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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

Asmelash Tassew[1*] Getaw Deresse[2] Essayas Gelaye[2] Molalign Bitew[2] Kibeb Legesse[3]
Muuz Gebru[4] Mulugeta Ftiwi[1]
1.Raya University, College of Agriculture and Natural Resources, Department of Animal Science; P.O.Box: 92; Maichew, Ethiopia
2.National Veterinary Institute; Bishoftu, Ethiopia, P.O.Box:
3.Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Biomedical Sciences; P.O.Box: 34; Bishoftu, Ethiopia
4.Mekelle university, College of veterinary Medicine

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.
Keywords: 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. MATERIALS AND METHODS

2.1 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

2.2 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.

2.3 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.

2.4 Study Methods

2.4.1 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.

2.4.2 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.

2.4.3 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 co 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 37co in 5% co2. 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).

2.4.4 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.

2.4.5 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.

2.4.6 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).

Log10= ((xo − (d/2) + d (ri/ni))

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

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

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

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

The summation was started at dilution Xo.

2.4.7 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^{o}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^{o}c for 5minutes and placed on ice for at least 1 minute. The cDNA synthesis mixture compounds 10X RT buffer (2 µl), 25mM $MgCl_2$ (4 µl), 0.1 DTT (1 µl), RNase OUT™ (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^{o}c for 50 minutes. Then the reaction was terminated at 85^{o}c for 5 minutes and chilled on ice and briefly centrifuged. 1 µl RNase H was added and incubated at 37^{o}c for 20 minutes. Finally the prepared cDNA was used immediately for PCR based on the 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'TGTAAAACGACGGCCAGTGCATGCGGTATGTGACGCTTGGTCAC-3'and Reverse primer: 5'CAGGAAACAGCTATGACCGAATTCGATCCTGTTGCCACTCTTTC-3' (OIE, 2016).

2.4.8 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 1^{st} denaturation at 95ºC for 30sec, annealing at 60ºC for 30sec, extension at 72ºC for 30sec and again followed by 20 cycles of 2^{nd} denaturation at 95ºC for 30sec ,annealing at 56^{0}c for 30sec ,extension at 72^{0}c for 30sec and final extension at 72ºC for 7 minutes for 1 cycleand hold 4 ºC until machine off (NVI SOP, 2012).

2.4.9 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.

2.4.10 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.

2.4.11 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.

2.4.12 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.

2.5 Data management and analysis

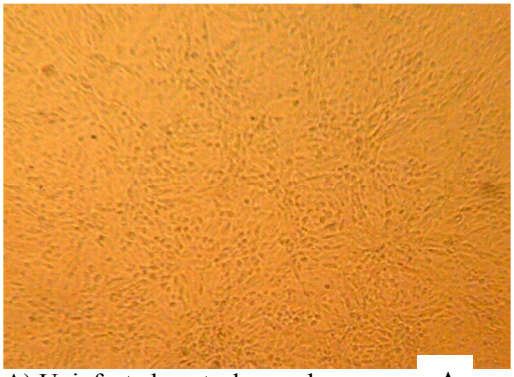
Serological data were encoded in MS-excel sheet and analyzed by using SPSS version 20 including decripive statistics (Percentage) and multiple sequence alignments were performed to align the sequences as codons using the Muscle algorithm in MEGA6.

3 RESULTS

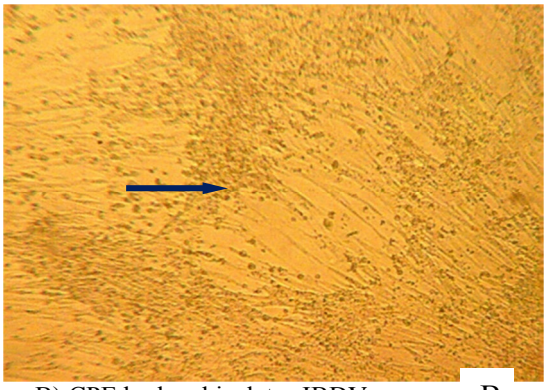
3.1 Isolation and adaptation of IBDV on cell culture

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.

