Adaptation of Newcastle Disease Virus Vaccinal Strain on Chicken Embryo Fibroblast and Vero Cell Lines and Evaluation of the Protective Efficacy of the Vaccine in Chickens

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A Thesis paper Submitted to the Department of Applied Biology

School of Applied Natural Science

Office of Graduate Studies

Adama Science and Technology University

October, 2024

Adama, Ethiopia

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DECLARATION

I declare that this thesis/dissertation paper entitled “Adaptation of Newcastle Disease Virus Vaccinal Strain in Chicken Embryo Fibroblast and Vero Cell Lines, and Evaluation of Protective Efficacy of vaccine in Chicken” is my own work and has not been submitted to any university for similar purpose. The references used in this thesis paper are duly recognized by proper citations.

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We hereby certify that the recommendation and suggestion given by the thesis paper review committee are appropriately incorporated into the final thesis paper entitled "**Adaptation of Newcastle Disease Virus Vaccinal Strain in Chicken Embryo Fibroblast and Vero Cell Lines, and Evaluation of Protective Efficacy of vaccine in Chicken "** prepared under my guidance by Megersa Mindaye.

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LIST OF ABREVIATIONS AND ACRONYMS

AIV	Avian influenza virus
ANOVA	Analysis of Variance
APMV-1	Avian Paramyxo Virus One
BMK-21	Baby Hamster Kidney
CEF	Chicken embryo fibroblast
CNS	Central nervous system
CPE	Cytophatic effect
Crbc	Chicken red blood cell
DIVA	Differentiating Infected From Vaccinated Animal
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribo Nuclic Acid
DPC	Days post challenge
EDTA	Ethylene Diamine Tetra Acetic acid
EID50	Embryo infective dose fifty
ELD 50	Embryo lethal dose fifty
EMEM	Eagle's Minimum Essential Medium
FCS	Fetal calf serum
FP	Fusion protein
GMEM	Glasgow minimum essential medium
GMT	Geometric Mean Titer
HA	Haem-Agglutination
HB1	Hitchner B1

HeLa	Henrietta Lacks
HI	Haem Agglutination Inhibition
HN	hem agglutinin-neuraminidase
ICF	Infected Cell Culture Fluid
ISO	International Organization for Standardization
MDT	Mean Death Time
MP	Matrix protein
mRNA	Messanger Ribonucleic Acid
ND	Newcastle Disease
NDV	Newcastle Disease virus
NP	Nucleocapsid Protein
NVI	National veterinary institute
OIE	Office International Des Epizooties
PBS	Phosphate Buffer Solution
QMS	Quality management system
RNA	Ribonucleic Acid
RPM	Revolution per minute
RT-PCR	Reverse transcriptase Polymerase chain reaction
SD	Standard deviation
SCDM	Soyabean Casein Digest Medium
SPF	Specific pathogen free
SPSS	Statistical Package for the Social Sciences
TPB	Tryptose phosphate broth
UV	Ultraviolet Light

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ABSTRACT

Newcastle disease is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) and is one of the most important poultry diseases that influence poultry production in the poultry industry worldwide. The main objective of this study was to compare Chicken Embryo Fibroblast (CEF) and Vero cell lines adapted thermostable ND I-2 vaccine and evaluate the efficacy of the vaccine on experimental chickens in order to control and prevent Newcastle disease. For this purpose, *in vitro* and *in vivo* experimental designs were employed to compare the adaptation and titration of NDV in CEF and Vero cell lines. The virus was serially passaged up to passages 8 and 15 in CEF and Vero cell lines, respectively. Besides, the cell culture-adapted experimental vaccine immunogenicity, efficacy, and safety were evaluated on eighty 14-day-old, unvaccinated, apparently healthy chickens. Chickens were randomly divided into four experimental groups, each having twenty (20) chickens. The study demonstrated the successful adaptation of the virus on Vero and CEF cell lines, exhibiting typical cytopathic effects. CEF and Vero cell lines infectivity titers rose from 3.9 log10 to 7.7 log10 TCID50/ml and 5.3 log10 to 6.7 log10 TCID50/ml, respectively. The mean antibody titers of experimental group 1, 2, and 3 showed 75.8 ±5.1, 50.0 ±19.5 and 86.5 ±41.2 respectively and the unvaccinated group was 0.8 ±0.4. These results confirmed the entire vaccinated group produces the best immunological response against challenge infection. All treatment and control groups were challenged with a velogenic ND virus with 10^5 ELD50 per chicken via the nasal route on the 35^{th} day. The current study showed that all of the chickens in the unvaccinated group were dead, whereas the chickens in groups 1 and 3 had induced a protective index of 100% and group 2 had induced a protective index of 95%. Hence, the CEF and Vero cell lines adapted NDI-2 vaccine as well as current ND vaccines produced at National Veterinary Institute induce a protective immune response against the circulating virulent ND virus using vaccination-boost and challenge protocols in experimental Specific Pathogen Free chickens. Finally, this study concluded that CEF and Vero cell lines were the most preferable cell lines for the production of Newcastle thermostable I-2 vaccine.

Keywords: *Adaptation, antibody titer, chicken embryo fibroblast, Newcastle disease, Newcastle disease vaccine, Newcastle disease virus, Vero cell lines*

1. INTRODUCTION

1.1. Background of the study

Poultry serve as a primary source of food for human consumption and source of income where they serve as one of the possible solutions to malnutrition, food security, and poverty in low- and middle-income countries. For these reason, maintaining the health of poultry is paramount important (Donma *et al.,* 2017). However, several infectious diseases challenge productivity and profitability of poultry farming. Newcastle disease (ND) is one of the most important poultry diseases that influence poultry production in poultry industry worldwide (Mauri *et al.,* 2021). Newcastle Disease primarily affects chickens, leading to significant economic losses because of high mortality and reducing egg production (Aljumaili *et al.,* 2020).

Newcastle disease virus (NDV) is a highly pleomorphic enveloped virus, which belongs to the genus *Orthoavulavirus* in the family Paramyxoviridae, subfamily Avulavirinae with a diversity of genotypes associated with severe economic losses in all poultry sectors. The subfamily is divided into three genera; *Metaavulavirus*, *Orthoavulavirus* and *Paraavulavirus*. The taxonomy of the three genera contains 21 species (Walker *et al.,* 2021). The virus can also be found in the eggs laid during acute vNDV infections and in every portion of the dead body. Aerosols and contaminated water or food can easily spread disease to chickens. NDV may come from infected hens and other domestic and wild birds (Du *et al.,* 2020).

All NDV isolates are serologically grouped into a single serotype because they share a fairly similar immune dominant epitopes. Thus, any strain of NDV could theoretically stimulate immune response capable of producing cross-protection against any other strain of the virus (Sultan *et al.,* 2020). Due to serious economic loss caused by ND (Aljumaili *et al.,* 2020), effective methods of prevention and control are needed to prevent the spread of such infectious disease within and from a flocks. One of the successful methods in poultry farm is implanting good biosecurity practices, vaccination program, and medication (Dimitrov *et al.,* 2017).Among these tools, vaccination of live attenuated lentogenic ND vaccine is the primary and most effective strategy for the prevention and management of virulence NDV (Ma remagae *et al.,* 2020).

According to Fentie et al.(2014) and Ferreira et al.*(*2021), an ideal vaccine against ND should reduce the transmission of virus among chickens in a flock and the spread of virus between flocks. However, the available information is limited about the effectiveness of vaccines producing in Ethiopia on their protection against infection and halting transmission of currently circulating ND Virus strains in village chickens. Naturally attenuated strains such as lentogenic Hitchner B1 (HB1) and La Sota strains were selected and developed as live virus vaccines and continue to be used extensively today to produce live and inactivated vaccines (Weedon, 2009). The thermostable I-2 vaccine of Australian origin has also been produced and distributed in Ethiopia.

According to Mehrabadi et al. (2020), killed vaccines should be applied to each bird while backyard poultries are often scattered. Thus, it is difficult and time-consuming to catch each of them. As a result, this type of vaccine is not a good option for vaccination of backyard poultry. On the other hand, commercial live vaccines are sensitive to temperature and vaccine delivery to villages and remote areas requires special equipment and high cost to maintain the cold chain. That is why the use of Newcastle vaccines produced by a lentogenic thermostable strains is advantageous in rural areas and can be a good alternative immunogenicity and avoid undesirable ones (genes responsible for pathogenicity) from many viruses, including NDV (Mohammed *et al.*, 2013).

Maternal antibody to NDV had a significant effect on the immune response to the vaccine strains regardless of whether the strains were administered by eye drop or aerosol methods. All live attenuated ND vaccines are known to stimulate both mucosal and systemic immune responses similar to those of the natural infection, because of their ability to replicate in chicken irrespective of the site of administration (Rauw *et al*., 2009).

1.2. Statement of problem

The National Veterinary Institute (NVI) was the sole veterinary vaccine company, primarily serving the animal health industry in the country. To combat animal disease, NVI currently produces nearly twenty-three (23) distinct types of veterinary vaccines against bovine, ovine, caprine, avian species, equine and dromedary diseases. Among avian vaccines, ND vaccine is one of the most widely used poultry vaccines (Sasipreeyajan *et al.,* 2016). Since ND causes devastating losses in all poultry industry, reducing losses of large numbers of chickens to virulent Newcastle disease is essential to improve the productivity of chicken through production of ND vaccine (Mesfin & Bihonegn, 2018). ND can be effectively controlled by the use of vaccines. Chickens that survive from virulent NDV infection develop a long lasting immunity to further similar infection (Orsi *et al.,* 2009). Furthermore, NDV of low virulence induces similar immune responses without causing severe disease (Ali *et al.*, 2022).

All strains of NDV can propagate in specific pathogen free (SPF) embryonated eggs that are used for isolating the virus, producing vaccine and antigen for serological tests. In addition to this, many cell culture systems have been reported to be able to support the growth of NDV (Miah *et al.,* 2006). Among those are African green monkey kidney (Vero) cell and chicken embryo fibroblast cells (CEF) are able to support the growth of NDV (Ahamed *et al.,* 2004). NVI is currently manufacturing ND vaccines since certified specific pathogens free (SPF) embryonated eggs. Usually, these SPF eggs are imported and large numbers of eggs are needed to produce a batch vaccine with 5.0 million doses.

Thus, the production requires a significant amount of foreign currency to propagate these SPF eggs. On the other hand, the institute necessitates establishing a complex modular system with aseptic procedure, which seems feasible to invest on it and ultimately makes the price of the vaccine unaffordable for backyard village poultry farmers. In addition, production process using embryonated eggs takes longer time and manpower demanding compared to that of cell line based viral vaccine production. Therefore, it is paramount important to transform the embryonated eggs based ND vaccine production to CEF cell culture and cell lines such as Vero cell line.

1.3. Objectives of the study

1.3.1. General objective of the study

- To compare the adaptation NDV vaccinal strain in chicken embryo fibroblast and Vero cell lines and to evaluate the protective efficacy of the adapted ND vaccine in chicken.

1.3.2. Specific objectives of the study

- To compare cytopathic effects (CPE) of NDV I-2 vaccinal strain on monolayer of CEF cell culture and Vero cell line
- To determine the incubation time of the adapted strains at which produce more 80% CPE on selected monolayer CEF cell culture and Vero cell lines;
- To determine the viral concentration of adapted I-2 vaccinal strain at time producing higher CPE on CEF cell culture and Vero cell line;
- To evaluate the immunogenicity, safety and efficacy of ND vaccine produced by adapted vaccinal strains *in vitro* cell cultures in chicken model.

1.4. Hypothesis

H0: a NDV vaccinal strain adapted on CEF cell culture and Vero cell line provide the same ND virus, adaptation and have almost equal titration and the vaccine does not have a significant effect.

Ha1: The titration of CEF and Vero cell lines adapted ND Vaccine have could increase from passage to passage.

Ha2: CEF and Vero cell lines adapted ND Vaccine antibody titer have a significant difference when compared to NDV I-2 Vaccine produced by NVI.

1.5. Research question

- ✓ Is it practicable for the ND Virus vaccinal strain to multiply and adjust on CEF and vero cell lines?
- ✓ Is the titer of the ND vaccine produced using CEF and Vero cell lines comparable?
- ✓ Are there any significant variations in the HI titers of the ND vaccine produced after adaptation on CEF and Vero cell lines in the experimental chicken groups?
- ✓ Is there a significant difference between the ND I-2 vaccine produced in NVI and the vaccine produced using CEF and Vero cell lines?

1.6. Significance of the study

The intention of the present study was to compare the adaptation of lentogenic NDV on CEF and Vero cells, and how the viral CPE can vary remarkably according to the kinds of cell substrate used. Besides, the goal of this study was to assess the methods by which the ND virus is cultured *in vitro* under controlled conditions using *invitro* cell culture system in order to yield a significant amount of viral antigen to produce ND vaccine that is pure, safe, reliable, and efficacious. Furthermore, this study meticulously examined which approach is safe, appropriate, and effective for producing the ND vaccine. It further determined the NDV titer for each technique and assessed the vaccine's potential to protect against disease before choosing the best approach for producing the ND vaccine.

1.7. Expected outcome of the study

The purpose of this study was to test the hypothesis that ND vaccine manufacturing based on cell culture is preferable to that of SPF embryonated chicken eggs. As a result, the CPE of the adapted NDV I-2 strain on CEF and Vero cell lines was well known; the optimal time to harvest vaccine bulks after inoculation was established; the viral concentration at which high CPE was observed was established; and safety, immunogenicity and protective efficacy of the adapted I-2 strains were estimated. Finally, the best feasible solution for the challenge with using SPF chicken embryonated eggs for the manufacturing of ND vaccine was produced.

1.8. Scope of the study

This research focused only on the generation of knowledge regarding the titration, CPE and adaptation of Newcastle disease virus on CEF and Vero cells compared to SPF egg based production, and evaluating the protective efficacy of ND vaccine against challenge infection. In addition to this, the research focused on selecting the best, safe and suitable method for Newcastle disease vaccine production.

1.9. Delimitation of the research

This study only compared and assessed thermostable I-2 strain virus's ability to proliferate, adapt, and titer in chicken embryo fibroblast and Vero cell lines. It also assessed the vaccine's ability to protect chickens against challenge viruses. Due to time and budgetary constraints, the propagation and adaption of other vaccine strains of the Newcastle disease virus, such as HB1 and the La Sota strain, on these cell lines were not investigated in the present study.

2. LITERARURE REVIEW

2. 1. History of Newcastle Disease Virus

The first outbreak of Newcastle disease occurred in 1926 in Java, Indonesia and Newcastle Tygne town in Great Britain, from which it takes its name. It was also described the same year in Korea (Dzogbema *et al.,* 2021). Doyle was the first person that discovered NDV via laboratory experiment and noted it as to not relate to the Avian Influenza Virus (AIV). The greater spread of the virus is linked to the commercialization of chicken and their feed (Yajie, *et al.*,2023).The virus is introduced and spread to Ethiopia along transport routes and occurred in and around seaports ports of the country and initially the disease was found in 1971 in Asmera. Even though the outbreaks of the disease were recorded in Addis Ababa in 1972, Harer, Shola, and Bishoftu poultry farms in 1974 NVI, in Ethiopia. Particularly, the first NCD virus reported was a velogenic type, which was classified according to the virulence of the strain and caused about 80% mortality (Sahlu, 2015).

2.2. Description of Newcastle disease virus

The disease is caused by virulent strains of avian paramyxovirus type 1 (APMV-1), also known as Newcastle disease virus (NDV) (Alemneh, 2019). At present, there are 21 serotypes of avian paramyxoviruses designated APMV-1 to APMV-21 (OIE,2021). NDV belongs to the serotype 1 of avian paramyxoviruses. The genome of Newcastle disease virus are negative sense, single stranded, non-segmented RNA and negative polarity with a capsid of helical symmetry genomes (Figure 1). All the avian paramyxoviruses APMVs are part of genus Avulavirus. It has a diameter ranging from 100 nm to 500 nm (Kumar *et al.,* 2020). The NDV genome encodes for six structural proteins in 3’ to 5’ direction (Miller and Koch, 2013). NP (nucleocapsid protein), P (phosphate polymerase cofactor protein), M (membrane associated matrix protein), F (fusion surface glycoprotein), HN (hem agglutinin-neuraminidase surface glycoprotein), and large RNA dependent RNA-polymerase (L) (Figure 1) and two non-structural proteins, the proteins W and V are additionally created within the P gene during transcription of mRNA at editing site by insertion of guanines (Abdisa & Tagesu, 2017). All genes likely play important roles in modulating the capacity of the virus to replicate and cause disease (Dimitrov *et al.,* 2017).

