian Dis.,* **11**: 430-438.

Bettridge, J. , Lynch, S., Brena, C. , Melese, K. , Dessie, T. , Gutu, Z., Taye, T., Hanotte, O., Kaiser, P., Wigley, P. and Christley, R. (2013)**:** Infectious bursal disease in Ethiopian village chickens.

Bewket, S., Martha, Y., Mesfin, S. and Yismashewa, W. (2011): Ethiopian animal health yearbook, Addis Ababa, Ethiopia.

Bumstead, N., Reece, R. L., and Cook, J. K. A. (1993): Genetic differences in Susceptibility of chicken's lines to infection with infectious bursal disease virus.*Poult.Sci*. **72**: 403-410.

Chaka, H., Goutard, F., Bisschop, S.P.R. and Thompson, P.N. (2012): Seroprevalence of Newcastle disease and other infectious diseases in backyard chickens at markets in Eastern Shewa zone, Ethiopia. *Poultry Science*, **91**: 862-869.

Chanie, M., Negash, T. and Tilahun, S.B. (2009): Occurrence of concurrent infectious diseases in broiler chickens is a threat to commercial poultry farms in Central Ethiopia. *Trop. Anim. Health Prod*, **41**: 1309-17.

Chavez, I., Resenberger, J., Cloud, S.and Rope, C. (2002): Characterization of the antigenic, immunogenic and pathogenic variation of infectious bursal disease virus due to propagation in different host system (bursa, embryo and cell culture). III. Pathogenicity. *Avian Pathol*; **31**:485–492.

Cosgrove, A.S. (1962): An apparently new disease of chickens-avian nephrosis. *Avian Dis*. **6**:385-389.

Cullen, G. A. and Wyeth, P.J. (1975): Quantitiation of antibodies to IBD. *The Veterinary Record* **18**: 315.

Dobos, P. (1979): Peptide map comparison of the proteins of infectious bursal disease virus. *Virol* **32**: 1046 - 1050.

El-Ebriary, E.A., El-Mahidy, S.S. and Khodeir, M.H. (1997): Trials of using cell cultures of evaluation infectious birsal disease virus vaccine in Egypt. *Assiu Vet. Med. J*., **36**:105-123.

Eterradossi, N., Arnauld, C., Toquin, D. and Rivallan, G. (1998): Critical amino acid changes in VP2 variable domain are associated with typical and atypical antigenicity in very virulent infectious bursal disease viruses. *Arch. Virol*., **143**: 1627–1636.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F. and Meng, X.J. (2004): Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. *The Journal of Viro.* **,78:** 13440-13446.

Fernando, L. and John E. A. (2014): Gumboro Disease Special*:* The rational use of vaccines and vaccinations for real control of Gumboro disease.

Gary, D.B. and Richard, D. M. (2003): Infectious Bursal Disease (Gumboro) in Commercial Broilers, University of Florida.

Gezahegn, A., (2009): Urban Physical Geography of Bishoftu town. Unpublished document, Pp.2.

Halima, H., Nesser, F.W., Van Marle-Koster, E., and De Kock, A. (2007): Village-based indigenous chicken production system in north-west Ethiopia. *Trop. Anim. Health Prod*., **39**: 189-197.

Hassan, M., Natour M., Ward, L. and Saif Y. (1996): Pathogenicity, attenuation and immunogenicity of infectious bursal disease virus. *Avian Dis;* **40**: 567–571.

Hassan, S.K, Ahmed, F. Siddique, N. (1998): A study on the Infectious bursal disease around Rausalpind/Islamabad. *Pak. Inter. Live. Res.*pp.**8**:84-88.

Hirai, K., and Shimakura, S. (1974): Structure of infectious bursal disease virus. *J Virol* **14**: 957 - 964.

Hitchner, S. B. (1970): Infectivity of infectious bursal disease virus for embryonating eggs. *Poult. Sci.* **49**: 511-516.

Hussain, I. and Rasool, M. (2005): adaptation of an indigenous very virulent infectious bursal disease virus on vero cell line. *PakistanVet. J., **25**(3)*

Hussain, I. and Rasool, M. (2006): Preparation and evaluation of Vero-cell infectious bursal disease vaccine in Pakistan. *Vaccine,* **24**: 2810–2814.

