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ADAPTATION OF NEWCASTLE DISEASE VIRUS VACCINAL STRAIN IN VERO CELL LINE AND EVALUATION OF VACCINE SAFETY IMMUNOGENECITY INCHICKEN UNDER LABORATORY CONDITION

MSc Thesis

By

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Department of Microbiology, Immunology and Veterinary Public Health

June 2018

Bishoftu Ethiopia

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A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial fulfillment of the Requirements for the degree of Master of Science in Veterinary Microbiology

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Adaptation of Newcastle Disease Virus Vaccinal Strain in Vero Cell Line and Evaluation of Vaccine Safety and Immunogenicity In chicken Under Laboratory Condition

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LIST OF TABLES **PAGE**

STATEMENT OF AUTHOR .... **I**

ACKNOWLEDGEMENTS .... **II**

LIST OF TABLES .... **III**

LIST OF FIGURES .... **VI**

LIST OF ANNEXES .... **VII**

ABBREVIATIONS .... **VIII**

ABSTRACT .... **IX**

1. INTRODUCTION **1**

2. LITERATURE REVIEW **4**

2.1. Description of the disease **4**

2.2. History **4**

2.3. Etiology **4**

2.3.1. Taxonomy 5

2.3.2. Morphology and structure of the ND virus 5

2.3.3. Virus entry 6

2.3.4. Replication Newcastle disease virus 6

2.3.5. Assembly and Release 7

2.4. Classification ND **7**

2.5. Epidemiology **8**

2.5.1. Distribution 8

2.5.2. Hosts range 8

2.5.3. Source of infection and Transmission ND 9

2.6. Clinical signs **9**

2.7. Pathology **11**

2.8. The Molecular Basis for Pathogenicity ....Error! Bookmark not defined.

2.9. Diagnosis **12**

2.9.1. Diagnosis based on clinical sign and lesion 12

2.9.2. Laboratory diagnosis 13

2.10. Control and prevention Newcastle disease **15**

2.10.1. Management and hygiene procedures 15

2.10.2. Vaccine and vaccination 16

2.10.3. Type of vaccine 16

2.10.4. Vaccination program 17

2.11. Status of Newcastle disease in Ethiopia **17**

3. MATERIALS AND METHODS **20**

3.1. Study area/location **20**

3.2. Study Design **21**

3.3. Study animals and husbandry **21**

3.4. Source of virus strain, cell line and culture media **22**

3.5. Study Methods **22**

3.5.1. Sub-culturing of vero cell adherent monolayer 22

3.5.2. Infection of vero cells monolayer 23

3.5.3. Virus harvesting 23

3.5.4. Adaptation of the virus 23

3.5.5. Identification and confirmation of the virus 24

3.5.6. Reverse-transcription polymerase chain reaction (RT-PCR) 24

3.5.7. Infectivity assay for virus yeild.. 25

3.5.8. Preparation of experimental vaccine 25

3.6. Experimental trail vaccine Safety test.. 25

3.7. Immunogenicity test.. 26

3.7.1. *Experimental layout* 26

3.7.2. Blood collection.. 26

3.7.3. Determination of antibody response 27

3.8. Data manegment and analysis 27

4. RESULTS 28

4.1. Cytopathic effect (CPE).. 28

4.2. Heamagulitination and haemagulitination inhibition test 29

4.3. RT-PCR for Cell Culture Adapted NDV I-2 Strain 29

4.4. Tissue culture infective dose 50 (TCID50) assay... 31

4.5. Safety test... 31

4.6. Antibody response evaluation.. 32

4.6.1. Maternal antibody antibody titer before vaccination 32

4.6.2. *Seroconversion Status of the Monitored chicken after Vaccination:* 32

5. DISCUSION 33

6. CONCLUSION AND RECOMMENDATIONS... 35

7. REFERENCES.. 36

8. ANNEXS 44

LIST OF FIGURES PAGE

Figure 1. The structure of single stranded, non-segmented, negative-sense RNA viruses genome 6
Figure 2. ND Source of infection 9
Figure 3. NDV infected chicken showing clinical sign 10
Figure 4.Pathological lesion of Newcastle affected bird 11
Figure 5. Map of the Study area (Bishoftu Town) 20
Figure 6. Cytopathic effects of Vero cells after infection with NDV I-2 virus 28
Figure 7. Slide Hemagglutination (HA) of cRBC for vero cell adapted NDV 29
Figure 8. Agarose gel electorophpresis (1.5%) of 1100-bp fragment of the F gene 30