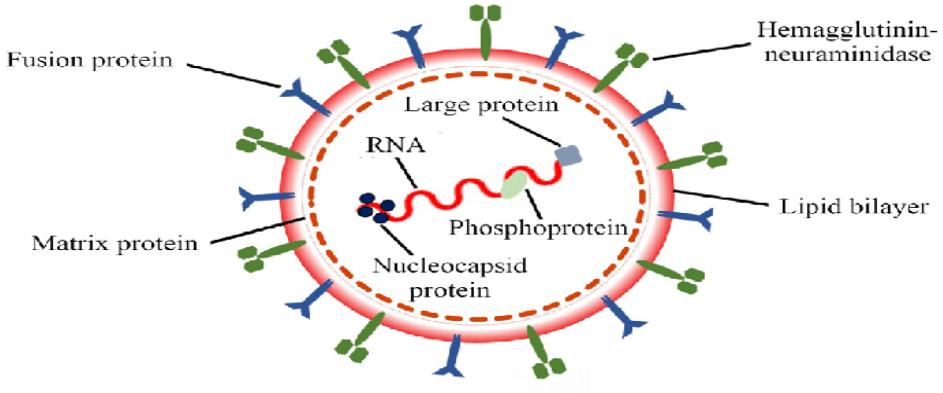

Figure 1: Schematic diagram of Newcastle disease virus structure

Source: Naz et al. (2022).

During gene expression the viral RNA dependent RNA polymerase binds the encapsidated genome at the leader region then sequentially transcribes each genes by recognizing start and stop signals flanking viral genes. The mRNAs are capped and polyadenylated by the L protein during synthesis. Most express a C protein by leaky scanning and V/W protein is produced through editing of mRNA (Figure 2) (SIB, 2019).

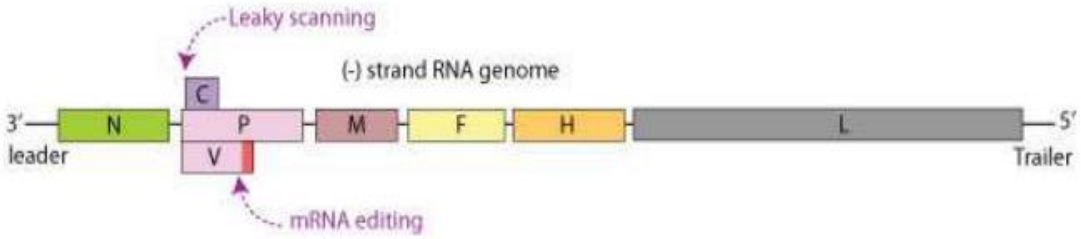

Figure 2: Genome organization of Newcastle Disease virus.

Source: SIB (2019)

2.2.1. Pathogenesis

Pathogenesis is the process by which infection leads to disease to the host leading to mortality and immunosuppression (Terefe *et al.*, 2015) . Viral replication occurs initially in the mucosa of the upper respiratory and intestinal tracts and the virus will be released and spread via blood as a secondary viremia to spleen and bone marrow causing infection of other organs such as lung, intestine and CNS (Yusoff & Tan, 2001). The extent of spread within the body relates to strain

virulence -determined by the amino acid sequence of F glycoprotein (Ashraf and Shah, 2014). The replication of lentogenic strains is confined to the respiratory and intestinal epithelia where suitable proteases are produced. This could increase the spread of virus in different which causes to pathognomonic clinical sign and death (Miller & Afonso, 2011). The vast majority of birds are susceptible to infection with ND virus, although the disease may vary enormously from one species to another species (Mossie, 2018).

2.2.2. Transmission and clinical sign of NDV

NDV is primarily transmitted via direct contact with secretions of infected birds; principally via inhalation or ingestion of virus shed in feces and fomites: feed, water, implements, premises, human clothing, boots, sacks, egg trays/ crates and respiratory secretions by infected birds for variable lengths of time (Brown & Bevins, 2017). Furthermore, virus has been found to be present in all parts of the carcass and is able to persist for many months on both chicken skin and bone marrow if kept at refrigerated temperatures. Virus is shed during the incubation period, during clinical stages and for a limited period during convalescence Fleas, rodent, insect and dog can also transmit ND virus mechanical from infected faeces (Chaka *et al.*, 2012). The incubation period varies from 3 to 8 days and the clinical consequences depend on factors such as: Virus type, Viral load ,Vaccination plan and type of vaccines, the age of host, environmental conditions and Immune status of birds (Alders *et al.*,2002). Chickens infected with virulent ND virus strains may die without showing any signs of illness. The chicken flutes its feathers and appears to have its coat dragging on the ground and normally ducks are resistant to the disease (El-Bagoury *et al.*, 2015).

Moreover, the major clinical signs of ND are depression, weakness, appetite loss, dehydration, inability to stand, cyanosis of comb, wattle, greenish watery diarrhea, nasal and eye discharges, decreased egg production, and loss of weight followed by death (Messaï *et al.*, 2019). The clinical findings for this species are further divided according to pathotypes. Mesogenic Newcastle viruses in field conditions cause mild clinical signs, mainly respiratory (Serbessa & Tucho, 2017). Field outbreaks with mesogenic strains also have been associated with a drop in egg production and misshapen eggs. Lentogenic Newcastle disease is generally accepted that

lentogenic viruses do not cause disease in adult chickens. Although some textbooks refer to La Sota as causing severe respiratory disease in very young animals, no peer reviewed references could be found in the scientific literature (Cattoli *et al.,* 2011).

2.2.3. Diagnostic techniques of Newcastle disease virus

Diagnosis of Newcastle disease involves consideration of flock history, clinical signs and postmortem lesions may establish a strong index of suspicion (Ranjith Babu *et al.,* 2015). There is no pathognomonic lesion and several birds must be examined before arriving at a tentative diagnosis (Roy, 2012). According to previous report Messaï et al.(2019), the serological techniques such as Hem-agglutination, hem-agglutination inhibition test for measuring NDV antibodies in chicken sera, virus neutralization test, Enzyme linked immunesorbent assay and plaque neutralization test and molecular techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) (Khurshid, 2018), can be used for confirmation of the ND virus. The surface of the NDV envelope exhibited both hem-agglutinin and neuraminidase activities. Hem-agglutinin will agglutinate different animals' red blood cells in vitro (mainly chicken red blood cells), and neuraminidase dissociates agglutinated red blood cells to release NDV (vita *et al.,* 2019; Abdel Rhman *et al.,* 2013). In addition to RT-PCR ,molecular techniques such as southern blotting, northern blotting and western blotting are used to diagnose ND and give the result within a short period of time, but PCR technique are more quick and accurate than the other molecular technique (Weedon, 2009).

2.2.4. Treatment, prevention and control of ND

There is no treatment for Newcastle disease, and, in many countries, infected and susceptible birds in the area of an outbreak are culled to contain transmission of the disease. Prevention is accomplished through vaccination and strict biosecurity (Betela *et al.,* 2023). However; strict biosecurity cannot achieve good results because of the difficulty in controlling the movement of people, chickens and wild birds as well as the control of the seasonal peaks in the marketing of the chicken. The use of vaccines could be one method that would be feasible in the control of the disease in village chickens (Mazengia, 2012). Thus, for the successful control of ND, new

vaccination technologies (such as the thermo stable vaccines that have been tried and tested and have scored successes in countries such as Ethiopia (Spradbrow, 2001).

2.3. Poultry industry and challenges of the ND

There are a number of risks associated with the introduction of infections, the ensuing development of disease, and the spread of diseases to other farms that are unique to each poultry farm. This risk profile is the result of a complex interplay between the degree of infection in a region, the disease control practices used on the farm, and other variables such as the number of farms in the area and connections to other farms and markets (Karcher & Mench, 2018). The production, immunity, and health of poultry pose a number of challenges to the poultry industry's ability to thrive in the future. The chicken industry's current state and strategic future will continue to be severely hampered by factors such as consumer confidence, product quality and safety, product types, and the introduction and reemergence of illnesses (Hafez & Attia, 2020).

Among several diseases impacting the poultry sector several domestic and wild bird species are susceptible to Newcastle disease, which is the most significant infectious bird disease that can spread to people (Abdisa & Tagesu, 2017). In many nations where agriculture is the main industry, the disease has spread quickly over the world and become endemic. According to the OIE (2021) all ND viruses under serotype 1 as well as the genus Avulavirus, sub-family Paramyxovirinae, and family Paramyxoviridae are the causes. Owing to the elevated rates of morbidity and death, this disease can have a catastrophic impact on poultry. Depending on the NDV's virulence, 100% of unvaccinated chickens may experience either morbidity or fatality. ND and its management in commercial (industrial) chicken production systems are well documented in the literature. Comparatively little information regarding ND control in village poultry has been reported, even though scientists are of the opinion that the disease is a major constraint to poultry development and productivity (Nwanta *et al.*, 2008).

Farm biosecurity measures, which include things like farm locations, physical infrastructure, and operational processes put in place, lessen but do not completely eliminate the danger of ND virus introduction or spread (Ashraf & Shah, 2014). Many countries employ both dead oil-based vaccinations and live viral vaccines to protect hens against illness. Long-term management of

this illness will depend on the creation of better vaccines against the newly developing ND lineages that can more successfully inhibit the reproduction of the virulent virus after infection (Bwala *et al.,* 2011).

2.4. Current status of Newcastle disease vaccines production in Ethiopia

Since ND was first identified, there have been a number of review articles published summarizing the current status of ND and the different control strategies available, however, ND still remains a threat to the poultry industry and backyard producers today. Several NDV strains have been cultivated which form the basis of many commercial vaccine seed stocks, and these include a range of avirulent, lentogenic and mesogenic strains. ND vaccines are available in the form of live, inactivated or attenuated and are licensed for use in many countries around the world (Mayers *et al.*, 2017).

Live attenuated vaccines, inactivated NDV vaccines, and recombinant vaccines have all been used to vaccinate birds against NDV. Unfortunately, the disease is still attacking and causing severe outbreaks. Two shots of primary vaccination with a live virus vaccine followed by booster vaccination with an inactivated vaccine also provided a better antibody response and reduced virus shedding following a virulent challenge as compared to both priming and booster vaccination with a live virus vaccine. Further, an inactivated booster vaccine prepared with a strain genotypically matching the challenge virus provides even better protection in terms of the prevention of virus shedding (Hossain *et al.*, 2023).

Table 1: The main APMV-1 strains utilized in current ND vaccine production

Virus strain	Phathotype	Vaccine type	Origin
Hitchner B1	Lentogenic	Live or inactivated	USA
LaSota	Lentogenic	Live or inactivated	USA
I-2	Avirulent	Live (thermostable)	Australia

Source: https://doi.org/10.1016/j.vaccine.2017.09.008

2.4.1. Conventional Newcastle disease vaccines

Commonly used traditional vaccines such as live attenuated vaccine and inactivated vaccine play important role in controlling economically devastating Newcastle disease from all over the world including Ethiopia (Orsi *et al.,* 2009). NDV strains that are low and medium in their pathogenicity such as mesogenic strains like Komorov and Mukteswar and lentogenic strains like HB1, LaSota, and I-2 or) were used to stimulate the immunity of birds against actual NDV infection (Bakhtiar *et al.,* 2022). However, each strain had their own advantages; for instance, HB1 had no post-vaccinal reaction in birds, while I-2 is more thermostable.

Mesogenic strains are more suitable as booster dose (Ali *et al.,* 2022). Although, factors such as maternal immunity, the virulence of a geographic locations endemic to NDV, the period between two subsequent vaccines, and the birds' lifetime can influence vaccination program which in turn causes adverse effect on the effectiveness of vaccine against ND (Hu *et al.,* 2022).However, previous studies showed that the live vaccines such as LaSota vaccine at a high dose could provide complete protection against mortality and clinical signs and significantly reduce challenge virus shedding to low levels despite the amino acid differences between the vaccine and challenge viruses (Ferreira *et al.,* 2021).

Currently in Ethiopia, live vaccines are commonly used in the poultry industry as they allow for the mass vaccination of a flock in the hatchery or the farm (Sasipreeyajan *et al.,* 2016). Live vaccines are easy to administer and can induce an early immune response, which induces cellular, mucosal, and antibody responses (Hossain *et al.,* 2023). The commonly used ND vaccines worldwide are live vaccine viruses of low virulence (lentogenic) that belong to genotype II (B1 and LaSota vaccines) (Igwe *et al.,* 2020). Live ND vaccines from lentogenic strains have been adopted to be used in the disease prevention since their first use until now. When ND vaccines are used as preventive measure, they must be carefully chosen according to the disease history of the birds (Asl Najjari *et al.,* 2017).

Another commonly used traditional vaccines in Ethiopia are inactivated vaccines which are used to control the outbreak caused by virulent Newcastle disease virus in chicken and turkey (Monir *et al.,* 2018). The conventional oil adjuvanted inactivated vaccines usually induce weak cell-

mediated immune response (Cheng *et al.,* 2016). In addition to this, the delivery of inactivated NDV vaccine through poly-lactic-co-glycolic acid (PLGA) nanoparticles in the natural host was safe and effective as evidenced by good seroconversion of the delivered antigens resulting in induction of high titers of HI and IgY antibodies that persisted for long duration as well as enhanced cell-mediated immunity (Aljumaili *et al.,* 2020). Inactivated vaccines, which can be formulated as genotype-matched vaccines, mount a stronger humoral immune response in birds than live vaccines and provide complete clinical protection, but they do not prevent virus shedding alone (Hossain *et al.,* 2023).

All inactivated vaccines are required to be checked for evidence of virus growth after inactivation to avoid the risk of their reversion to virulence (Ananda Kumar *et al.,* 2023). The inactivated NDV vaccine induces a high and prolonged immune response in vaccinated chickens and good protection efficacy against prevalent viruses (Hongzhuan *et al.,* 2020). The live and inactivated vaccines have their own merits and demerits (Zhao *et al.,* 2017). Live vaccines are relatively inexpensive and lend themselves to mass application. Local immunity is being stimulated by vaccination with live viruses and protection occurs very soon after application (Waheed *et al.,* 2013) whereas Inactivated ND vaccine with stringent vaccination schedule is more effective than attenuated live to minimize/eradicate ND viral shedding as well as its multiplication (Ali *et al.,* 2014).

2.4.2. Novel Newcastle disease vaccines

Veterinary vaccines need to have desired characteristics, such as being effective, inexpensive, easy to administer, suitable for mass vaccination and stable under field conditions. Accordingly, recent developments in the field of recombinant DNA technology have made it possible to create recombinant vaccine by cloning a gene encoding an immunogen or group of neutralizing epitopes into an expression plasmid (Cheng *et al.,* 2016). When the cell containing the recombinant plasmid is expressed and then the expressed protein is purified. This protein can be administered to the animal along with a suitable adjuvant and cause a protective immune response (Shafaati *et al.,* 2022).This recombinant vaccine have been used as potential solutions for poultry diseases since they are subunit vaccines with no risk of infection to virulence. In

addition to this, these vaccines are relatively easy to design and inexpensive to manufacture, store and effective delivery is required to induce a strong and long-lasting immune response. This can produce high and sustained levels of antigen production at targeted sites and has the ability to elicit both cellular and humoral immune responses (Jazayeri & Poh, 2019).

Another novel vaccine for prevention and control of ND is virus vectored vaccines. This vaccine depends on uploading the most important genetic materials (F and HN genes) of the NDV on another vector (carrier) virus (Ferreira *et al.,* 2021). The carrier virus is usually large in its genome size and has the ability to genetically express foreign genes. Among several virus vectored vaccine, Adenovirus vectors are highly efficient for gene transfer in a broad spectrum of cell types and species. Adenoviruses often induce humoral, mucosal and cellular immune responses to antigens encoded by the inserted foreign genes and induce potent immune responses in various cell lines as well as in chickens (Maremagae *et al.,* 2020).