Ignjatovic, J. (2004): Very Virulent Infectious Bursal Disease Virus

Islam, K. M. D., (1999): Adaptation of reovirus on Vero cell line. BSc Thesis, Biotechnology Discipline, Khulna University, Khulna, Bangladesh.

Islam, M. R. (2002): Developing vaccines. Georg Forster.

Islam, M.R. (2005): A manual for the production of BAU 404 Gumboro vaccine Submitted to the Department of Livestock Services, Dhaka, Bangladesh.

Ismail, N.M. and Saif, Y.M. (1996): Differentiation between antibodies to serotype 1 and 2 infectiuos bursal disease virus in chicken's sera. *Avian Diseases*, **34**:1002-1004.

Jackwood, D., Saif, Y.and Hughes J. (1987): Replication of infectious bursal disease virus in continuous cell lines. *Avian Dis*; **31**: 370-375.

Jackwood, D.J. and Niseln, C.K. (1997): Detection of infectious disease viruses in commercially reared chickens using the transcriptase/polymerase chain reaction-restriction endonuclease assay. Avian Diseases.

Jenbreie, S, Ayelet, G., Gelaye, E., Kebede, F., Stacey, E. Lynch, and Negussie, H. **(**2012): Infectious bursal disease: seroprevalence and associated risk factors in major poultry rearing areas of Ethiopia**.** *Trop. Anim. Health Prod. Volume* **4 (3)**.

Jenbreie, S, Ayelet, G., Gelaye, E., Kebede, F., Stacey, E. Lynch, Asmare, K. and Negussie, H. (2014): Genetic characterisation of infectious bursal disease virus isolates in Ethiopia. *Acta Tropica*, **130***: 39– 43*.

Jonathan, A.A. and John T. (2013): Epidemiology of Gumboro Disease.

Kassa, S.A. and Molla, W. (2012): Seroprevalence of infectious bursal disease in backyard chickens of North West Ethiopia. *Sci. J. of Crop Sci.*, **1**:20-25.

Kibenge F., Dhillon, A. and Russell R. (1988): Growth of serotype-I and II and variant strains of infectious bursal disease virus in Vero cells.*Avian Dis*; **32:**298–303.

Kibenge, F .S. B. and McKenna, P. K. (1992): Isolation and propagation ofinfectious bursal disease virus using the ovine kidney continuous cell line. *Avian Dis.* **36**: 256-261.

Lasher, H.N. and Shane, M. (1994): Infectious bursal disease. Journal of World Poultry Sci., **50**: 133-166.

Le Nouën, C., Rivallan, G., Toquin, D., Darlu, P., Morin, Y., Beven, V., De Boisseson, C., Cazaban, C., Comte, S., Gardin, Y. and Eterradossi, N. (2006): Very virulent infectious bursal disease virus: reduced pathogenicity in a rare natural segment B-reassorted isolate. *J. Gen. Virol*., **87**: 209–216.

Lee, L. and Lukert, P. (1986): Adaptation and antigenic variation of infectious bursal disease virus. *J Chin Soc Vet Sci;* **12**:297–304.

Lin, Z., Kato, A., Otaki, Y., Nakamura, T., Sasmaz, E. and Ueda, E. (1993): Sequence comparisons of a highly virulent infectious bursal disease virus prevalent in Japan. *Avian Dis*., **37**: 315–323.

Lukert, P. D. and Saif, Y. M. (1997): Infectious bursal disease. In: Diseases of Poultry. Iowa State University Press, Ames, Iowa, pp.721-738.

Lukert, P. D. and Saif, Y. M. (2003): Infectious bursal disease. In: Diseases of Poultry. **11**th ed. Pages 161-180. Iowa State University Press, Ames.

Lukert, P., Leonard, J. and Davis, R. (1975): Infectious bursal disease virus: antigen production and immunity. *Am J Vet Res*; **36**:539–540.

Lukert, P.D. and Davis, R.B. (1974): Infectious bursal disease virus: growth and characterization in cell cultures. *Avian Disease* **18**:243-250.

Marian, B. (2001): Eating well: Shopping for Antibiotic-Free Meat.

Maris, P. (1986): Désinfection des bâtiments: le vides sanitaire en aviculture. *Point vét.* , **18**: 635-639.