LIST OF ANNEXES **PAGE**

Annex 1. Formulation of growth and maintenance Cell Culture Medium 44
Annex 2. Preparation Trypsin Solution (0.25%). 44
Annex 3. Growth and maintenance media 45
Annex 4. Procedure for Inoculating Preformed Monolayer's 45
Annex 5. Haemagglutination test 46
Annex 6. Haemagglutination inhibition test 47
Annex 8. Calculation of the geometric mean titre (GMT) 50
Annex 9. Procedure Preparation of 4 HA units of ND virus antigen suspension 51
Annex 10. Procedure to preparation of positive sera 51
Annex 11. List of picture taken during laboratory work 52

ABBREVIATIONS

AB	Antibody
BP	Base pair
CEF	Chicken Embryo Fibroblast
cRBC	Chicken Red blood cell
DEMEM	Dulbecco‘s Modification of Eagle‘s Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethyl diamidetriacetic acid
EID50	Egg infective dose fifty
ELISA	Enzyme linked immune sorbent assay
FCS	fetal calf serum
GEMEM	Glasgow minimum essential medium
ICPI	Intracerebral Pathogenicity Index
ML	Mililitre
ND	Newcastle disease
NDV	Newcastle disease virus
NVI	National veterinary institute
NT	Nucleotide
OIE	Office International des Epizooties
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFU	Plaque-forming unit
PI	Post Infection
RNA	Ribonucleic acid
SCDM	Assoyabean-Casein Digest Medium
SPF	Specific pathogens free
TCID50	Tissue culture infective dose fifty
VP	Virus Protein

ABSTRACT

Newcastle disease (ND) is a highly infectious disease causing considerable economic losses to poultry industry worldwide. Traditionally, substrates for production of ND vaccines have been embryonated eggs, a method which has the disadvantages of being labor-intensive, time consuming and requires large area for the incubation of eggs. The present study was conducted to develop Vero cell-adapted, NDV I-2 vaccine and evaluate its safety and immunogenicity in chicken under laboratory condition. For this study, healthy and confluent monolayer of vero cell were further sub-cultured to prepare semi confluent monolayers then infected with 0.25 ml of Newcastle disease virus I-2 vaccine strain. The passage 1(P1) virus was harvested and used for the next passage in this way virus was given 12 serial passages on Vero cell line, where characteristic cytopathic effects (CPEs) were observed starting from fourth passages. Typical syncytium (irregularly shaped cells), gaint cells, clustering of infected cells, death of cells (plaque) were noticed on passage 4 from 72hourspost-infection. The positive hemagglutination and hemagglutination inhibition test confirmed that all vero cell adapted NDVI-2 adapted virus was Newcastle disease virus.The infectivity titer of adapted virus increased from $\log10^{3.2}$ to $\log10^{7.2}$ tissue culture infected dose/ ml.From the vaccine strain NDVI-2, Vero cell adapted live vaccine was produced. The antibody titer of experimental vero cell adapted live vaccine was determined in chicken by comparing with conventional live commercial vaccine (NDV I-2). Before experimental vaccination of the chicks, the level of antibody titre was very low (1.8±0.6). Following experimental vaccination, antibodies production were gradually increase after day 7 of post vaccination and the mean antibodies of the two vaccines (groups) were increased across each week of the followed up. The peak antibody titer was observed in the both groups at day 21 of post vaccination. After vaccination the two group chickens had antibody titres of >4 log2 starting from day and remain within protective range at day 35 final sera collection.The vaccine stain of NDV was well adapted to Vero cell line after successive passages and appeared equally immunogenic.