Ads can efficiently infect a wide variety of cell types, have the capacity to grow to high titres *in vitro,* have high levels of transgene expression, lack integration into the host genome and show genetic stability. Thus, adenoviruses have become a vector of choice for delivery and expression of foreign proteins for vaccination (Ferreira *et al.,* 2005).In addition to this, cell culture technologies appear as the most promising strategies for the rapid development of new vaccine candidates to rapidly respond to circulating Newcastle disease genotypes (Farnós *et al.,* 2020). Moreover, another technology is Formulation of Nano-encapsulated vaccine tablet which is a novel technique for the delivery of ND vaccine to village chickens. Vaccine tablets were prepared using gelatin, trehalose and casein as thermo stabilisers and binders, respectively, and each vaccine tablet contained a nominal oral dose of Newcastle disease virus strain I-2 for a single chicken (Wambura, 2011).

2.5. Immunogenicity of Newcastle Disease Vaccines

In developing countries like Ethiopia, ND outbreaks are frequent despite immunization, causing significant economic losses. Concerns regarding the safety of live-attenuated vaccinations have been raised in light of the ND outbreaks in the immunized farms. This is because there have been insufficient investigations on the comparative immunogenicity of commercial vaccines

(Marahatta *et al.,* 2023). There are several potential causes of ND outbreaks in chicken farms, particularly following ND vaccination. Regardless of the delivery method eye drop or aerosol maternal antibodies to NDV significantly influenced the immunological response to the vaccine strains (Omony *et al.,* 2017).

Due to their ability to reproduce in chickens regardless of the location of administration, all live attenuated ND vaccines are known to induce systemic and mucosal immune responses similar to those of the natural infection. Incorrect dosage of vaccine is another potential. In order to address the issue of virus shedding linked to conventional ND vaccines, more advanced and economically viable immunization strategies are needed. On the other hand, tissue tropism has a significant role in determining vaccine efficacy (Illango *et al.*, 2005).

2.6. Cell Culture methods to produce ND vaccine

A significant amount of cells are needed in order to generate high titres of ND vaccines. Creating suspension cells at different scales is one way to increase the quantity of cells producing the ND vaccine (Ravindraa *et al.,* 2009). Small batch sizes are preferred for the flexibility of vaccines throughout the development and small-scale manufacture of ND Vaccine employing primary CEF, and adherent cultures are usually the most common. Production of ND vaccine in Vero cell line switches to bioreactors in the form of stirred tanks, rocking waves, and fixed bed bioreactors with multi-layer flasks straddling the intermediate scales when bigger and more consistent batch sizes are required (Perry & Rayat, 2021).

2.6.1. Growth and Adaptation of ND Virus on CEF

Primary cultures of CEF are widely used for the cultivation of various viruses for avian vaccine production typically for propagation, growth and adaptation of lentogenic ND vaccinal strain. CEF are derived from 10-12 days old embryonated egg/host tissues/ and generally will not subculture more than a few passages, depending on the host and the tissue. Some viruses may not plaque or grow in continuous cell lines, which is why the use of primary cell cultures is still employed (Hernandez & Brown, 2010).The virulent Newcastle disease viruses showed strong hem agglutination activity and the embryo died within 24 hours showing hemorrhage, congestion and encephalitic lesions (Miah *et al.,* 2006).

Different types of cells are used to cultivate and propagate NDV; CEF cells are the most popular throughout the world to grow NDV. Some of the velogenic and mesogenic strains produced a clear cytopathic effect (CPE) on the CEF cells, but without adaptation passages, lentogenic strain did not produce plaques on CEF cells. The ability of NDV to induce CPE is considered a reliable indicator of virulence. lentogenic strain of NDV has a cytopathic effect but fails to produce plaques in CEF cells within 96 h in the absence of magnesium and DEAE dextran (Mehrabanpour, 2017).

2.6.2. Growth and Adaptation of Newcastle disease Virus on Vero cell lines

ND virus can grow within different animal cells including primary cell culture and established cell line Arifinet *et al.*, 2011). Commonly used cell lines to replicate NDV are rabbit, pig, calf, chicken, monkey kidney cells, CEF, chicken embryo kidney (CEK), baby hamster kidney (BHK-21) and HeLa cells. Among commonly used cell lines, Vero cells fibroblast like cells from African green monkey kidney, are anchorage dependent in culture (Anam *et al.*, 2021).

According to Ahmed et al. (2004), it took five consecutive passages to adapt NDV to Vero cell lines. The NDV on Vero cells was maintained and Vero cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with nutrients. In passages one and two, wild NDV did not exhibit any definite signs of CPE; however, in passage three, alterations in the properties of the cell monolayer were noted. Within 50 to 60 hours of infection, during the fourth and fifth passes, distinct and consistent CPE were seen.

3. MATERIALS AND METHODS

3.1. Description of study area

The study was carried out from September 2023 to September 2024 at the NVI, located in Bishoftu town, Ethiopia. Bishoftu town is located 45 Km South East of Addis Ababa in Oromia Regional State (Figure 3). The area is located at 9^{o}N latitude and 40^{o} E longitude at an altitude of 1850 meter above sea level in central high land of Ethiopia with annual rainfall of 866 mm (National metrology Agency/NMA, 2010). The poultry population in Ethiopia is estimated to be about 48.96 million. According to the report of CSA (2020), indigenous breeds were estimated to be about 81.71%, while hybrid and exotic breeds share from the total population 10.86% and 7.43%, respectively. NVI is a public enterprise established in 1964 under the Ministry of Agriculture to produce veterinary vaccines and drugs as well as biologicals. The institute is accredited with ISO/QMS 9001:2015 for vaccine and drug production and ISO/IEC 17025:2017 for diagnostic laboratories.

Figure 3: Location of the study area.

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

3.2. Study materials

3.2.1. Cell, Media, solutions and culture condition

The media used for CEF and Vero cells replication were growth medium and maintenance medium, which were prepared from Glasgow minimum essential medium(GMEM) (Annex 1) with 10% tryptose phosphate broth(TPB) (Annex 2) and 2% sterile fetal calf serum (FCS)(Annex 3), respectively. Furthermore, CEF and Vero cell lines were prepared using working solutions such as Trypsin 0.05%/EDTA 0.02% solution (Annex 4), fetal calf serum, and phosphate buffered saline (PBS) (PH 7.2) (Annex 5), TPB, and 70% ethyl alcohol. Both CEF and Vero cell lines were cultured at 37^{o}C in humidified CO_2 incubator (5% CO_2). Additionally, a variety of tools and supplies during this research used were tissue culture flask, centrifuge, microscopes, sterile pippetes (multichannel, single channel, and glass pippetes with varying volumes), candling lamps, egg holders, magnetic stirrers, sterile magnetic bars, sterile beakers, sterile funnels and guaze, petridish, forceps, scissors, and others used throughout this study. NVI provided all of the media, functional solutions, and supplies needed to prepare CEF and Vero cell lines. The working solution and media were prepared in accordance with the manufacturer's instructions (Annex 1,2,3,4, and 5).

3.2.2. Source of the virus, CEF and Vero cell lines

Throughout the investigation, NVI's quality control (QC) laboratory provided working seed of the NDV thermostable I-2 strain with a titer of $10^{9.5}$ EID50/ml, CEF cell culture, and Vero cell line at passage 32. The working seed that was obtained from the QC laboratory was regenerated and subcultured in order to boost the virus's potency. This new working seed was then utilized to infect and adjust to the CEF cell culture and Vero cell line that would be required (Reed and Muench, 1938).

3.3. Experimental animals

The study used 80 unvaccinated, apparently healthy, 14-day-old Saso breed chickens hatched from SPF eggs (Annex 6) and reared together in a disease-free house in the NVI (Bishoftu, Ethiopia) until grouping for the experiment. After the chickens reached 14 days old and the

clinical trials were begun, Chickens of all age group, without sex and breed limit were assigned randomly using the complete random design approach and grouped into four (4) experimental groups each having twenty (20) chickens in each treatment group (Annex 6). Groups one, two, three and four were designated as CEF-adapted NDV I-2, Vero cell line-adapted NDV I-2, commercially available NDV I-2, and negative control, respectively.

3.4. Study design

In vitro and *in vivo* experimental designs were employed to compare the CPE, adaptation, and titration of NDV in CEF cell culture and Vero cell line. In addition, the cell line-based experimental vaccine's immunogenicity, efficacy, and safety were evaluated in chickens using three main procedures. These procedures further examined at the virus's adaptation and CPE in the two cell lines. First, the NDV I-2 strain was allowed to adapt to CEF primary cell culture and Vero cell line monolayers through a serial passages. Second, the NDV-Infected Cell culture Fluid (ICF) of each passage was assayed using HA and HI tests with RT-PCR to validate the identity. After trial vaccines were prepared, the immunogenicity, safety and efficacy of the CEF cell culture and Vero cell line adapted ND I-2 vaccines were evaluated. Chicken handling and experiments were approved by Ethical clearance certificate Ref. No. NVI/778 /26/09/2023 held according to the animal handling and use guidelines set by Animal Research Ethics Committee of NVI.

Eighty exotic saso breed chickens were randomly assigned into four groups of each containing 20 chickens. All chickens were raised in isolation bio-secure house, supplied multivitamins with water and feed administered ad libitum. Experimental chickens group one to three were immunized with CEF cell culture and Vero cell lines adapted ND thermostable I-2 vaccine and commercially available ND I-2 vaccine through ocular route. For 63 days until the end of the experiment, vaccinated chickens were inspected twice a day for clinical symptoms and mortalities, and data were recorded. The adverse vaccine reactions in 14,21,28,35,42 and 49-day-old chickens were assessed by the number of chickens presenting with clinical signs and survived within six consecutive weeks period.

HI tests was performed after serum sample was collected weekly for six consecutive weeks at 0, 7,14,21,28 and 35 days post immunization (dpi) from each vaccinated and control groups to evaluate the antibody titer. The experiment groups 1 – 3 vaccinated at day zero (age of 14 days) and a boosted at 21 days (age of 35 days old) with CEF adapted ND I-2 vaccine, commercially available ND I-2 vaccine and Vero cell line adapted I-2 vaccine respectively. On day 35 (at age of 49^{th} days), the vaccinated and unvaccinated chicken from groups 1, 2, 3 and 4 were challenged by inoculating the wild ND virus. Each chicken were challenged through the nasal route by inoculating 0.2 ml of ELD 50 of the challenge ND virus.

Then, the chicken was followed for 14 days post challenge (dpc) by supplying feed and clean public tap water ad libitum. After all groups of experimental chickens were challenged by velogenic NDV, postmortem examination was performed and Brain, spleen and tracheal sample from unvaccinated and challenged chickens showing clinical sign of NDV and died within six days of post challenges were collected using sterile surgical blade, universal bottles and gloves. Samples were transported in medium of sterile phosphate-buffered saline (PBS) pH 7.2 with antimicrobial additives (penicillin 10,000 units, streptomycin sulfate 10 mg and amphotericin B 25 µg per ml) to the National Veterinary Institute Research and diagnostic laboratory and stored at -80^0C until processing.

3.5. Preparation of CEF and ND Virus Cultivation

3.5.1. Preparation of CEF

A CEF primary cell culture monolayer was prepared from ten-day-old SPF avian embryonated eggs. To check fertility, the eggs were candled and the location of the air sac was marked.The embryos were identified as dead, infertile, or fertile, as shown in Figure 4.

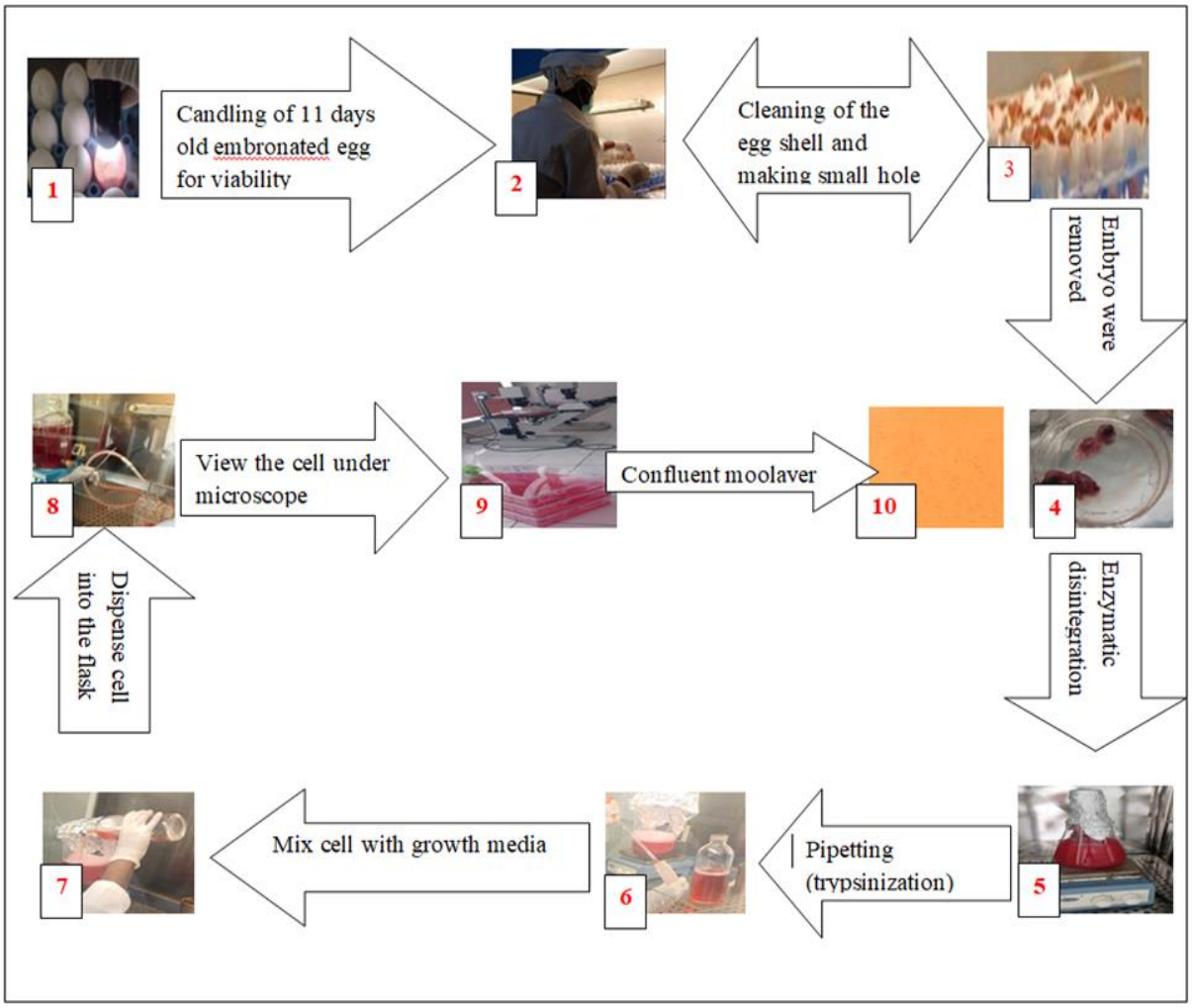

Figure 4: CEF preparation protocol.

The eggs that contained dead or non-viable embryos were discarded while the embryos were aseptically extracted from viable eggs and put into a sterile petri dish, as depicted in Figure 4, after the eggs were disinfected with 70% ethanol. The embryos' head, legs, wings, and viscera were severed and discarded after sanitised in cooled PBS with the required antibiotics. The remaining body part was further washed three times with PBS then mechanically chopped with scissors into tiny pieces. The washed minced tissue were transferred to Erlenmeyer trypsinizing flask containing sterile magnetic bar and trypsinized and shaked gently with 10 ml of prewarmed trypsin/EDTA solutions per embryo for 15 minutes at 37 ^{0}C (Annex 7). Trypsin was inactivated by adding 0.5 ml fetal calf serum and the cell suspension was filtered through two layers of sterile cheese cloth to another conical tubes and the supernatant containing the cell suspension

was transferred to a tube and centrifuged for 10 min at 2500 rpm at room temperature. The cell sediment was trypsinized with a pippete and mixed with growth complete medium. Finally, the cell was counted using a hemacytometer as previously described by Strober (1997). According to Hernandez & Brown (2010), a single embryo should yield approximately 1.0×10^6 to 10^7 viable cells/ml based on this number, the number of cells were adjusted to 5.4×10^7 cells/ml (Annex 8).