Marquardt, W., Johnson, R.B., Odenwald, W.F. and Schlotthober, B.A. (1980): An indirect enzyme linked immunosorbent assay (ELISA) for measuring antibodies in chickens infected.

Mazengia, H., Bekele, S.T.and Negash, T. (2010): Newcastle Disease and Infectious Bursal Disease are Threats to Village Chicken Production in Two Districts of Amhara National Regional State, Northwest Ethiopia. IUP *J. Life Sci.*, **4**(2): 62-72.

Mazengia, H., Tilahun, S.B. and Negash, T. (2009): Incidence of infectious bursal disease in village chickens in two districts of Amhara Region, Northwest Ethiopia. *J. Livest. Res. Rural Dev.* **21**:12.

McFerran, J.B., McNulty, M.S., Killon, E.R., Conner, T.J., McCracken, R.M., Collins, D.S. and Allen, G.M. (1980): Isolation and serological studies with infectious

bursal disease from fowl, Turkey and duck: Demonstration of a second serotype. *Avian Patho,* **9**:395-404.

MOA (2012): Ethiopia animal health yearbook 2011.

Muller, H., Scholtissek, C. and Becht, H. (1979): The genome of infectious bursal disease virus consists of two bursal disease virus consists of two segments of double-stranded RNA. *J. of Viro,* **31**: 584-589.

Naqi, S., Thompson, G., Bauman, B., and Mohammed, H. (2001): The exacerbating effect of infectious bronchitis virus infection on the infectious bursal disease virus-induced suppression of opsonization by Escherichia coil antibody in chickens. *Avian Dis*., Jan-Mar; **45**(1):52-60.

Nawzad, A. R., Nahla, M. S., Shilan, F. M., and Zana, H. M. (2016): Int'l Journal of Advances in Chemical Eng., & Biological Sciences (IJACEBS) *Vol. **3**, Issue 1 ISSN* 2349-1507 EISSN 2349-1515.

Negash, T., Gelaye, E., Petersen, H., Grummer, B. and Rautenschlein, S. (2012): Molecular evidence of very virulent infectious bursal disease viruses in chickens in Ethiopia. *Avian Dis.*, **56**: 605-610.

OIE (2003): Homepage of http://www.oie.int/hs2/ci_ pays.asp.

OIE (2016): Infectious bursal disease (Gumboro disease); Version adopted by the World. Assemply of Delegates. of the OIE.

Ozel, M., and Gelderblom, H. (1985): Capsid symmetry of viruses of the proposed birnavirus group. *Arch Virol* **84**:149-161.

Paul, B.K., Das, S. K., Badhy, S. C., Amin, M. R., Aminand, K. M. and Banik, S. C. (2004): Effect of existing and imposed vaccination on body weight against Gumboro in broiler under farm condition. *Inter. J. of Poultry Sci.*, **3**(10): 655-657.

Peilin, W., Boaxiang, C. and Jinsong, L. (1997): Adaptation and propagation of infectious bursal disease virus in Vero cells. *Chin J. Vet Res Tech*; **27**:23–24.

Pejkovski, C., Davelaar, F. G. and Kouwenhoven, B. (1979): Immunosuppressive effect of infectious bursal disease virus on vaccination against infectious bronchitis. *Avian Path.,* **8**:95-106.

Peter, R. (2007): Molecular Detection and Differentiation of Infectious Bursal Disease Virus, Avian Diseases, Vol. **51**, No. 2. pp. 515-526 Published by: American Association of Avian Pathologists, University of Arkansas.

Pettit, J. R., Gough, A. W. and Gagnon, A. N. (1983): Infectious Bursal Disease of chickens. (Gumboro Disease). www.gov.on.ca\OMAFRA.

Rabee, J. I. (2014): Review of infectious bursal disease (gumboro), University of Diyala, College of Veterinary Medicine, Iraq

Rautenschlein, S., Kraemer, Ch., Vanmarcke, J.and Montiel, E. (2001): Protective Efficacy of Intermediate and Intermediate plus Infectious Bursal Disease Virus (IBDV) Vaccines against Very Virulent IBDV in Commercial Broilers. *Avian Dis.*, ***49*** (2):231-237.