Key words: *Adaptation, Antibody Titer,Cytophatic Effect, Newcastle Disease Vaccine, Vero Cell*

1. INTRODUCTION

Newcastle disease (ND) is a highly contagious and devastating disease of poultry caused by the virulent strains Newcastle disease virus (NDV) or avian paramyxovirus type 1, (APMV-1). It is classified under the genus Avulavirus of family Paramyxoviridae (Mayo, 2002 Miller *et al*., 2009).The disease was its first outbreaks in 1926,in Java, Indonesia (Kraneveld, 1926), and in Newcastle-upon-Tyne, England (Doyle, 1927).It infects a wide range of domestic and wild bird species resulting in heavy economic losses to the poultry industry. Chickens are the most susceptible host in which the severity of the disease may vary from mild infection with no apparent clinical signs to a severe form causing 100% mortality (Wajid, *et al.,* 2016).

The virus is single stranded, non-segmented, enveloped RNA virus with negative polarity. It composed of six genes and their corresponding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin neuraminidase (HN), and the RNA polymerase (L). RNA editing of the P protein produces two additional proteins, V and W. The HN and F are glycoproteins that allow binding and fusion of the virus to the host cells to initiate a NDV infection. Antibodies to HN and F are neutralizing and represent the primary protective component induced by ND vaccines (Xiao *et al*., 2012; OIE, 2012).

Based on the clinical signs and severity of the disease NDV strains are classified into three main pathotypes: Lentogenic, mesogenic and velogenic; Lentogenic strains cause mild or virulent infections that are largely limited to the respiratory system. Mesogenic strains are of intermediate virulence causing respiratory infection with moderate mortality, while velogenic strains are highly virulent causing mortality in chickens. Velogenic strains can be further categorized into two types: Viscerotropic and neurotropic. Viscerotropic velogenic strains produce lethal hemorrhagic lesions in the digestive tract, whereas neurotropic velogenic strains produce neurological and respiratory disorders (Alexander and Sene, 2008b;OIE, 2012).

The Sources of infection for NDV are exhaled air from infected birds and contaminated feed and water and transmission is mostly via aerosol. Feces, eggs lay during clinical diseases, and all parts of the carcass during acute infection and at death can also act as sources of infection. Chickens infected with virulent NDV may die without showing any clinical sign of illness though young chickens are more susceptible and show sign sooner than older ones. Much of the spread of ND in village is probably via human agents (Ashraf and Shah, 2014; Wajid, *et al*., 2016).

Until now, there is no treatment or method to eradicate ND. Prevention is include biosecurity, importing birds from disease free flocks or through vaccination that must continue throughout the life of the bird. Traditionally, ND vaccines have been produced by growing vaccine virus strains in embryonated chicken eggs. NDV is harvested from the allantoic fluid and processed to create a vaccine (Gallili and Nathan, 1998, Alexander, 1992; OIE, 2012). This traditional method however poses some drawbacks, such as poor quality control, high labor-intensity, time consuming, needs high amount of specific pathogen-free eggs and requires big area for the incubation of eggs. Besides the process is slow and difficult to scale-up, so large strategic stocks must be kept to respond in cases of epidemics (Souza, *et al.*, 2009).

In Ethiopia, there is only one center that produces vaccines for ND; the National Veterinary Institute (NVI). The center use the specific pathogen free embryonated chicken eggs for propagation of the vaccine strains and the eggs are imported from abroad which has resulted in insufficient amount ND vaccines production required to provide immunities to almost over 65 million chickens (CSA, 2016).To fulfill high local demand of ND vaccines, Ethiopia has to import SPF eggs and vaccines from overseas with high foreign currency and importing vaccine poses a great risk because newer strains may be introduced to the local poultry industries in addition to loss of foreign currency.