3.5.2 Cultivation of NDV I-2 strain into CEF monolayer

Total of seven flasks (75 cm^2) with confluent monolayer of both CEF cell culture and Vero cell line were prepared (six for infection and one control) and labelled for inoculation with ND virus I-2 strain into cell suspension using face to face and adsorption of virus inoculation method, respectively. The cell suspension was distributed into each tissue culture flasks and ready for virus inoculation. Based on this, 0.5ml, 1ml and 1.5ml of the renewed working seed of NDV thermostable I-2 strain with a titer of $10^{9.5}$ EID50/ml were inoculated into 50 ml of three culture flasks containing cell suspension using face to face method at the right time, place and materials. Similarly 0.5ml, 1ml and 1.5ml of virus was inoculated into 50ml of another three culture flasks containing cell suspension using adsorption method. The virus supplemented with growth medium was homogenized using magnetic stirrer and the suspension was incubated at 37 ^{0}C for 18 to 24 hours to get a full monolayer.

Following this protocol, eight subsequent passages were conducted and the characteristic CPE such as growth rate, morphology and the change in culture media were carefully examined in all passage. The time for the appearance and intensity of CPEs was also be observed from inoculation date until the growth media was changed to acidic PH and harvested blindly and recorded in each passage daily. Tissue culture flask was visually inspected and CPE of 80% infectivity were subjected for harvest. According to Ratta, et al. (2008), the acidic PH may trigger the release of virion into the cytoplasm and that is why the viral suspension should be harvested blindly until CPE was observed. While viruses were just beginning to adapt and infect CEF cells in the first and second passages, no discernible changes in CEF cell shape or early CPE were seen in the third passage. During the fourth passage, the form and nucleus of the cells began to gradually change, and some CEF cells began to collect on the monolayer in various

locations. Syncytium, large cells were formed 48, 72, 96, and 108 hours after infection in each passage, indicating the typical CPE. Flasks with suspension/bulk were harvested and stored at -20ºC until they are pooled and tested for sterility (Annex 9).

3.6. Sub-culturing of Vero cell adherent monolayer and cultivation of ND virus

3.6.1. Sub-culturing of Vero cell lines

The cell was sub-cultured to confluent monolayer in 75 cm^2 tissue culture flasks in side biosafety cabinet. The growth media from the flask containing Vero cell was removed and followed by washing with sterile PBS 3 times. Then 5 ml of trypsin (0.25%) were added into the flask and stay at room temperature for 30 second, until the action of trypsin was started, then 3ml of trypsin were discarded and the remaining 2ml were incubated for 5-10 minutes to detach cell from the flask wall. After adding 10ml of the growth media, gentle pipetting was done to detach from the flask wall. The mix was transferred to a centrifuge tube and centrifuged at 2500 rpm for 10 minutes after which the supernatant was discarded to get the cell pellate.

Ten ml of the cell was poured in the new culture flask containing 150ml growth media and distributed and dispersed to three new culture flask each containing 50ml of cell suspension (figure 5) and incubated at 37ºC in the presence of 5% CO_2 and cultured cells were observed carefully under inverted microscope until the formation of 70 or 80% confluency monolayer development (figure 5). According to (El-Bagoury,et al.,2018 and Sheets, 2000), the optimum anchorage dependent cells of the Vero cell is 1.5 $\times 10^5$ to 10^7 cell/ml is required for the propagation of the virus. Based on this, the cell was counted using a hemacytometer and adjusted to 1.47 $\times 10^6$ cell/ml (Annex 10).

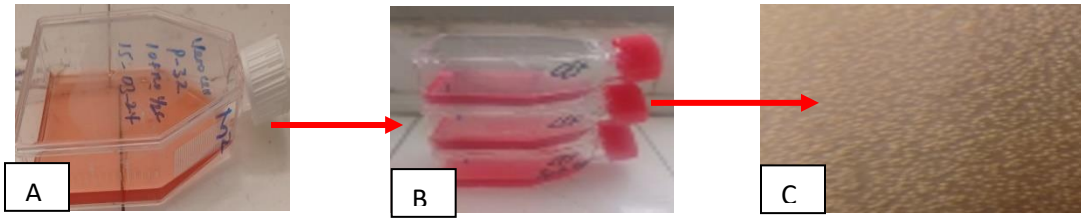

Figure 5: Sub culturing of Vero cell lines.

(A) 27 hrs of confluent monolayer of vero cell lines,**(B)** vero cell line after subculturing and **(C)** morphology of vero cell lines under inverted microscope after subculturing.

3.6.2. Cultivation of thermostable I-2 strain in to Vero cells monolayer

Normal and confluent monolayer of Vero cells at 27 hours after sub culturing were used for infection with NDV I-2 strain. Old growth medium from the flask of 75 cm^2 containing Vero cell with confluent monolayer was removed and cell monolayer was washed with sterile pre warmed PBS three times. Then confluent monolayer Vero cells was inoculated with ND virus with the volume of 0.5ml, 1ml, 1.5ml, 2ml and 2.5ml of ND I-2 strain with sterile working seed of a titer of $10^{9.5}$ EID50 /ml virus in GMEM Supplemented with cell culture fluid over the surface of confluent monolayer and the virus inoculums was spread uniformly over the monolayers and incubated in humidified CO_2 incubator at 37°C for 1 hour with intermittent rotation to allow adsorption.

One flask was kept as un-inoculated for control. Sterile maintenance medium with 2% of serum was added to each flask. The flasks were incubated at 37 ^oC in the presence of 5% of CO_2 in CO_2 incubator and monolayers was examined twice daily under inverted microscope for evidence of CPE from inoculation date up to five days and harvested blindly passed until evidence of CPE were observed for fifteen consecutive passage. The characteristic CPE was carefully examined and the time for the appearance and intensity of CPEs was also recorded in all passage. An infected monolayer was removed from the flasks and transferred to another tubes for further processing (Hussain and Rasool, 2005).

3.7. Detection of NDV I-2 strain

3.7.1. Haemagglutination test

The red blood cells was collected in 1:1 ratio with alsever's solution, centrifuged at 2500 rpm for 10 min to separate alsever's solution and other debries then washed with PBS (pH 7.2) and centrifuged at 2500 rpm/min for three times and finally a 10% and 1% Chicken RBC were prepared from chickens blood in order to perform rapid haemagglutination test and micro haemagglutination (HA) test in a V/U-bottom micro well plate respectively for agglutination of

chicken red blood cells according to OIE protocol (OIE, 2021a).This allows proving the presence of any hemagglutinating agents in the harvested infected cell culture fluid (ICF). Those test positive for HA were confirmed by HI test (Annex 11).

3.7.2. Haemagglutination inhibition test

According to OIE (2021), haemagglutination inhibition test (HI) was used to test both Antibody titer and virus or antigen potency. The harvested infected cell culture fluid (ICF) that were tested positive by HA was subjected to HI. The test was based on the inhibition of haemagglutination by monoclonal specific anti sera against NDV. If the collected infected cell culture fluid have the suspected ND virus, nucleic acids of the viruses encoding hemagglutinin proteins expressed on the surface of the virus bind to or clump erythrocytes creating a lattice, which settle irregularly in the bottom of the microtiter well. Specific antibodies inhibit such agglutination. In the absence of ND virus, the erythrocytes do not clump; instead they form a compact button (Florman, 1947; OIE, 2021). Serum samples were inactivated at 58^0C in water bath and two fold serial dilution of NDV I-2 vaccine virus titer were prepared up to dilution 12 in micro titration plate and 8 HA titer of the virus were obtained.

According to NVI test protocol eight Haemagglutination unit (HAU) of the antigen were prepared for the HI test. A 25 µl PBS were added per each well of the micro titration plate, then 25 µl of the serum samples were added in the first well of the V-bottom micro titration plate from 1 to 10 11^{th} column positive control and 12^{th} column negative control, and two fold serial dilution were done up to well No A to H vertically. A 25 µl of 8HAU antigen/I-2 was added per each well and incubated at room temperature for 15 minutes. Finally, 50 µl of 1% chicken RBC were added per each well and incubated at room temperature for 30 minutes. The agglutination was judged by tilting the plates. The wells with RBCs stream at the same rate as the control wells (positive serum, virus and PBS controls) were considered to show inhibition (Annex 11). A cut off value $\geq Log2^3$ were used as a standard positive (OIE, 2021).

3.7.3. Molecular Identification and confirmation of the virus

To detect the presence of NDV, tissue culture supernatant was subjected to slide HA test following the standard procedure (Alexander, 2009). One drop of collected infective culture fluid was taken on a clean glass slide and two drops of 10% freshly prepared cRBC suspension was added and mixed thoroughly. The appearance of clumping of the cRBC on the glass slide within 1 to 2 minute was recorded as the presence of hem agglutinating virus in the infective cell culture fluid. The positive haemagglutinating cell culture fluids were confirmed in Hyperimmune chicken anti-NDV serum, raised in chickens by repeated inoculation with a NDV vaccine virus. In addition to this, the presence of NDV in tissue culture supernatant was further reconfirmed by RT-PCR.

RNA extraction

RNA extraction was performed from ICF harvests confirmed positive by HA and HI test and extracted according the procedure described by the manufacturer (QIAGEN, Germany) RNeasy Mini Kit. ICF was thawed and vortex to mix. A 100 µl volume of the homogenized ICF harvests and 350 µl of RLT buffer were transferred to Eppendorf tube and then homogenized by vortex then centrifuged at 13,000 rpm for 30 second. After centrifugation 250 µl of 96% ethanol alcohol were added to the homogenized lysate and mixed by pipetting. A volume of 700 µl of the sample were transferred to RNeasy mini column membrane to bind nucleic acid into membrane and centrifuged for 30 sec at 13,000 rpm, the supernatant/liquid after centrifugation were discarded.

A volume of 500 µl Buffer RW1 were added to each RNeasy spin column membrane, then centrifuged for 30 second at 13,000 rpm and discarded the flow-through liquid. Again a volume of 500 µl Buffer RPE were added to each RNeasy spin column membrane, and then centrifuged for 2 minute at 13,000 rpm and the supernatant was discarded. The mini column tube were opened and centrifuged for 1 min at 13,000rpm to evaporate alcohol and the supernatant were discarded. Finally, RNeasy spin column were placed in a new clean tube and 40 µl of RNase free water were added on to each membrane of mini column and centrifuged for 1 min at 13,000 rpm and RNA template were collected.

Master Mix preparation

Master Mix was prepared in a separate room. The reaction mix for selected ICF at the required passage was conducted. The following sequence primers were used for amplification of the M-gene. An amplicon size around 121bp primers APMV1 forward, 5pm/µl 5' AGTGATGTGCTC GGACCTTC 3' a volume of 2 µl and NCDV APMV1 Reverse, 5 pm/µl 5' CCTGAGGAGAGG CATTTGCTA - 3' a volume of 2 µl targeting to amplify the matrix were used. RNase free water 8 µl, 5X RT-PCR buffer 5 µl, Q – solution 5 µl, 10mM dNTPs mix 1 µl, one step RT-PCR enzyme mix 1 µl and Template RNA extracts 5µl were mixed for one reaction volume of 29 µl of master mix were prepared.

Reverse transcriptase polymerase chain reaction

In amplification room, the template RNA volume of 5 µl was added to the Master Mix in biological safety cabinet. Amplification of the M-gene was performed in a 29 µl reaction volume in Applied Biosystems 2720 Thermal Cycler (Table 2).

Table 2: One step RT-PCR process

	Temperature	Time	Cycle
cDNA synthesis	50^{oC}	30 minutes	1 cycle
Initial denaturation	95^{oC}	15 minutes	1 cycle
1^{st} denaturation	94^{oC}	30 second	
Annealing	55^{oC}	30second	40 cycle
Elongation	72^{oC}	30second	
Final elongation	72^{oC}	5minutes	1 cycle
put the product at	4^{oC}	Until machine off	

Gel electrophoresis

A 1.5% agarose gel was prepared, and 4 µl gel red with loading dye were added. Then 10 µl of each PCR product and 10 µl of molecular marker (ladder) started at 50 and 100 bp plus were loaded in the gel. Electrophoresis was conducted in 1X Tris-Borate-EDTA (TBE) buffer in

agarose gel electrophoresis System (BIO - RAD - USA, Thermo EC - 2060) for one hour and 20 minutes at 120V electric current supply. A DNA ladder started with 50 and 100-bp increments was used as a molecular weight marker. Thereafter, gels was visualized under UV light using UV trans illuminator (Uvitec UK, Cambridge CB4 - 1QB - UK) and photographed by a digital camera (Uvitec UK).

3.8. NDV adapted on CEF and Vero cell lines infectivity titration in microplate

The titer of CEF and vero cell adapted NDV in each serial passage were measured. 9ml of complete GMEM with 10% calf serum and 10% TPB were dispensed into each test tube and 1 ml of virus containing fluid (supernatant) was serially passed in ten tubes containing 9 ml of growth medium (GMEM) for each passage separately. After serially distributing 1ml of viral suspension to each test tube, 10-fold dilutions of virus previously prepared in glass test tubes were transferred to the microplate. One hundred µl CEF and Vero cells containing 10^6 cells per ml were dispensed into all wells of the first eight rows of the Microtitration plate leaving 11^{th} column as a line of demarcation.

One hundred µl of GMEM was added in to the first eight wells of 12^{th} column for control. 100µl serially diluted virus was added to all wells of eight rows up to 10^{th} column starting from low concentration to higher one. This was the same procedure for all passages to determine the titer of each passage. The microplates were then covered by microplate sealer and were incubated 37^oC in the presence of 5% of CO_2 in CO_2 incubator until CPE were observed and examined twice daily for CPEs. Cell growth and the condition of the tissue cultures after infection were observed with the aid of an inverted microscope. The titers for each virus passages were determined according to Spearman Karber formula (Karber, 1931).

3.9. Preparation of trial vaccine

The culture supernatant CEF and Vero cells adapted NDV I-2 10 passage were harvested by 3 times freeze-thaw cycles, clarified by centrifugation at 3000 rpm for 10 minute. Then sterile supernatant mixed with 10% sucrose and 5% lactalbumin hydrolysate (annex 12) was added to help preserve infectivity during storage. The formulated vaccine were tested and confirmed free

of viable bacterial and fungal contaminants that could be harmful to chickens receiving the vaccine during experiments. Sterility was checked by using sterility media such as soyabean-casein digest medium (SCDM) for aerobic bacteria and fungi incubated at 37^0c and 25^OC 14 days and broth, thioglycollate broth for culture of anaerobic bacteria and incubated at 37^0c for 7 days (OIE, 2021).

3.10. Embryo mean death time (MDT) determination

The MDT is the minimal lethal dose required to kill embryos in the meantime, which is measured in hours. Parthiban et al. (2022) report that the virulence of the isolated Newcastle disease virus was classified as mesogenic strains/moderately virulent, which take 60–90 hours to destroy an embryo; lentogenic strains/low virulent take longer than 90 hours; and velogenic/highly virulent strains require less than 60 hours to destroy an embryo. In order to determine the MDT for embryo death, allantoic fluids of the locally isolated Newcastle disease virus which was stored in research and development laboratory of NVI were used. Next, in a level II biological safety cabinet, tenfold serial dilutions of the allantoic fluid of the viral isolates were generated using 11-day-old SPF embryonating chicken eggs. According to Hanson and Brandly's (1955) instructions, 0.1 ml of the dilutions between 10^{-5} and 10^{-10} was injected into 5 eggs per dilution in the allantoic cavity. The small holes were then filled with melted paraffin wax, and the eggs were incubated at 37 °C in a humidified egg incubator. The highest dilution at which all embryos die was considered as mean lethal dose (MLD).

3.11. ND vaccine efficacy evaluation on experimental chicken and management

The efficacy of the ND vaccinal strains, Thermostable (I-2) produced in CEF and Vero cells, was assessed from April 2024 to July 2024 at Research and Diagnostic Laboratory of NVI Animal Experiment facility. Before experiment chicken rooms were cleaned and disinfected by 10% sodium hypochlorite then kept free for two weeks. A total of 80 chickens were hatched from SPF eggs and were assigned randomly in to four experimental groups each having twenty chickens.The four groups except the control group, each with 20 chickens were vaccinated through the intraocular route (Table 3). The experiment groups 1 – 3 vaccinated at day zero (age of 14 days) by ND virus produced in CEF, SPF Embryonated egg and Vero cells (Annex 13) and

each group were inoculated a booster at 14 days with commercially available ND I-2 vaccine. Before each vaccination blood was collected from each vaccinated and control groups to evaluate the HI titer.