Rinaldi, A., Cessi, D., Cervio, G. and Iodetti, T. (1972): Attenuation of infectious bursal disease virus and vaccination trial under laboratory and field conditions. *Avian Path*., **3**: 51-57.

Roberto Soares (2008): Passive immunity: part 2, Issue No.**19**

Rooney, C.S. and Freund, R.C. (1988): A comparison of infectious bursal disease antibody titers using different antigens in the serum neutralization and enzyme-linked immunosorbent assay tests.In Proc. 37th Western Poultry Disease Conference, 29 February - 2 March, Davis, California. University of California, Davis, 17-20.

Rosenberger, J. and Cloud, S. (1986): Isolation and characterization of variant infectious bursal disease viruses. Proceedings of the 123rd annual meeting of American Veterinary Medical Association;

Rosenberger, J., Saif, Y. and Jackwood, D. (1998): Infectious bursal disease.Isolation and identification of avian pathogens. *Am Assoc AvianPatho*: 215–218.

Saif, Y.M. (1998): Infectious bursal disease and hemorrhagic enteritis. *Poult. Sci.*, **77:** 1186-1189.

Shane, S. M., Lasher, H. N. and Paxton, K. W. (1994): Economic impact of infectious bursal disease.Proceeding of the second International symposium on infectious bursal disase (IBD) and Chicken infectious anaemia (CIA), 197-205.

Sivanadan, V. and Sasipreeyajan, J. A. (1986): Histopathological changes induced by serotype II of IBDV in specific-pathogen free chickens. *Avian dis.* **30**(4): 709-715.

Skeeles, J.K., Slavik, M., Beasley, J.N., Brown, A.H., Meinecke, C.F., Maruca, S. and Welch, S. (1980): An age-related coagulation disorder associated with experimental infection with infectious bursal disease virus *Am. J. vet. Res*., **41** (9): 1458-146.

Sofei, D., Avran, E., Danes, M., Malinetescu, M., Plamadeala, S., Bucur, E. and Vior, E. (1996): Gumbovivac-CC live vaccine against infectious bursal disease prepared on cell cultures.Studies and Researches in Veterinary Medicine, **4:**122-126.

Solomon, D. (2007): Poultry sector country review Ethiopia: Jimma, Ethiopia, food and agriculture organization of the United Nations 2007, Re-edited September 2008.

Spearman, C. (1908): The method of 'right and wrong cases' ('constant stimuli') withoutGauss's formulae. *Brit. J. Psychol.,* **2**:227-242.

Stram, Y., Meir, R., Molad, T., Blumenkranz, R., Malkinson, M. and Weisman, Y. (1994): Applications of the polymerase chain reaction to detect infectious bursal disease virus in naturally infected chickens. *Avian Dis*., **38**: 879-884.

Synder, D.B., Lana, D.P., Cho, B.R. and Marquardt, W.W. (1988): Group and strain specific neutralization sites of infectious bursal disease virus defined with monoclonal antibodies. *Avian Dis.*, **32**:527-534.

Synder, D.B., Marquardt, W.W., Malkinson, M., Russe, K., Cohen, E.T.E., Savage, P.K. and Allan, D.C. (1986): Rapid serological profiling by enzyme-linked

Immunosorbant assay. IV Association of infectious bursal disease serology with broiler flock performance. *Avian Dis.,* **30**:139-148.

Tadelle, D. and Ogle, B. (2001): Village poultry production systems in the central highlands of Ethiopia. *Trop. Anim. Health Prod*., **33**: 521-537.

Tadelle, D. Million, T. Alemu, Y. and Peters, K.J. (2003): village chicken production systems in Ethiopia: 2. Use patterns and performance valuation and chicken products and socio-economic functions of chicken. *Livest. Res. Rural. Dev*. **15**:1.

Tamiru, N., Alkie and Silke, R. (2016): Veterinary Medicine: Research and Reports downloaded from https://www.dovepress.com/ by 213.55.95.160 on 16-Sep-2016. University of Veterinary Medicine Hannover, Germany.

Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution **30**: 2725-2729.

Teshager, N. (2015): Pathological and seroprevalence studies on infectious bursal disease in chickens in and around Bahir Dar, North West, Ethiopia. Addis Ababa University, College of Veterinary Medicine and Agriculture.