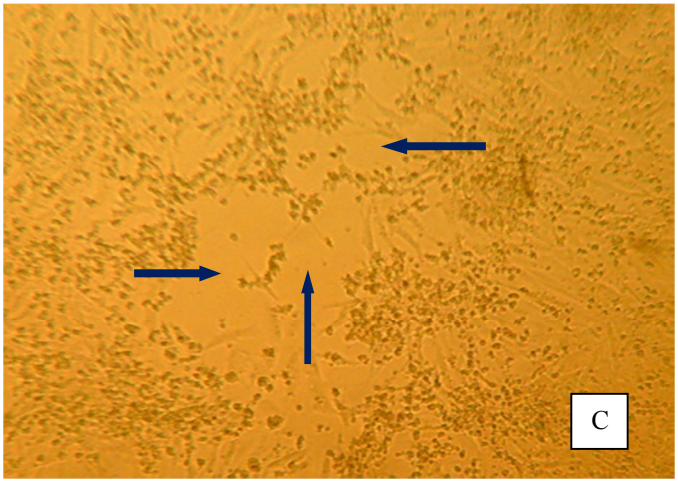
Vero cell monolayers inoculated with the bursal homogenates and reconstitute vaccine at separately using similar conditions resulted in isolation of the virus. Local isolate and LC-75 vaccine strains were adapted to cell culture for several successive passages. Typical aggregation, rounding and granulation 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

3.2 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.

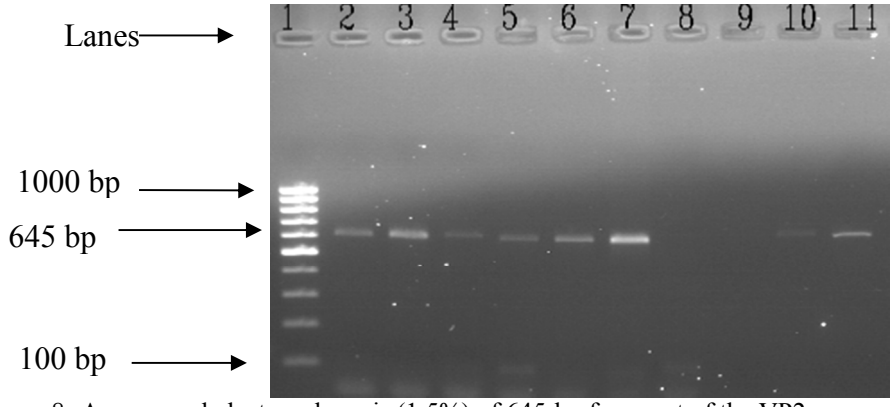


Figure 8**:** Agarose gel electorophpresis (1.5%) of 645-bp fragment of the VP2 gene.
1= 100-1000 bp DNA ladder marker; Lanes 2 to 4 = Local isolate IBDV strain adapted on Vero cell (positive; band at 645 bp); Lanes 5 to 7 = LC-75 IBD vaccine strain adapted on Vero cell (positive; band at 645 bp); Lane 8= Extraction control (RNase free water); and Lane 9=negative control; Lanes 10 and 11 positive controls (Known IBD vaccine, band at 645 bp).

3.3 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.

3.4 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.

3.5 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.