Table 3: Immunization schedule of experimental chicken

Experimental group	Type of vaccine administered	Age of chickens **1st vaccination**	**booster**	**Route of vaccination**	**Dose of the vaccine**
1	CEF-NDV-I-2	14 days	35 days	Intraocular	$10^{6.5}$ TCID50/ml
2	VERO-NDV-I-2	14 days	35 days	Intraocular	$10^{6.5}$ TCID50/ml
3	C-NDV-I-2	14 days	35 days	Intraocular	$10^{6.5}$ TCID50/ml
4	Not vaccinated	14 days	-		

Where: CEF-NDV-I-2 = CEF adapted Newcastle disease thermostable vaccine

C-ND-I-2-V= commercially produced NVI Newcastle disease thermostable vaccine

Vero-NDV-I-2=Vero cell lines adapted Newcastle disease thermostable vaccine

$TCID_{50}$=tissue culture infective dose fifty

3.12. Serum Collection

Blood samples of 3ml were collected from each experimental chicken by using sterile 3ml disposable syringe (guage 22Gx1¼"). Each chicken were handled gently, the site of blood collection were disinfected by 70% ethyl alcohol and blood were collected from the wing vein. A volume of 2-3 ml blood was collected from each chicken; the syringes was kept in horizontal position for 3-4 hours at room temperature. Finally the serum was decanted in to sterile cryovial and stored at -20^{o}C until HI test conducted to evaluate the antibody titer. The chickens were supplied multivitamins after each blood collection. Blood was collected weekly for six (6) consecutive weeks starting from day 0, age of 14 days to day 35,age of 49 days before and after each vaccination from each vaccinated and control groups to evaluate the antibody titer (Annex 14).

3.13. Chicken challenge model

On day 35 (at age of 49th days), the vaccinated and unvaccinated chicken from groups 1, 2, 3 and 4 were challenged by inoculating the wild ND virus. Each chicken were challenged through the nasal route by inoculating 0.2 ml of ELD 50 of the challenge ND virus. Then, the chicken was followed for 14 days post challenge (dpc) by supplying feed and clean public tap water ad libitum. Recording of the clinical signs, mortalities were performed and samples for postmortem examination were collected from chickens showing clinical signs and from the control groups. Laboratory confirmations were performed for the collected samples to examine any of the clinical signs and mortalities. Chickens protection from the virulent virus challenge was determined by absence of clinical signs or absence of death within 14 dpc.

3.14. Data Management and Analysis

Collected data from laboratory investigations and experimental trial were coded and imported into Microsoft Excel for window 2010. The statistical Data were analyzed by calculating geometric mean titers (**GMTs**), mean ± SD through Statistical Package for the Social Sciences (SPSS) version 25 software. To analyse (GMT + SD) for both the vaccinated and unvaccinated groups, a Microsoft spreadsheet was used. The repeated measure ANOVA was performed to assess whether there were any significant differences in the HI titres of chickens in a group following the first and second vaccinations on the basis of research objectives. During statistical analysis, the significance level was set at a p-value of 0.05 for detecting a significant difference. After the statistical analysis, the results were presented in tables, figures and graphs.

3.15. Ethical clearance

The experimental animals were treated according to the protocol of ethical guidelines of the Animal Welfare Committee of NVI. They were kept and managed in the animal facility of NVI and used only for this experiment. For this purpose, ethical clearance was obtained from Animal Research Ethics Review Committee of NVI (Annex 15).

4. RESULTS

4.1. Adaptation of NDV I-2 strain on CEF and vero cell lines

The NDV I-2 strain was adapted on CEF cell culture through six consecutive passages. The Haemagglutination (HA) test and molecular confirmation for the identity of NDV were done for each passage and each passage were positive for NDV and this indicates the presence of ND virus on CEF monolayer. However; in the first to second passages, virus did not produce any clear change in CEF cells and viruses were just started to adapted and infect the CEF primary cells, but in the third passage change in cell morphology and early CPE characterized by rounding of cells were observed as shown in Figure 6, nevertheless, CPE of I-2 strain of NDV on CEF characterized by rounding of cells, aggregation and syncytial formation were obviously observed on the fourth, fifth, sixth, seventh, and eighth passages.

Thus, the development of CPE indicates multiplication of the thermostable I-2 strain of NDV and can be another indication for its successful adaptation on the CEF. The virus showed the same type of CPE in Vero cell line in the form of aggregation, rounding of cells and formation of syncytial. Moreover, the mean titer at passage three, four, five, six, seven, and eight were $10^{3.9}$, $10^{5.5}$, $10^{6.5}$, $10^{6.7}$, $10^{7.5}$ and $10^{7.65}$ TCID50/ml; respectively, as shown in Table 4 and figure 7. This indicates that the mean titer of NDV adapted on CEF were increase from passage three to passage eight as shown in Figure 6 and this results was in agreement with OIE (2021) recommendation, where the minimum titer for producing ND Vaccine is $10^{5.5}$ EID50/ml.

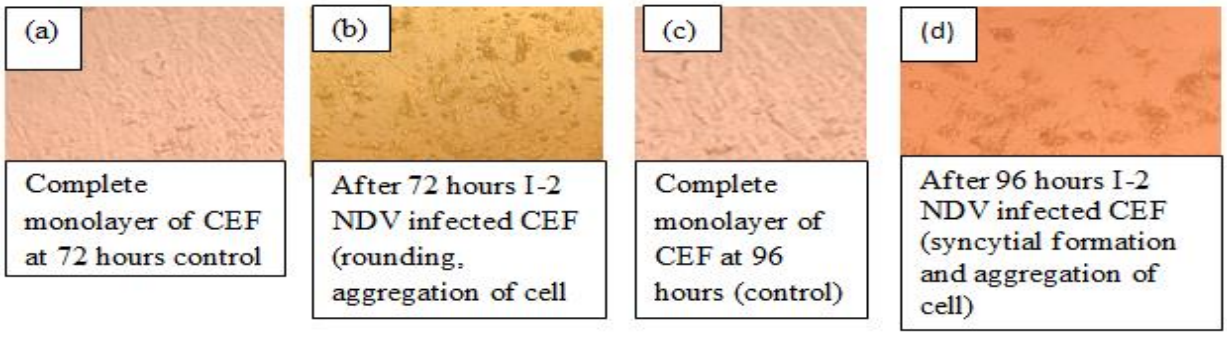

Figure 6: Morphological alteration of CEF after infection with ND I-2 virus.

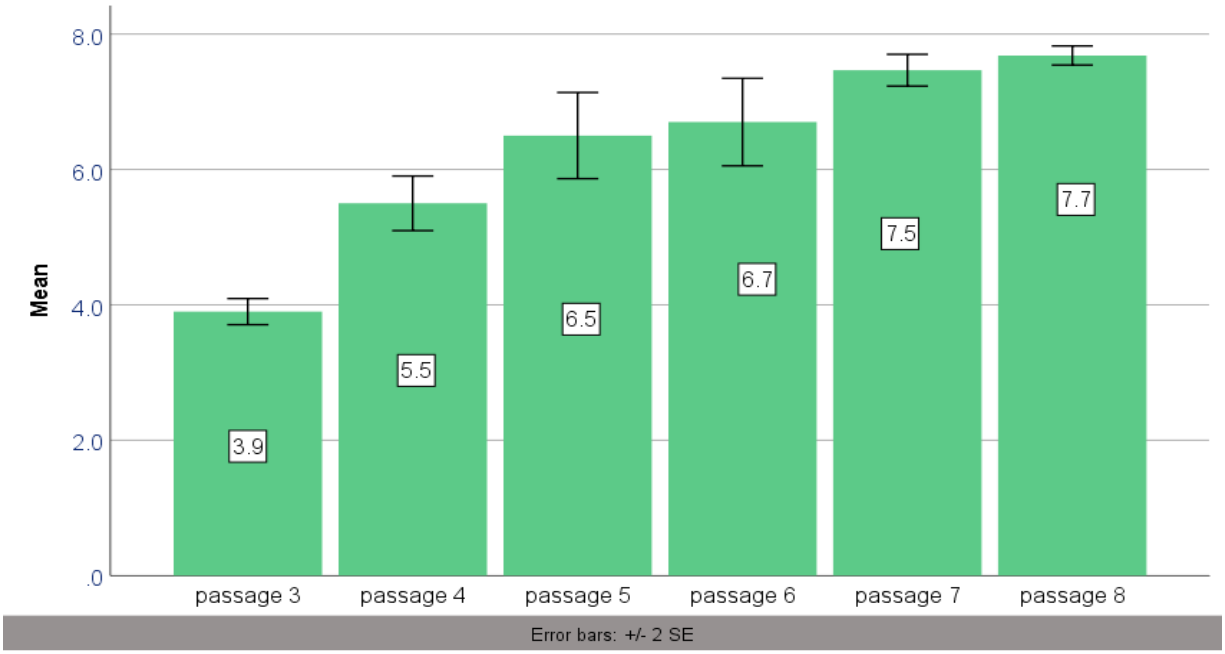

Figure 7: Error bar graph showing the incremental trends of titer/ml of CEF adapted ND I-2 vaccine.

Another cell lines used for adaptation of NDV I-2 strain was Vero cell line. Fibroblast like confluent monolayer of Vero cells was established after 27 hours of incubation using GMEM growth medium. The NDV I-2 strain was adapted on Vero cell line through starting from ten passages. However, there were no any CPE were observed on Vero cell line until the 10^{th} passage. It may be due to adjustment of virus on Vero cells, where during passage 10, few alterations on Vero cell monolayer were observed after 48 hour of inoculation. Consistent and clear CPEs were appeared on passage 11 after 72 hours of inoculation. The cells gradually started to change in shape and size. CPE was charaterized by granularity in cytoplasm, clustering of infected cells to develop syncytial rounding and separation of cells from monolayer accompanied by death (Figure 8).

CPE was initiated at passage three in CEF cell culture, however it was initiated at passage ten in Vero cell lines. The characteristics of CPE were comparable in Vero cell lines and CEF cell culture. Thus, the thermostable I-2 strain of NDV confirmed its successful adaptation in CEF and Vero cell lines which tried to answers for the research question that, whether or not Newcastle disease virus thermostable I-2 strain propagate and adapt on CEF and Vero cell lines. Moreover,

the mean titer of passage ten, eleven, twelve, thirteen, fourteen and fifteen were $10^{5.3}$, $10^{5.6}$, $10^{5.7}$, $10^{6.1}$, $10^{6.4}$ and $10^{6.6}$ TCID50/ml respectively (Table 4 and Figure 9). The finding of this study was also tried to answers the research question whether CEF adapted ND virus have comparable titration or does not have equal titration with Vero cell lines adapted ND virus.

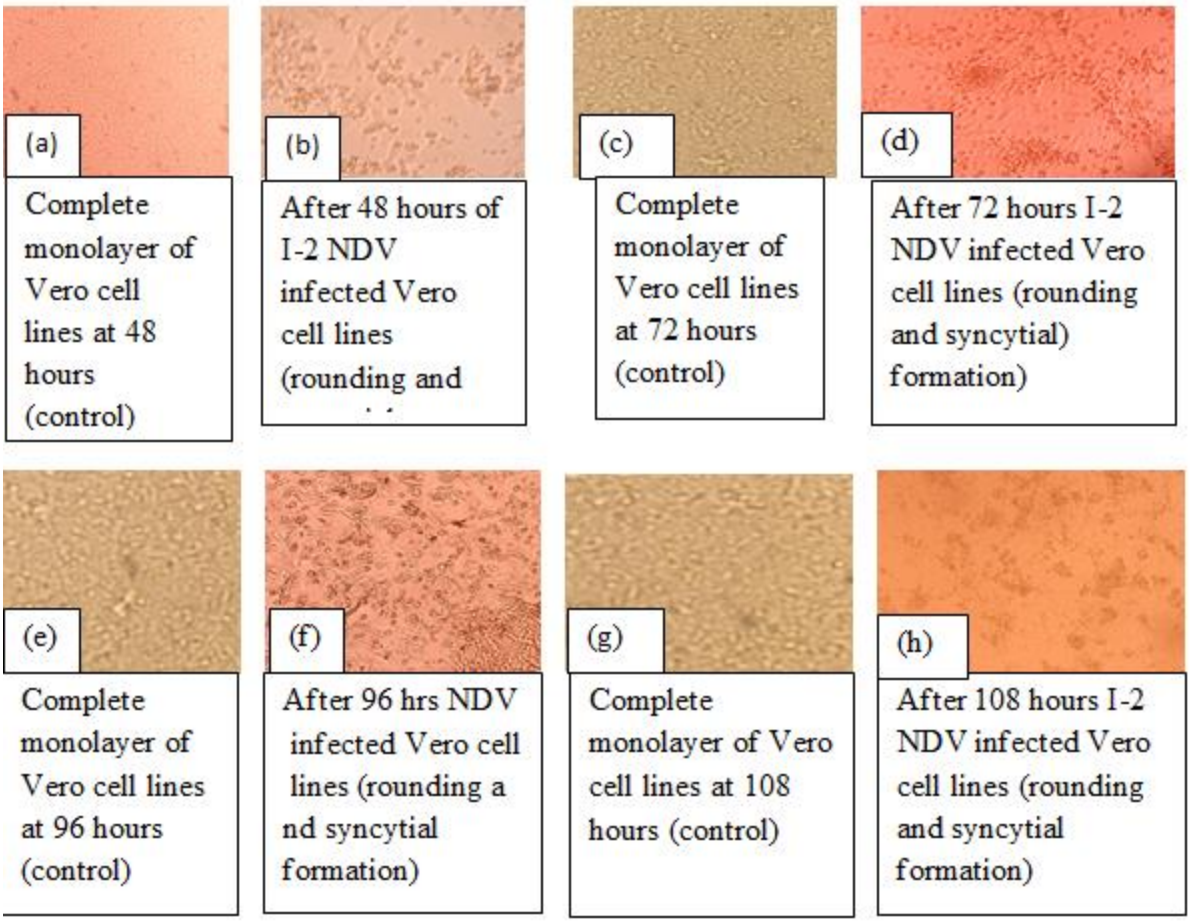

Figure 8: Morphological alteration of Vero cell lines after infection with ND I-2 virus

Table 4: TCID50 % (log10/ml) CEF and Vero cell line adapted ND I-2 strain

	CEF Adapted ND I-2 vaccine		Vero cell adapted ND I-2 vaccine	
No of flask	passage	Mean titer	Passage	Mean titer
6	3	3.9	10	5.3
6	4	5.5	11	5.6
6	5	6.5	12	5.7
6	6	6.7	13	6.1
6	7	7.5	14	6.4
6	8	7.7	15	6.6

Key: TCID50% : Tissue culture infective dose fifty.

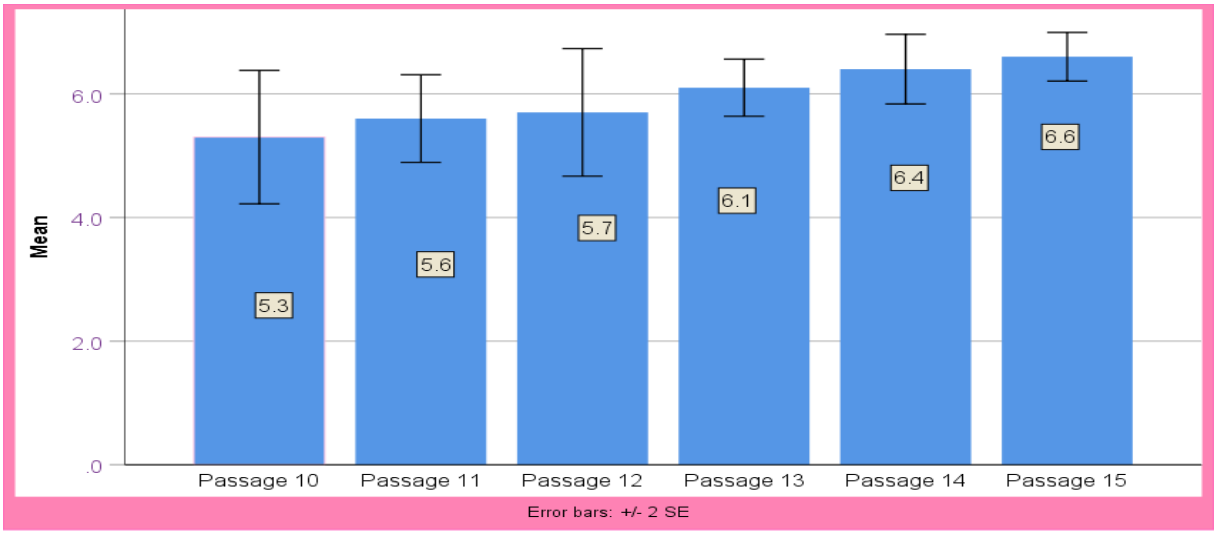

Figure 9: Error bar graph showing the incremental trends of titer/ml of Vero cell adapted ND I-2 vaccine.