Tham, K.M., Young, L.W. and Moon, C.D. (1995): Detection of infectious bursal disease vims by reverse transcription-polymerase chain reaction amplification of the virus segment a gene. *J. Virol. Meth*. **53**, 201-212.

Tian, C. and Keping, C. (2009): Investigation and Application Progress of Vero Cell Serum-free Culture. *Inter. J.Vol*.**1**:No.2.

Van den Berg, T.P. (2000): Acute infectious bursal disease in poultry: a review. *Avian Pathol*. **29**: 175-194.

Van Den Berg, T.P., Eterradossi, N., Toquin, D. and Meulemans, G. (2000): Infectious bursal disease (Gumboro disease), p.529

Van den Berge, T.P., Morals, D. Entradossi, N., Reivallan, G., Toquin, D., Rave, R. and Zeinberge, M.F. (2004): Assessment of genetic, antigenic and pathogenic criteria for the characterization of Infectious bursal disease virus strain.

Wafaa, A.E. (2006): Infectious Bursal Disease (IBD) Gumboro disease, Cairo University.

Weisman, J. and Hitchner, S.B. (1978): Virus-neutralization versus agar-gel precipitin tests for detecting serological response to infectious bursal disease virus. *Avian Dis*., **22**: 598-603.

Winterfield, R. W. and Hitchner S. B. (1962): Etiology of an infectious nephritis-nephrosis syndrome of chickens. *Amer. J. of Vet.Res.* **23**:1273-1279.

Woldemariam, M. and Wossen A. (2007): Infectious bursal disease: case report at region. *Ethio. Vet. J.*, **11**: 151.

WSU (2014): Infectious bursal disease. Veterinary medicine extension.

Wu C.C., Lin, T.L., Zhang, H.G., Davis, V.S. and Boyle J.A. (1992): Molecular detection of infectious bursal disease virus by polymerase chain reaction. *Avian Dis*., **36**:221–226.

Yannick, G., Vilmos, P., Marcelo, P., Christophe, C., Branko, A., Fernando, L., John, El. A. (2014): Gumboro disease special. Ceva Animal Health

Zeleke, A., Gelaye, E., Sori, T., Ayelet, G., Sirak, A. and Zekarias, B. (2005): Investigation on infectious bursal disease outbreak in Debre Zeit. Asian Network for Scientific Information. *Int. J. Poult. Sci.* **7**: 504-506.

Zeleke, A., Yami, M., Kebede, F., Melese, N., Senait, B., Gelaye, E., Sori, T., Ayelet, G. and Berhanu, B. (2002): Gumboro: An emerging disease threat to poultry farms in Debre Zeit. *Ethio. Vet. J.*, pp. 1-7.

Zeryehun, T. and Fekadu, G. (2012): Seroprevalence of infectious bursal disease in chickens managed under backyard production system in Central Oromia, Ethiopia. *Afri. J. of Micro.Res.* **6**, 6736-6741.

Zierenberg, K., Raue, R. and Müller, H. (2001): Rapid identification of "very virulent" strains of infectious bursal disease virus by reverse transcription-polymerase chain reaction combined with restriction enzyme analysis. *Avian Pathol*.**30:** 55-62.

8. ANNEXES

Annex 1: Growth and maintainance media

Growth medium

GMEM	80%
TPB	10%
Fetal calf serum	10%
Antibiotic solution (penstrep)	0.2%

Maintainance Medium

GMEM	84%
TPB	14%
Fetal calf serum	2%

Annex 2: Solutions

Tryptose phosphate broth

Glucose	2g
Nacl	5g
Tryptose	20g
$Na_2HPO_4.2H_2O$	2.5g
Distill water	upto 1000ml

Dulbecco's phosphate buffered saline (PBSA)

Nacl	8g
Kcl	0.2g
KH_2PO_4	0.2g
$Na_2HPO_4 .2H_2O$	2.16 g
Distilled water	up to 1000 ml

Trypsin 0.05%/EDTA 0.02% solution for sub culture

Trypsin 2.5% stock solution	20 ml
PBSA	970 ml
EDTA 2% stock solution	10 ml
Antibiotic solution (if required)	2 ml

Source: NVI tissue culture viral vaccine production issue number 2, 2012.