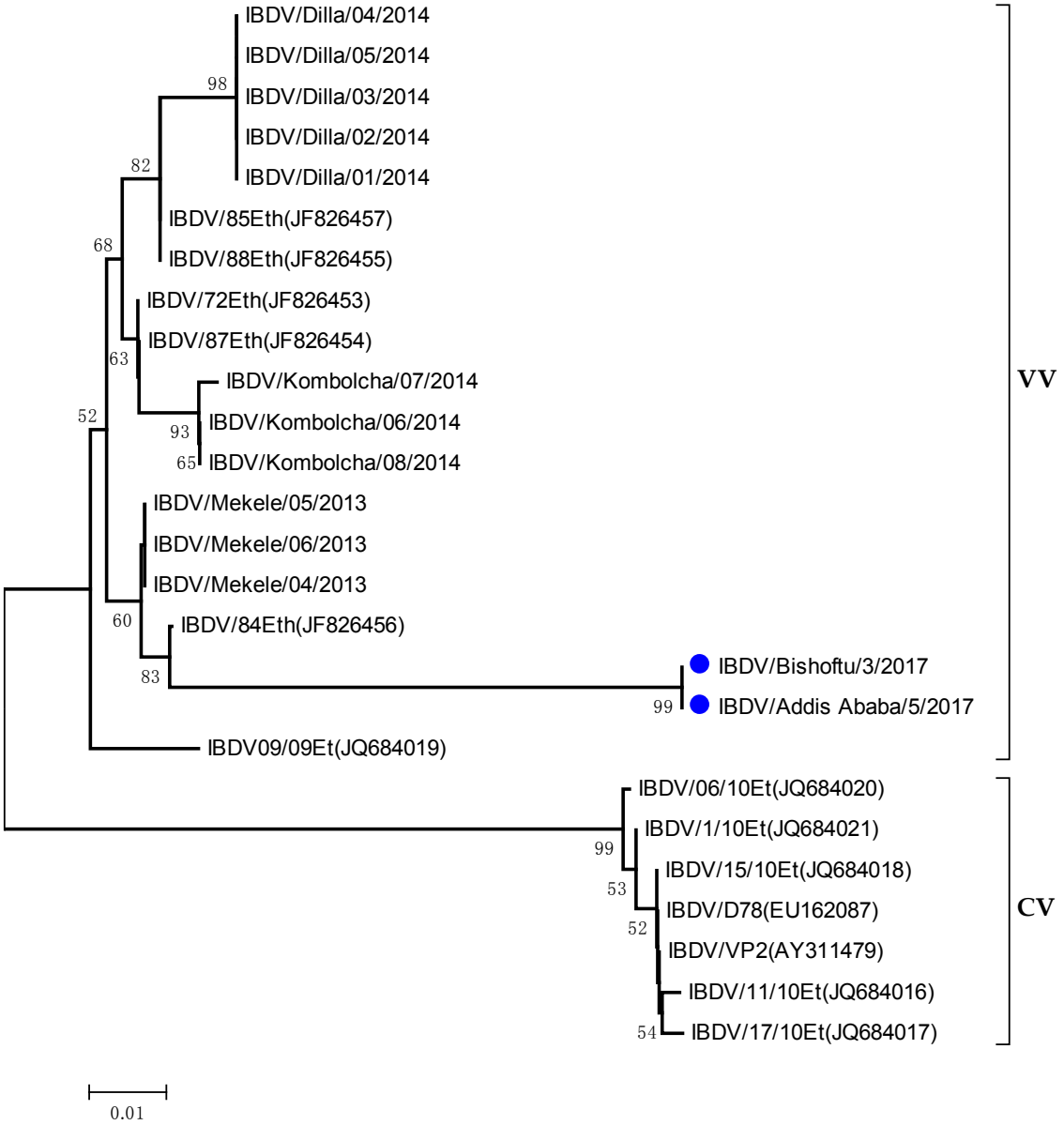


Figure 9: Phylogenetic analysis of the VP2 hypervariable coding sequence of IBDV isolates.
VV-very virulent IBDV, CV-classical virulent IBDV

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.

3.6 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.

3.7 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.

4 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).

5 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.

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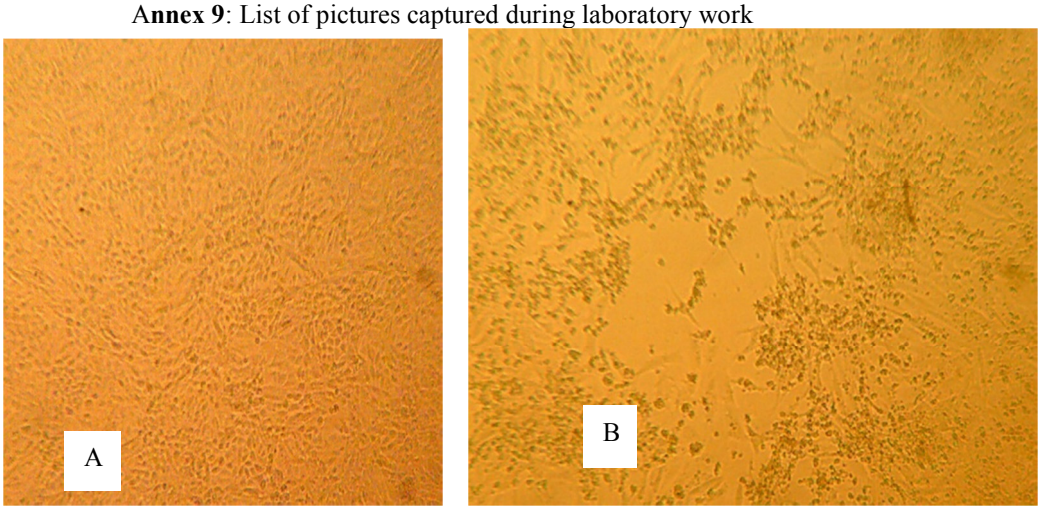
Annex 7: Nucleotide sequences of the VP2 variable domain in LC-75 IBD vaccine virus aligned with Vero cell adapted vaccinal strain

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

A**nnex 9**: List of pictures captured during laboratory work



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