. The findings of this experiment showed that the values of titration of ND thermostable I-2 strain adapted on CEF was significantly different from Vero cell lines and even have significant difference of titration from passage to passage which accept the alternate hypothesis that CEF and Vero cell line adapted ND I-2 virus has significant difference of titration from passage to passage.

4.2. Molecular Identification

One step RT-PCR was conducted by synthesis of cDNA. Six sample of CEF adapted Newcastle thermostable I-2 vaccine were tested from each passage and four samples of Vero cell lines adapted Newcastle thermostable I-2 vaccine from each passage were tested to validate identity. The result confirmed eight infected cell culture fluid harvests of CEF adapted Newcastle thermostable I-2 strain and fifteen infected cell culture fluid harvest of Vero cell lines adapted Newcastle thermostable I-2 strain were positive around 121 bp for APMV-1 primer designed to amplify the matrix of ND virus (Figure 10a and 10b).

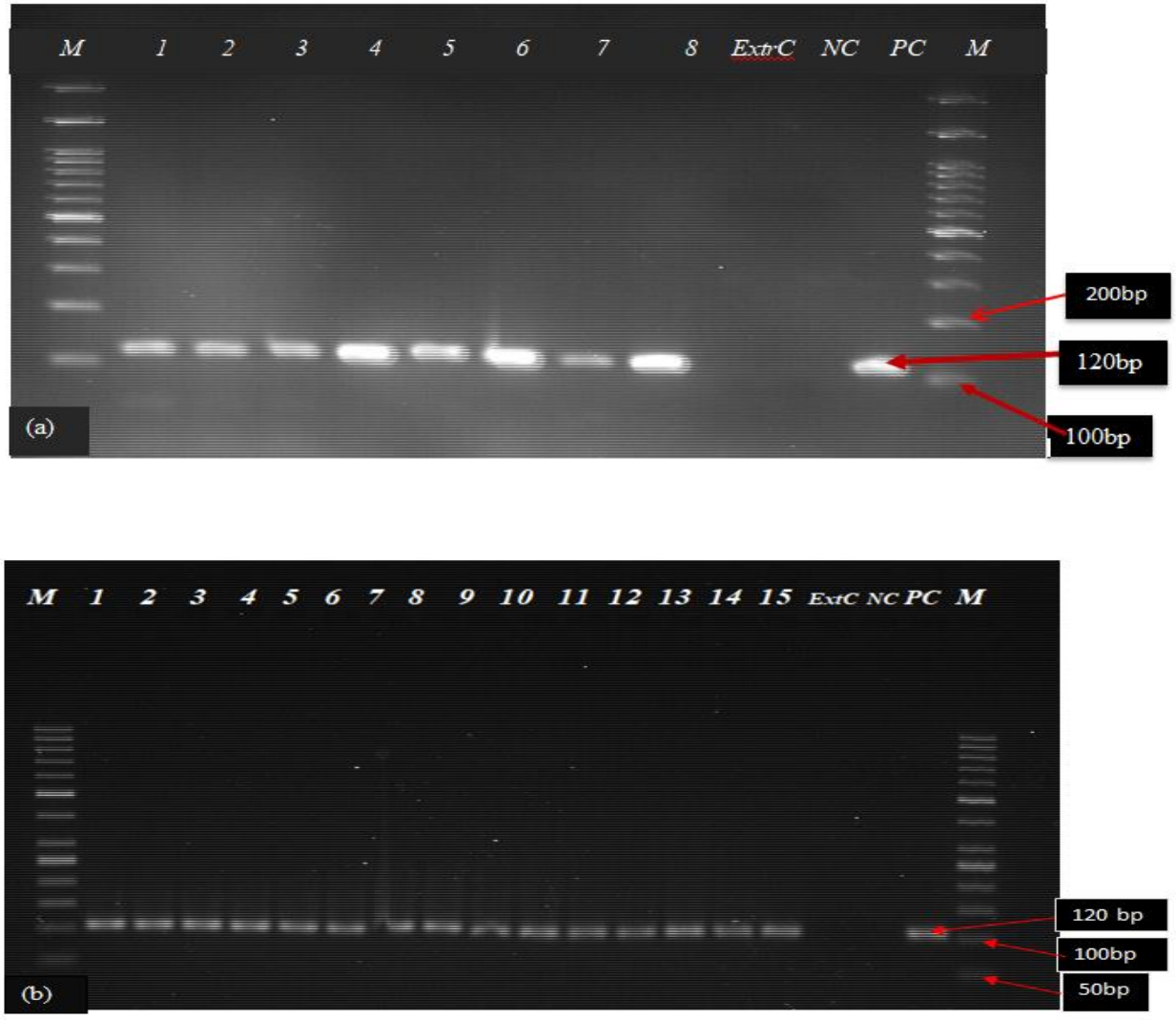

Figure 10: Gel picture of CEF (Panel a) and Vero cell lines (Panel b) adapted NDV-I-2 samples.

(10a) the first and last lanes molecular ladder 100 bp plus (termostat, USA) lanes 1-8: gel picture of samples of CEF NDV I-2 strain from passage 1-8; ExtC: extraction control; NC negative control and PC : positive control. (10b): the first and last lanes: molecular ladder 50 bp plus (termostat , USA) lanes 1-15 :gel picture of samples Vero cell adapted NDV I-2 from passage 1-15;ExtC : extraction control; NC negative control and PC : positive control from NVI collections.

4.3. Immunogenicity and safety of Newcastle thermostable vaccine

4.3.1 Maternal Antibody response

The level of maternally derived antibody of all the experimental chickens before vaccination were evaluated by measuring antibody titers at age of 14 days old for group 1-3 and at age of 14, 21, 28, 35, 42 and 49 days old for unvaccinated groups. The mean titer recorded for the group 1, 2, 3 and 4 chickens at day 0 (age of 14 days old) were 4.1± 1.89, 5.9 ±3.4, 5.0±2.4 and 4.9 ± 2.2 respectively and the mean titer for unvaccinated group at day 7,14,21,28 and 35 were 0.00±0.00 as shown in Table 5 and Figure 11. This result showed that statistically significant differences were observed in HI titres before vaccination and after vaccination in all groups and which is significant at p (<0.05) in our case (sig=0.000).

4.3.2 Immune response of Newcastle disease thermostable vaccine

The result of HI test Antibody titers at 0, 7, 14, 21, 28, and 35 day post- vaccination have been presented in Table 5 and Figure 11.The mean and standard deviation of log2 HI titres after first vaccination were 13.6±5.9, 40 ± 16.8 and 67.2 ± 22.98 at day 7, day 21 and day 28 of experimental chicken group 1 respectively. After 2nd vaccination of group 1 experimental chickens, the mean and standard deviation of log2 HI titres were 124.8 ± 38.7 and 204.8 ± 96.5 at day 28 and day 35 respectively. In addition to this, the mean and standard deviation of log2 HI titers of experimental chicken group 2 after first vaccination were 21.2±9.5, 50.4±17.4 and 83.2±30.1 at day 7, day 14 and day 21 respectively. After second vaccination, the mean and st. deviation of log2 HI titres were 137.6±66.6 and 220.8±120.1 at day 28 and day 35 respectively.

Finally, the mean and st. deviation of log2 HI titers of experimental chicken group 3 after first vaccination were 15.2±6.8, 32.0±8.99 and 51.2±16.1 at day 7, day 14 and day 21 respectively. After second vaccination, the mean and SD of log2 HI titers were 75.2±28.0 and 121.6±54.5 at day 28 and day 35 respectively. Notably, significant differences in HI titres were also observed between the group with (P=0.000). The overall mean HI titer ± SD of experimental chicken groups 1, 2 and 3 were 75.75±5.1, 86.5±41.2 and 50.0±19.5 respectively. This result revealed that the CEF adapted NDV I-2, Vero cell lines adapted NDV I-2 and a Newcastle thermostable vaccine which is produced in NVI used for immunizing the experimental SPF chickens induce a protective immune response. Additionally, until the end of the experiment, no clinical signs, mortality, or adverse reactions were seen in any of the experimental chicken groups. This demonstrated the safety of the Newcastle thermostable vaccine.

This research was also designed to answer the research question and testing the formulated hypothesis in order to achieve the objective of the study. The first research question of the study regarding vaccine efficacy was to find answers for the question whether or not the HI titer of ND vaccine produced by adapting CEF and Vero cell lines have a significant difference among the groups of experimental chickens. According to the finding of this experiment, the antibody titer among experimental group and even before and after each vaccination of all expermental chickens were significantly difference and accept the alternate hypothesis that CEF and Vero cell lines adapted ND vaccine antibody titer have significant difference when compared to ND thermostable I-2 vaccine produced by NVI with p-value <0.05(0.000) (Table 5).

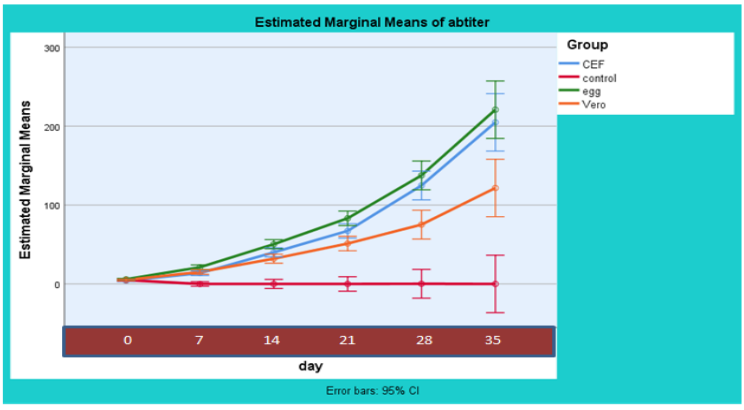

Figure 11: Overall HI titer mean of each group of experimental chickens at different days.

Table 5: Mean HI titer and SD of Newcastle disease vaccine

HI titer at age of	**Experiment group 1 (CEF -I-2,CEF -I-2)**			**Experiment group 2 (NVI -I-2 -NVI-I-2)**			**Experiment group 3 (Vero- I-2,vero -I-2)**			**Experiment group 4 (Negative control)**		
	Mean	SD	Sig.	Mean	SD	Sig.	Mean	SD	Sig.	Mean	SD	Sig.
14	4.1	1.89		5.9	3.3		5.0	2.4		4.9	2.2	
21	13.6	5.86		21.2	9.5		15.2	6.8		0.00	0.00	
28	40.0	16.82		50.4	17.4		32.0	9.0		0.00	0.00	
35	67.2	22.98		83.2	30.1		51.2	16.1		0.00	0.00	
42	124.8	38.7		137.6	66.6		75.2	28		0.00	0.00	
49	204.8	96.5		220.8	120.1		121.6	54.5		0.00	0.00	
HI titer of the group	75.75	30.1	0.000	86.5	42.1	0.000	50.0	19.5	0.000	0.81	0.35	0.000

4.4. Protection efficacy of Newcastle thermostable vaccine

Before challenging both the vaccinated and unvaccinated experimental chickens, the wild virus concentrations for selected allantoic fluids were determined. The lethal dose fifty and the titration of the challenge virus were $10^{7.9}$ LD50/ml and$10^{7.5}$ EID50/ml respectively (Table 6).

Table 6: Embryo mean death time determination for challenge virus

Dilution	24 hours	48 hours	60 hours	72 hours	96 hours
-5	0	5	5	0	0
-6	2	2	4	0	0
-7	2	1	3	0	1
-8	2	1	3	0	0
-9	2	1	3	0	0
-10	0	0	0	0	0
	Median dose log10=$10^{7.9}$ LD50/ml				

LD50: Lethal dose fifty.

4.4.1 Vaccinated and challenged experiment groups

According to this study, the mean antibody titer of experimental chickens group 1, 2 and 3 were increased from day 7 post vaccination to 21 days post vaccination in vaccinated group. This chicken were also challenged with very virulent NDV at day 35 to evaluate whether the CEF, VERO cell lines and egg adapted NDV I-2 strain was protective or not protective against challenge infection. Before the challenge, no clinical signs were observed for the first 35 dpi in all groups. In vaccinated groups, varied antibody responses relative to vaccine groups were induced except for the vaccine-negative control group. After challenge at 35 dpi, clinical signs and mortality were observed in control groups as compared to vaccine groups. The mean HI antibody titre in the vaccinated groups was significantly different ($P < 0.05$) at 35 dpi as shown

in table 7 and Figure 9. From vaccinated and challenged experiment group, one (1) chickens in group 2 showed clinical sign of depression, sneezing, wing and leg paralysis.

This chicken was unable to feed since day 7 after challenge. On day 12th, 5th day to the onset of clinical signs, this chicken was euthanized for post mortem examination to find lesion in internal organs and whether that lesion was due to Newcastle disease or other disease and confirm using molecular technique. Therefore 95 % in this group showed no clinical sign and survived. Experimental group 1 and group 3 chickens showed no clinical signs until 14 dpc and 100 % survived as shown in figure 12a and 12b.

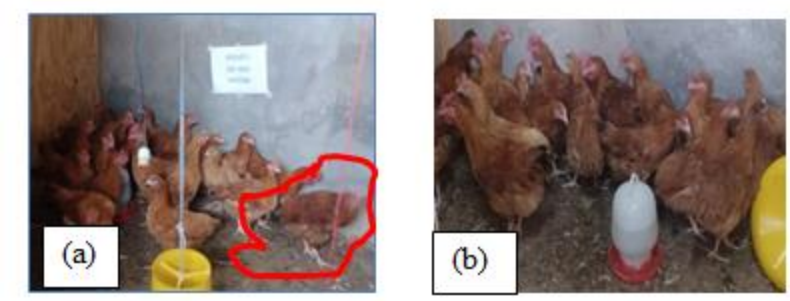

Figure 12: Vaccinated and challenged experiment group.

(a) Vaccinated and challenged chickens showing clinical sign (b) vaccinated and challenged experimental chickens showing no clinical sign (100% survived)

4.4.2 Unvaccinated and challenged experiment group

Relative morbidity and mortality in unvaccinated groups was shown in Figure 13, 14. All birds in group 4 challenged with virulent ND virus showed morbidity from 3 dpc (Figure 13a) and 100% mortality by 6 dpc (figure 13 and 14). These chickens showed severe depression, sneezing, nervious sign such as wing and leg paralysis, ruffled feather, loss of appetite and diarrhea associated with ND from 3 to 6 day post challenge as shown in Figure 13. At 4th dpc, 5 chickens died (Figure 13b), on 5th dpc, 9 chicken died (Figure 13c) and finally on 6th dpc, the left 6 chicke ns were died and 100% of unvaccinated (control) group died by challenge virus within 6 days of post challenge (Figure 13d).

This result was also associated with maternal antibody titers and challenge infection. In our experiment the maternal antibody was declined and eventually disappeared after the age of 21 days old in unvaccinated experimental chickens and the chickens were challenged with vvNDV and 100% of unvaccinated experimental chickens were died (Figure 13 and 14).This indicates that the reduction and eventually disappearing of mean maternal antibody titer after the age of 21 days old and challenging the chickens with vvND virus was correlated with the protective efficacy of maternal antibody in unvaccinated group in which the unvaccinated experimental chickens were not protective against challenge infection since maternal antibody is not persisted with chickens for a long period of time.

Figure 13: Unvaccinated and challenged experiment group.

(13a) all chickens were showed clinical sign at 3rd dpc, (13b) 5 chickens were dead at 4th dpc, (13c) 9 chickens were dead at 5th dpc and (13d) all chickens were dead at 6 dpc.