Annex 3: Procedures for subculturing of vero cell from confluent monolayer

- Remove all growth medium from confluent monolayer of vero cell.
- Wash adhering monolayer with small volume of PBS free from Ca^2 amd Mg^2
- Add enough amounts of trypsin and gentle shaking. Then discard the trypsin and incubate the flasks upto the cells start disperses.
- Add some amount of complete media and trypsinize upto the cell detached completely from the bottom surface of the flask.
- Distribute an appropriate amount of complete media (GMEM) into fresh flasks.
- Incubate the cell suspension and monitor the every day up to they make confluency for the next sub culturing.

Annex 4: RNA extraction from Vero cell adapted IBDV

- Lysate preparation

Add lysing buffer to sample containing tubes and ethanol alcohol for RNA to be bind and precipitate to spin mini column kit

- Transfer the lysate to the 2ml size spin column (containing silica)
- Wash the spin column to remove contaminants from extracted RNA(2-3 times)
- Elution of the total RNA using RNase free water (DEPC-treated water)
- Harvest the elute to new tube and store -20 c^o up to use

(Incubate and Centerfuge between)

Annex 5: Agarose Gel Electrophoresis procedure for PCR product

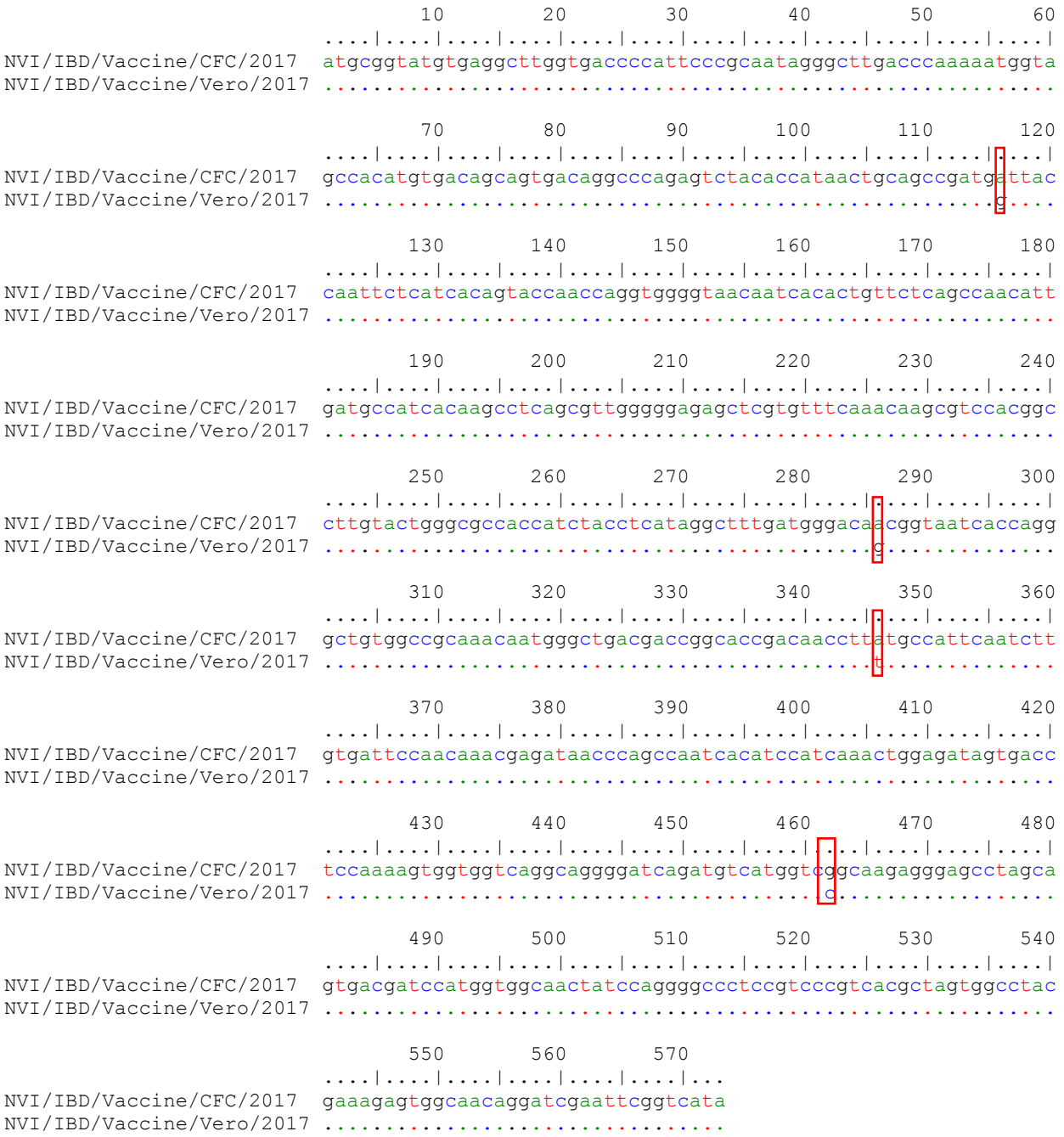
- Mix agarose powder with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted
- Add the red gel (final concentration 1.5 %) at this point to facilitate visualization of the band after electrophoresis.
- After cooling the solution to about 60 ^{0}C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.
- After the gel has solidified, the comb is removed
- The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer.
- Samples containing RNA product mixed with loading buffer are then pipeted into the sample wells, the lid and powern leads are placed on the apparatus, and a current is applied.
- Visualize RNA, after the gel is placed on an ultraviolet transilluminator and capture the image, safe and print for documentation.

Annex 6: Indirect ELISA procedures for IBDV

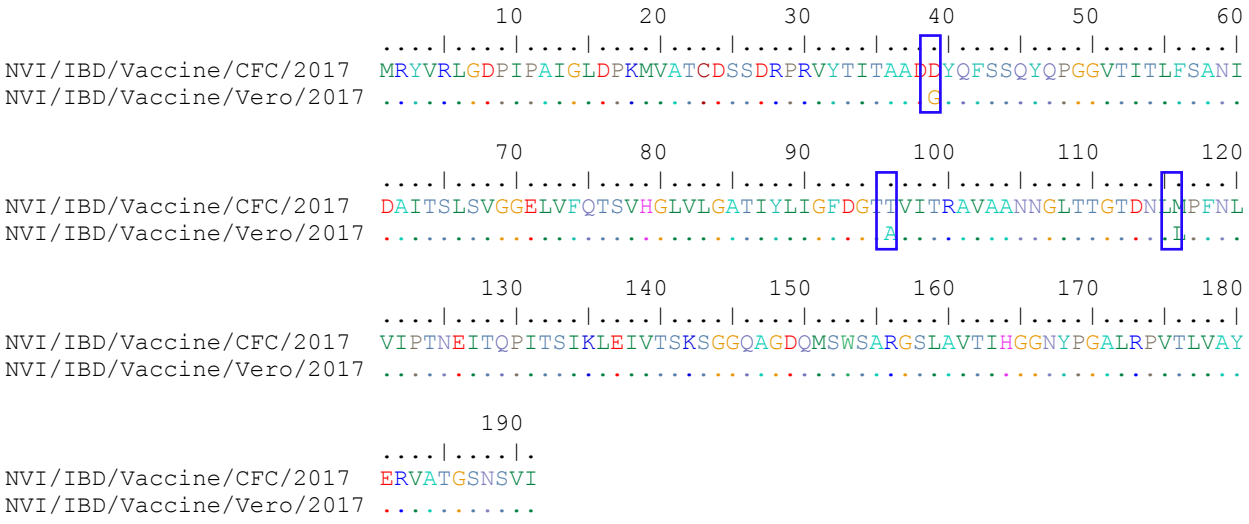
- Dilute the test samples to five hundred fold (1:500) dilution.
- Add 245µl of dilution buffer 14 to each wells of microplate.
- Add of 5µl of negative control to wells A1 and B2 and 5µl of positive control to wells C1 and D1 and add 5 µl of each samples to be tested in the remaining wells.
- Then disperse 90 µl of dilution buffer 14 and 10 µl of the pre diluted samples in to the appropriate 96-well plate coated with IBDV viral antigen and covere the plate and incubate at room temperature for 30 minutes.
- Wash the plate 3 times with 300 µl of the wash solution 1x at the end of incubation period followed by addition of an anti chichen horseradish peroxidase conjugate into each well. Allow the plate to incubate at room temperature for 30 minutes

- Wash the plate 3 times with 300 µl of the wash solution 1x again before adding 100 µl of the substrate solution to each test well which then incubate for 15 minutes at dark place of room temperature.
- Finally, add 100 µl of stop solution in to each well to stop the reaction and read the absorbance at 450 nm.