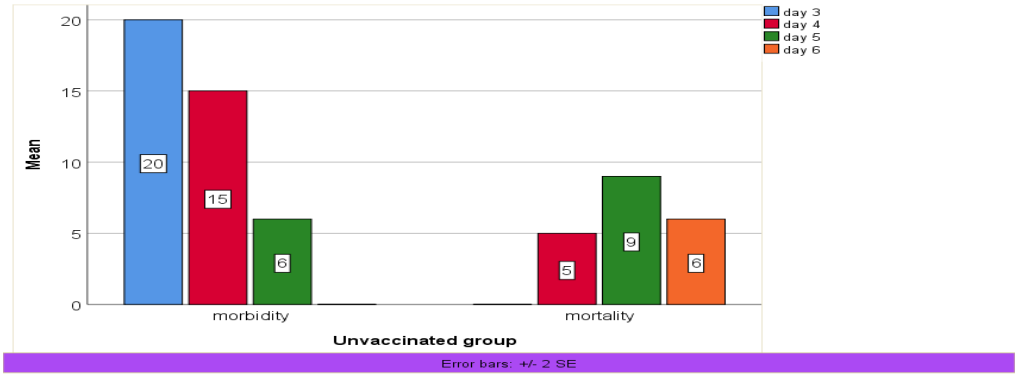

Figure 14: Morbidity and mortality rate of control group after 35 DPC

4.5. Postmortem examination

The spleen, trachea and brain sample from vaccinated, unvaccinated and challenged experimenta l group chickens showing clinical sign of depression were tested by RT-PCR. The results of the spleen, trachea and brain samples from vaccinated and unvaccinated group followed by challenged chicken were confirmed ND positive at around 420 bp for velogenic NDV by amplifying ND virus M-gene (Figure 16). Enlarged spleen (Figure 15a) in 1 chicken from vaccinated group 3 and unvaccinated group, hemorrhage of the proventriculus in 3 chickens (Figure15c) and lesion on liver (Annex 16) were observed.

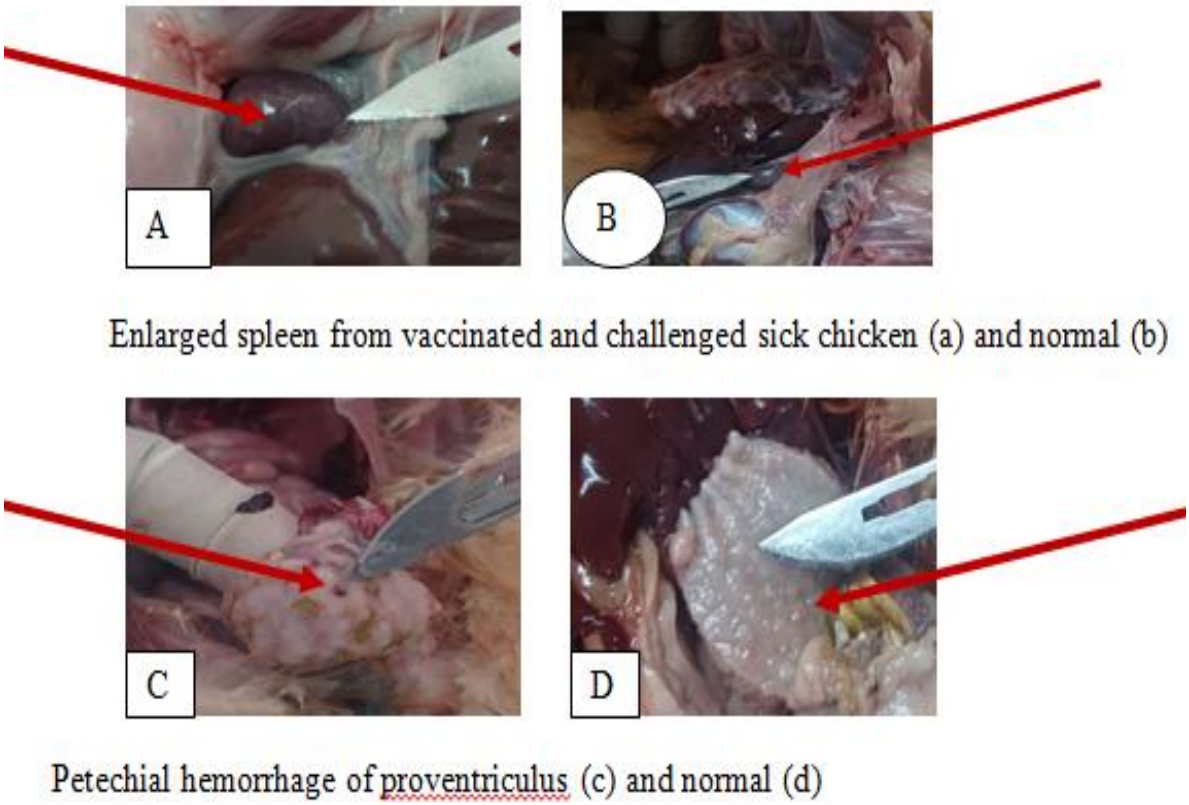

Figure 15: Petechial hemorrhage of proventriculus and enlarged spleen

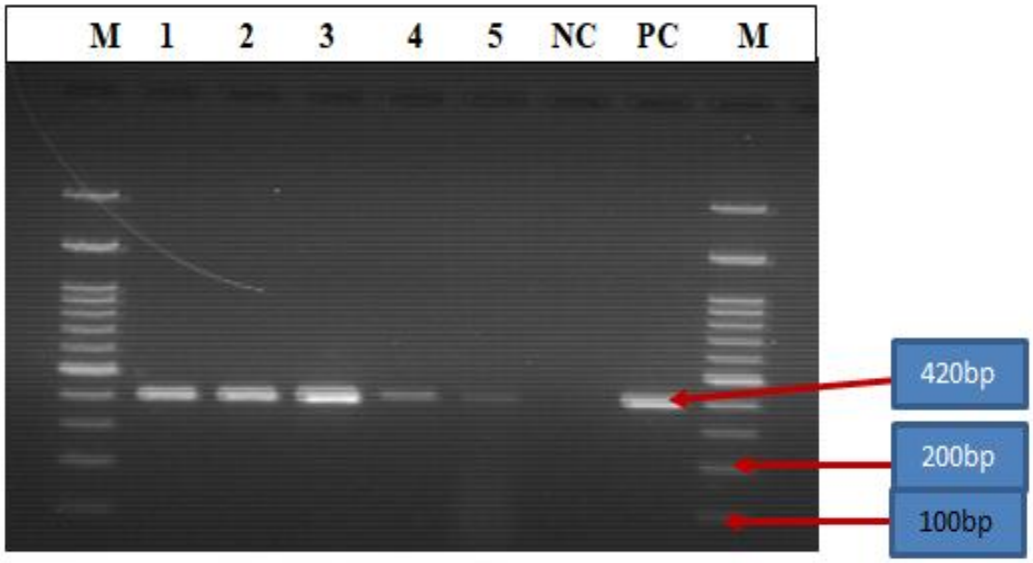

Figure 16: Gel picture of tissue suspension from unvaccinated and vaccinated sick.

The first and last lanes: molecular ladder 100 bp plus (thermostat, USA) lanes 1-5: gel picture of samples from brain, spleen, trachea, spleen and brain respectively from 1-5; NC negative control and PC: positive control from NVI collections.

5. Discussion

The result of *invitro* experiment showed that, CPEs was started to appear 48 hours post infection and 72 hours post infection during the 3rd and 4th passage, respectively in CEF and passage 10^{th} and 11^{th} passage in Vero cell lines. This indicates that as the incubation period of infected cell culture fluid increases, the ability of the virus to infect the cells monolayer from time to time and passage to passage increases.

Prominent CPEs such as cytoplasmic granulation, cell rounding, aggregation, detachment, and floating were observed at 96 hours,108 hours post infection of passage 5,6,7 and 8 in CEF whereas typical CPE were observed at 96 hours,108 hours and 120 hours post infection of passage 12,13,14 and 15 in Vero cell lines. The CPEs observed on CEF and Vero cell lines cells in this study, were consistent with CPEs reported when NDV grew on CEF (Hossain & Bhattacharjee, 2006; Ahamed *et al.,* 2004; Siddique *et al.,* 2017; Ratta *et al.,* 2008) and Vero cell lines (Siddique *et al.,* 2017; Ahamed *et al.,* 2004) respectively. Confirmation of virus identity in CPE positive cultures from passage 1 to passage 8 in CEF and passage 1 to 15 in Vero cell lines was accomplished using one-step RT-PCR by amplifying M-gene.

Both CEF cells and Vero cell lines have the ability to grow rapidly but Vero cells grow more slowly than CEF cells as the cell density of CEF cells increases more rapidly than Vero cells after each 48 hours. The main difference observed in their morphology was that CEF cells were more longer spindle shaped than Vero cell lines. Regarding the CPE of the viruses, NDV showed similar type of CPE in both CEF and Vero cell lines causing rounding, aggregation of cells and syncytial formation. These results were found to be similar with the findings of previous study (Ahamed et *al.,* 2004 ;Anam *et al.,* 2021). Aggregated cells with round shape and formation of multicellular large nuclei were observed as CPE of NDV in both Vero cell lines and CEF cells. A previous study also supported these results (Kamal *et al.,* 2015).

Another finding in this study was that, the titer of CEF adapted NDV I-2 were 3.9log10, $10^{5.5}$, $10^{6.5}$, $10^{6.7}$, $10^{7.5}$and $10^{7.7}$ TCID50/ml of passage 3, 4,5,6,7 and 8 respectively. According to other recent studies, a $10^{6.89}$ TCID50 /mL titer was seen when NDV propagated in CEF cell culture (Anam *et al.,* 2021).In addition to this, the titer of Vero cell lines adapted NDV I2 were $10^{5.3}$, 10^{5}

$^{.6}$, $10^{5.7}$, $10^{6.1}$,$10^{6.4}$ and $10^{6.6}$ TCID50/ml in passage 10,11,12,13,14 and 15 respectively indicating that NDV grow more rapidly with high value of titer and have more potential to grow in CEF than Vero cell lines. In other recent studies, when NDV propagated in Vero cell lines, 10^9 TCID50 /mL titer has been observed (Siddique *et al.*, 2017). It was concluded that CEF may be considered as another avian cell lines suitable for propagation of NDV with high titer. NDV has potential to grow in chicken embryonated eggs and different avian cell lines. In our study, NDV cultured in CEF cells showed higher titer than Vero cells. Maximum titer of the virus (7.7 Log10TCID50 /ml) was observed after 72 hours Post infection in passage 8 of CEF. This indicates that NDV I-2 strain was successfully adapted on CEF and Vero cell lines.

Another finding of this study showed that the immunological response of control (unvaccinated) group at the age of 14 days old (day 0) were (4.9±2.2) and age of 21st days old (day 7) were (0.000±0.000) in which GMT were significantly declined. The decline in GMT values can be attributed to the disappearing of maternal antibody levels in the chicks as they grow older. This suggests that the passive immunity acquired from the mother may not be sufficient to protect the chicks from NDV as they continue to grow. According to our findings MDA were significantly declined after the age of 21 days old which is consistent with those of Ezzulddin et al. (2022), Kapczynski et al. (2013) and Oberlander et al. (2020), who also observed that MDA persisted up to 27 days of age, Cvetic et al. (2021), who reported that MDA persisted for up to 28 days of age and Geletu, (2024),who reported maternal antibodies in chickens were persisted up to 31 days of age in which they are inherited from parent stock. In other recent studies, mAb against NDV were depleted by 10 day of age (Gharaibeh & Mahmoud, 2013). After maternal antibodies were declined, notably, statistically significant differences were observed among vaccinated groups (sig. = 0.000).

The HI titer in geometric mean of Log2 ± SD of each group vaccine used in experimental groups was considered to compare the immune response for ND vaccines. Primary immunization of the experimental chickens with the CEF adapted, Vero cell lines adapted and egg adapted ND I-2 vaccines revealed that the MDA was decreased from it was detected at age of 21days and statistically significant increament of active immunity. After the first booster vaccination it was observed that booster vaccination by CEF adapted ND I-2 vaccine for group 1, Vero cell lines

adapted for group 2, and egg adapted ND I-2 vaccine for group 3 gives a higher mean antibody titer as compared to all groups of NDI-2 vaccine vaccinated after first vaccination.

The current findings were in agreement with the previous reports in which vaccination of chickens with lentogenic strains followed by booster produces higher antibody titers (Banu *et al.*, 1970).In all groups, CEF adapted, egg adapted and Vero cell lines adapted NCTH-I-2 first booster vaccinations, the p-value (< 0.05) indicates there was significance difference in HI titer due to booster vaccination. Previous study indicated that primary vaccination and secondary vaccination of lentogenic ND vaccines induced high immune responses and protection against the velogenic NDV challenge, making it possible to significantly decrease viral replication and shedding with high levels of antibodies (Ebrahimi *et al.,* 2023).Therefore, both CEF and Vero cell line adapted ND I-2 strain as well as commercially available ND I-2 vaccine were produce the best immunological response. All groups of experimental chickens except control group were vaccinated and boosted with Newcastle thermostable I-2 vaccine. The result showed that CEF adapted ND I-2 vaccine produces significantly greater antibodies than Vero cell lines adapted Newcastle thermostable I-2 vaccine and this result was in agreement with previous reports (Siddique *et al.*, 2018).

Embryonated egg adapted Newcastle thermostable I-2 vaccine were produces greater antibodies than other vaccine groups. Vero cell line adapted Newcastle thermostable I-2 vaccine produces less antibodies than other vaccine groups but it produces the best immune response as compared to unvaccinated group of experimental chickens. This finding was in agreement with the findings of Banu et al. (2009), who reported that lentogenic strain provided superior antibody production after first and second vaccination. In comparison of individual groups, each groups of experimental chickens revealed a significance difference with P-value (< 0.05). Generally, in the current study maternal antibodies, choice of strains, and vaccine titer were considered as a key factor for antibody production and protection against challenge virus. All the groups vaccinated by CEF adapted NDV I-2, Vero cell lines adapted thermostable I-2 vaccines and SPF egg adapted ND I-2 Vaccine yields mean HI titer that can protect chickens from virulent ND.

After challenging with velogenic Newcastle virus, the chickens that were under control exhibited various signs, such as reduced feed intake, depression, and gasping, sneezing and respiratory

problems nervous signs like wing droop and leg paralysis. The clinical signs of ND observed in this study are consistent with the findings of (Abdisa & Tagesu, 2017). All vaccinated group shows no any clinical sign and 100% survived whereas all the chickens (100%) were showed clinical sign and died within six days of post challenge from unvaccinated groups as shown in Table 13. The morbidity and mortality of our experimental chickens were associated with the antibody titres. This indicates that the induction of faster and high protective antibody titer after vaccination followed by challenging was highlighting better protection potential against challenge infection. The differences in survival rates of the birds following challenge with a local virulent NDV may suggest the difference between viruses (Abolnik *et al.*, 2004).

6. Conclusions and recommendations

In this study the adaptation of Newcastle disease virus I-2 in CEF and Vero cell lines were done. The study demonstrated the successful adaptation of the virus on CEF and Vero cell lines and showed rapid growth and similar patterns of CPE. CEF cells showed higher titer of NDV I-2 compared to Vero cells lines. Another finding of this study was showed that both CEF and Vero cell line adapted ND I-2 strains produces the best immunological response. However, group 1 chickens produces more antibodies than Vero cell lines adapted ND I-2 vaccine, while group 3 chickens produce greater antibodies than other vaccine groups. After challenging with the velogenic ND virus, the chickens under control showed ND clinical signs which could lead to 100% death whereas the vaccinated groups were survived. Therefore, the study concluded that CEF and Vero cell lines adapted Newcastle thermostable I-2 vaccine and the current ND vaccines can protect chickens from virulent ND virus. However, if we produce the CEF and Vero cell line adapted NDV I-2 strain vaccine, NVI will save a minimum of 8000 SPF eggs per batch. In addition to this, in Ethiopia, majority of small scale poultry farm customer needs 200, 300 or 400 dose per vial (cell culture based vaccine) rather than 800 or 1000 dose per vial (NVI produced ND vaccine) to immunize their chickens. This may increase the capacity of producing ND vaccine and customer satisfaction. Thus, CEF and Vero cell lines adapted ND I-2 vaccine were the best alternative and solve the problems encountered with currently ND I-2 vaccine produced by NVI.

Therefore, based on the above conclusion the following recommendations are forwarded:

- To overcome the problems encountered with currently produced ND I-2 vaccine in NVI, it's better to shift from conventional method of ND vaccine production into cell culture based production of ND vaccine.
- There is need for further research to investigate another strain of Newcastle disease virus such as La Sota and HB1 whether they are adapted or not on different kinds of cell lines and evaluate its protective efficacy of vaccine on chicken.
- Detailed investigation of the CEF and Vero cell lines adapted ND viruses based on gene sequencing and sequence analysis is recommended to understand the evolutionary relationship of the former and the current circulating viruses.

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Annex 1: Formulation of tissue culture media

1.1 Growth Medium

Glasgow modification of Eagle's Minimum essential Medium (GMEM)

Formulation

GMEM	80%
TPB	10%
Calf serum	10%
Antibiotic solution	0.2%

Preparation procedure

- Weight out the required amount of GMEM powder containing $NaHCO_2$ for the preparation the intended volume
- Add the GMEM powder into 5 or 10L of container
- Pour distilled water into the container up to the intended volume
- Dissolve the powder using magnetic stirrer
- Sterilize GMEM solution by filtration
- Dispense the solution into l000 ml volume of the sterile plasma bottles
- Incubate the solution at 37 ^{0}C for 24 hr to check the solution sterility
- Label the type of solution, its Lot No. and Date of preparation on the surface of plasma bottle
- Store the bottles at 4^{o}C

Just before use,

- Add aseptically the required amount of calf serum, Tryptose phosphate broth (TPB) and antibiotic solution based on the formulation.

Annex 2: Tryptose phosphate broth (TPB)

Formulation

Glucose	2g
NaCl	5g
Tryptose	20g
$Na_2HPO_4.2H_2O$	2.5g
Distill water	Up to 1000ml

Preparation procedure

- Weight the required amount of each ingredient for preparation intented volume of TPB
- Add distilled water up to the intended volume
- Mix well using magnetic stirrer for 30 minutes
- Dispense into the final containers
- Sterilize by autoclaving at 121^0C for 15 minutes
- Label the type of chemical, Lot No. And date of preparation on the container
- Store at room temperature

For complete formulate TPB powder,

The TPB powder has the followings ingredients in g/L

Tryptose	20.0
NaCl	5.0
D-glucose	2.0
Disodium phosphate	2.5

For such formulated TPB powder the preparation formulation is :

TPB powder	29.5g
Deionised water	Up to 1000ml

Préparation procédure

- Weight out the required amount of the powder for the preparation of the intended volume of the broth
- Add deionised water (conductivity < 10µs) up to the intented volume
- Heat to dissolve
- Distribute into screw cap containers/bottles
- Sterilize by autoclaving at 121 ^{0}C for 15 minutes

Label the type of chemical, Lot No. and date of preparation on the container

Annex 3: Calf serum

Calf Serum is used as a general growth supplement in cell culture medium. Before use, it should be screened for the absence of bacteria, mycoplasma, viruses and viral antibodies, of particular concern is BVD. The sterility of Calf Serum is checked using broth culture. Moreover, the serum's ability to support cell growth must be approved before use.

Preparation procedure

- Thaw Calf Serum the stored unopened stocks at -20°C
- Filter the serum using ESK 0 filter to remove unnecessary coagulates
- Filter the serum again using ESK 2 filter to sterilize
- Aseptically dispense into sterile bottles
- Label Calf Serum, Lot No. and Date of preparation on the surface of the bottles
- Store at +4°C until use.

Annex 4: Formulation of enzyme solutions for dispersing cells

4.1. Trypsin 2.5 %(W/V) Solution (l0X Stock Solution)

Formulation

NaCl	8g
KCl	0.4g
Na_2HPO_4	0.0475g
KH2PO4	0.06g
NaHCO3	0.35g
Trypsin (1:250)	25g
Deionized and distilled water	Up to 1000ml

Preparation procedure

- Calculate the amount of each ingredient required for preparation of intended volume
- Weight out the ingredients and add into sterile bottle
- Pour deioned and distilled water into the bottle up to the intended volume
- Dissolve by stirring overnight at 4^oC.
- Adjust pH to 7.8 by the addition of sterile 1M NaOH.
- Sterilize by filtration through a Seitz EK pad or a 0.2 um membrane filter.
- Distribute aseptically into 50-l00 ml volumes.
- Take sample for sterility tests.
- Label the type of solution, Lot No. and date of preparation on the surface of the bottles
- Store at -20^oC until use.

For use,

- Add l00 ml to 900 ml sterile PBS

4.2. EDTA (Versene) 2% Stock Solution

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
EDTA di-sodium salt.$2H_2O$	22.14g
Phenol red	1.0g

Preparation procedure

- Calculate the amount of each ingredient required for preparation of intended volume
- Weight out the ingredients and add into sterile bottle
- Pour deionized distilled water into the bottle up to the intended volume
- Dissolve well.
- Distribute in 100 ml volumes in screw cap bottles.
- Autoclave at 121^{o}C for 15 min.
- Take sample for sterility tests.
- Label the type of solution, Lot No. and date of preparation on the surface of the bottles
- Store at 4^{o}C until use.

4.3. Trypsin 0.05%/EDTA 0.02% Solution for Sub-culturing

Formulation

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

Preparation procedure

- Calculate the required volume of each solution to prepare the intended volume of final solution
- Add aseptically all the required volume of solution into sterile final container

Annex 5: Formulation of balanced salt solutions

5.1: PBSA (Dulbecco's phosphate buffered saline)

Formulation

NaCl	8.0g
KCl	0.2g
KH_2PO_4	0.2 g
$Na_2HPO_4.7H_2O$	2.16 g
or $Na_2HPO_4.2H_2O$	1.44g
Distill water	Up to 1000ml

Preparation procedure

- Calculate the amount of each ingredient required for intended volume of the solution
- Dissolve well
- Adjust the pH to 7.2 at 20^0C or 7.4 at 36.5^0C with sterile 1M NaOH.
- Dispense into 500- 1000ml bottles.
- Mark liquid level before autoclaving.
- Autoclave the solution at 121^oC at 100 KPa (or 15 lb/sq.in. or 1 bar) for 10-15 min.
- Make up to mark with sterile deionized distilled water before use, if evaporation has occurred.
- Label the type of solution, Lot No. and date of preparation on the surface of the bottles

- Store the solution bottles at room temperature.

5.2 Sodium Bicarbonate Solution (7.6%)

Formulation

NaHCO3	75g
Phenol red 0.2%	50g
Deionised water	Up to 1000ml

Preparation procedure

- Dissolve $NaHCO_3$ in 800 ml of deionised water.
- Make up volumetrically with phenol red and rest of the water.
- Gas with CO2/air mixture for 12 minutes.
- Check pH = 8.0
- Dispense into bottles using a siphon, filling to overflowing.
- Tighten tops as hard down as possible.
- Autoclave at 10 p.s.i. for 10 minutes.
- Check sterility by broth culture.
- Store at +4°C.

Annex 6: Hatching of expemental chicken after 21 days of incubation

Hatching of chicken after 21 days of incubation at 37^0c

4 days old after hatching

Annex 7: Preparation of primary cell culture

A

Pipetting of cell (trypsinization process)

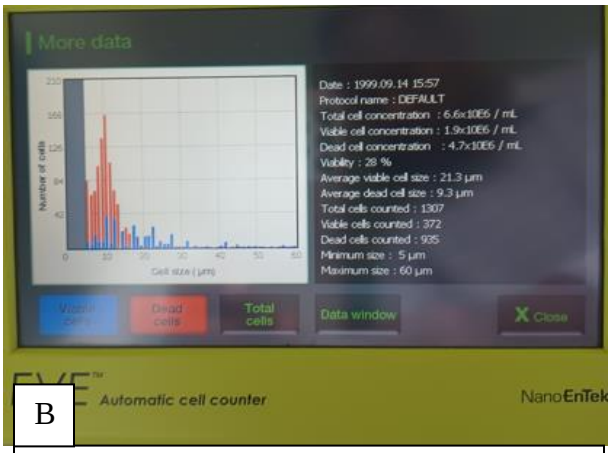
B

Observation of cell morphology under inverted microscope

Annex 8: Counted chicken embryo fibroblast cell

A

Counted cells by EVE by automatic cell counter



B

Graphs showing live and dead cells after counted

Annex 9: Sterility testing media

9.1: Tryptic soya broth

Formulation

The tryptic soya borth powder was prepared with the following ingredients in g/L

Casein peptone	17.0
Soya peptone	3.0
NaCl	5.0
Dipotassium phosphate	2.5
Dextrose	2.5

Preparation procedure

- Weight 30g of the tryptic soya broth powder
- Add the measured powder in 1000ml of distilled water
- Dissolve well using magnetic stirrer
- Distribute 8ml of the solution into sterile test tubes
- Sterilize by autoclaving at 121 ^{0}C for 15 minute
- Incubate at 37 ^{0}C for 24 hours to check its sterility
- Store at

Precaution:

→ Whatever the case, read carefully the instruction of the manufacture before starting preparation if there is any change in the procedure.

9.2: Thioglycollate USP fluid Medium

Formulation

The tryptic soy borth powder was prepared with the following ingredients in g/L

Peptone for casein	15,000
Yeast extract	5,000
Dextrose	5,500
NaCl	2,500
Sodium thioglycollate	0,500
L-cystine	0,500
Resazurine	0,001
Agar	0,750

Preparation procedure

- Weight 30g of the thioglycollate fluid medium powder
- Add the measured powder in 1000ml of distilled water
- Slowly bring to the boil
- Stir until complete dissolution
- Distribute 8ml of the solution into sterile test tubes
- Sterilize by autoclaving at 121 ^{0}C for 15 minute
- Incubate at 37 ^{0}C and check its sterility after 24 hours
- Store at 37^{0}C

Precaution:

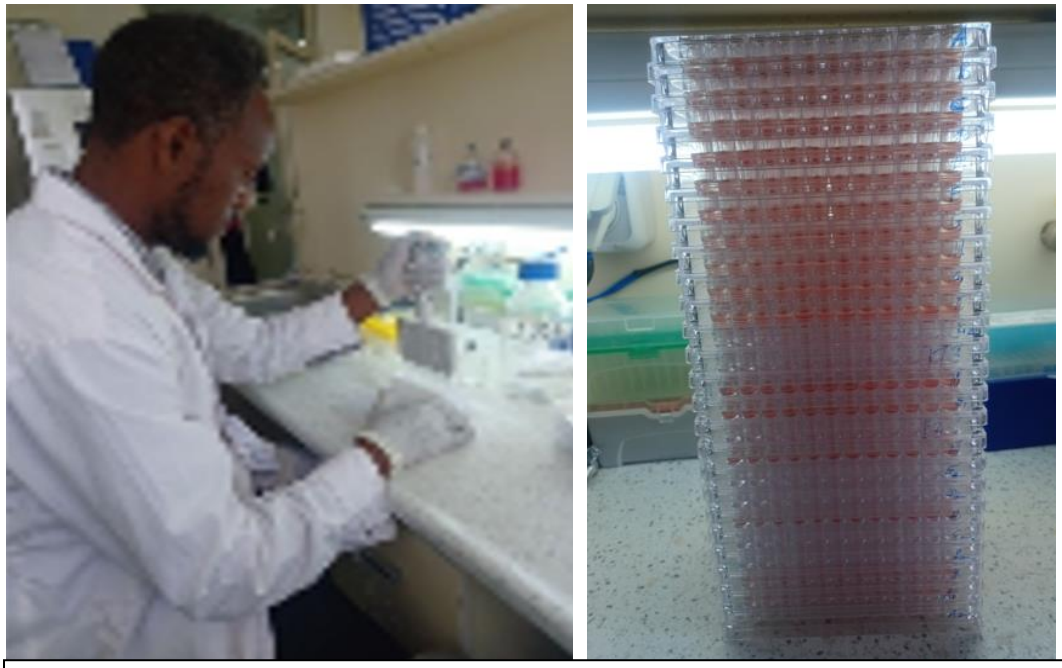
→ Whatever the case, read carefully the instruction of the manufacture before starting preparation if there is any change in the procedure.

Annex 10: Counted Vero cell lines

Counted cells by countess II automatic cell counter

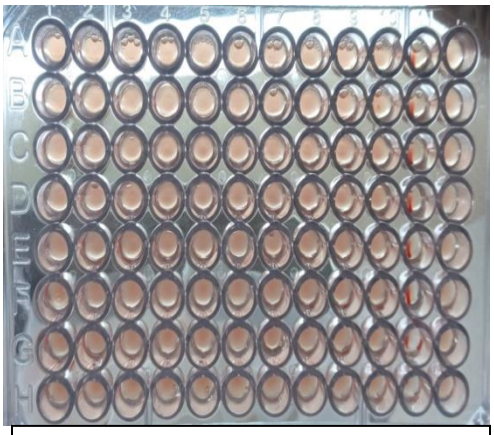
Graphs showing live and dead cells after counted

Annex 11: Conducting HA and HI test



Conducting HI test

HI Positive



HI negative

Annex 12: Preparation of freeze drying stabilizer

Formulation

The following formulation is used for getting 5% LAH and 10% Sucrose of freeze dying medium solution

Lactalbumin hydrolysate powder	50g
Sucrose	100g
Distilled water	Up to 1000ml

Preparation procedure

- Weigh out the required amount LAH for preparation of the intended volume
- Pour it into a sterile container containing two third of the intended volume of distilled water.
- Place the flask on a magnetic stirrer/hot plate and start gentle stirring and heating. Do not boil!
- Stop heating once the LAH is dissolved
- Allow cooling while continue stirring for 15 minutes.
- Weigh out the required amount of sucrose and
- Add the sucrose into the solution.
- Add distilled water to bring volume to the intended the level,
- Cool the solution to room temperature.

- Adjust the pH to 7.2 with 1M KOH.
- Sterilize the liquid by filtration through a 0.22µ filter pad into final containers
- Take sample to check the solution sterility
- Label the type of solution, Lot No. and Date of preparation on the containers
- Store at $+4^0$C until use.

Note:

→ LAH/Sucrose stabilizer may be stored at 4^0C up to one year.

Annex 13: Vaccinated experimental chicken

Vaccination of experimental chickens

Vaccinated and challenged experimental chicken 100% survived

Annex 14: Serum sample collection

Collecting serum sample from experimental chicken

Collected serum sample

Annex 15: Ethical clearance

NATIONAL VETERINARY INSTITUTE

Ref.No: NVI/778
Date: 26/09/2023

To: Megersa Mindaye

Subject: <u>Approval for Ethical Clearance</u>

The research ethical committee of the National Veterinary Institute reviewed and discussed your research project entitled "**Evaluation of Newcastle Disease Virus Vaccinal Strain Adaptation in Chicken Embryo Fibroblast and Vero Cell Lines, and Evaluating Protective Efficacy of vaccine in Chicken**" on September 26, 2023. After review and discussion of your project proposal it is scientifically and ethically sound from relevance, originality and technical competence of view. Hence the project allowed to be executed provided that:

1. All procedure, condition stipulated in the proposal are respected and any deviation, variation or change may be made only in consultation between reported to the committee.
2. All comments given by the committee should be considered and fulfilled by the researchers
3. The project activity is open for occupational supervision by the committee whenever this is deemed necessary.

The committee expects to be informed about the progress of study with any changes in protocol.

Sincerely,

Mirtneh Akalu(DR.)
R&D Directorate Director

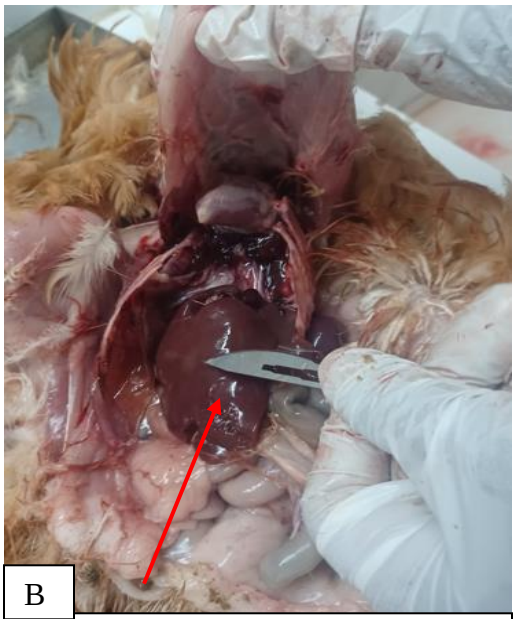
<u>Cc</u>
Director General
Deputy Director General

Bishoftu, ETHIOPIA
FAX 011 433-93-00
Website: www.nvi.com.et

Annex 16: Working on postmortem examination

A

Postmortem examination



B

Lesion on liver