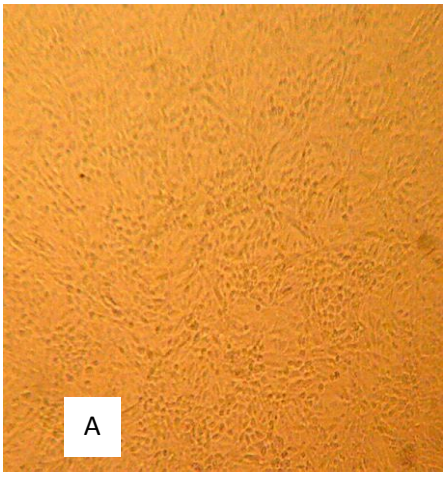
Annex 7: Nucleotide sequences of the VP2 variable domain in LC-75 IBD vaccine virus aligned with Vero cell adapted vaccinal strain



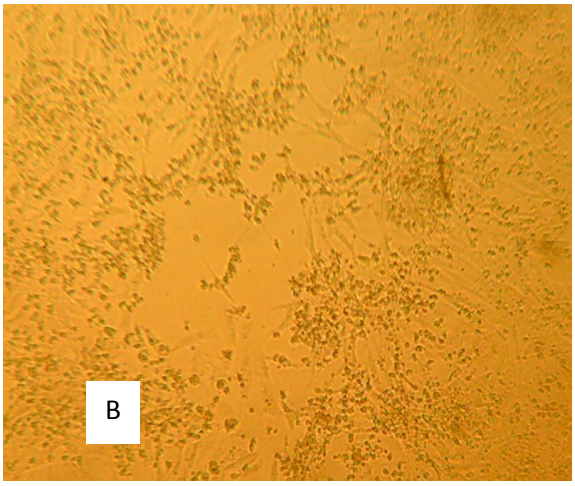
Annex 8: Amino acid sequences of LC-75 IBD vaccine before and after adaptation on Vero cell



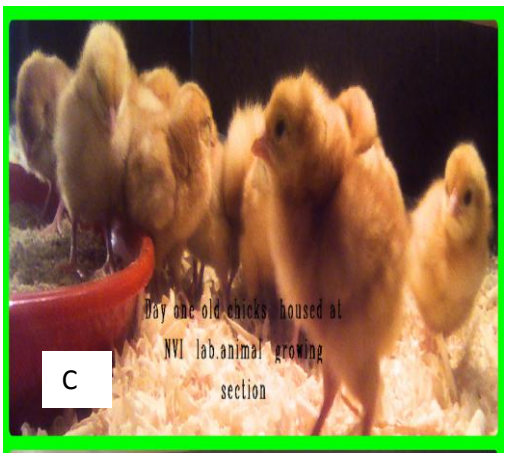
Annex 9: List of pictures captured during laboratory work



A) Confluent Vero cell

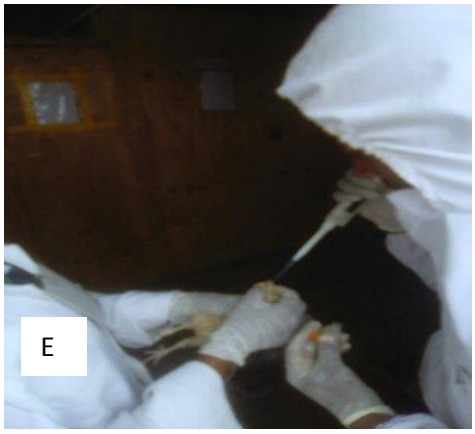


B) The effect of IBDV on the vero cell (CPE)



C) Chickens at day 1

D) Chickens at day 21



E) Infection of chicken with Vero cell adapted and attenuated IBDV through orally.