One method that has high possibility to overcome all the problems mentioned is by producing ND vaccines by using animal cell culture. Animal cell culture offers many advantages over the traditional chicken eggs method. The method is rapid, convenient,

and less expensive than eggs, supports easy scale up and also it allows evidence of viral proliferation to be examined microscopically. Many cell substrate systems have been reported to be able to support the growth of NDV. Among cell substrate systems that have been identified are African green monkey kidney (Vero) Vero, Chicken fibroblast cell cells (CEF) and Douglas Foster one (DF-1) cells (de Leeuw and Peeters, 1999; Napoli, *et al.,*2007; Ravindraa, *et al.,* 2008). In addition, more mechanized cell culture methodology allows virus particles to be produced with the highest control and quality. In Ethiopia vaccine production using vero cell line was not studied. Therefore, this work was initiated with the following objectives.

General Objective

- To develop Vero cell adapted Newcastle disease virus vaccine (I-2srain) as an alternative to the traditional embryonated chicken eggs based vaccine

Specific objectives

- Adaptation of Newcastle disease virus I-2 vaccinal strain on Vero cell line.
- To evaluate safety and immunogenicity of Vero cell adapted liveNDI-2 trail vaccine in chicken by comparing commercially available ND I-2 vaccine.

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2. LITERATURE REVIEW

2.1. Description of the disease

Newcastle disease (ND) is an extremely important viral disease of poultry and wild birds worldwide. Is an acute viral disease affecting many domestic and wild avian species with respiratory, gastrointestinal, and central nervous system involvement. The disease classified as a list A disease by the World Animal Health Organization, Office International des Epizooties, (OIE) because it is highly contagious and responsible for severe disease and high mortality in susceptible birds (Alexander and Sene, 2008b;OIE, 2012).

2.2. History

Newcastle disease was first recognized in Java, Indonesia, in 1926. At about the same time, a disease with the same symptoms was observed in England, where it was recognized in Newcastle by Doyle, hence the name. Within ten years, the disease had spread to middle Korea, Japan, India, Philippines, East Africa, and Australia. This disease was first called pneumoencephalitis but later was shown to be caused by a virus which was indistinguishable immunologically from Newcastle disease virus (NDV) (Alexander, 1991). Within a few years numerous NDV isolations that produced either a mild or no disease were made around the world. Such isolates were used later as live vaccines. Newcastle disease is now recognized in most of the major poultry producing countries. Its virulent form is one of the most devastating diseases of poultry, causing 100% mortality in chickens (De Leeuw *et al.,* 2005).

2.3. Etiology

Newcastle disease (ND) is caused by virulent strains of Avian Paramyxovirus Type1 (APMV-1) of the genus Avulavirus belonging to the family Paramyxoviridae.The disease is characterized by the lesions in the respiratory tract, visceral organs and brain and causes moderate to severe mortality and morbidity in susceptible flocks (OIE, 2012).

2.3.1. Taxonomy

Newcastle disease virus belongs to order Mononegavirales, family Paramyxoviridae and subfamily Paramyxovirinae (Cattoli *et al*., 2011). The subfamily is divided into five genera: Mor-billivirus, Respirovirus, Henipavirus, Rubulavirus, and Avulavirus (Miller *et al*., 2009); all the avian paramyxoviruses APMVs are part of genus Avulavirus. The virus exists in 10 serotypes; APMV-1 to APMV-10 (Waheed et al., 2013), but all NDV isolates belong to serotype 1 (APMV-1). Most of these serotypes appear to be present in natural reservoirs of specific feral avian species, although other host species are usually susceptible. Only APMV-2 and APMV-3 viruses have made a significant disease and economic impact on poultry production (Briand *et al.,* 2012). APMV-1 is synonymous with NDV (Cattoli *et al*., 2011; Miller *et al*., 2009).

2.3.2. Morphology and structure of the ND virus

NDV Virions are roughly spherical; 150 nm or more in diameter and filamentous (Catroxo *et al*., 2011). The genome single stranded, non-segmented, negative-sense RNA is about 15.2 kb in length (Cao *et al.,* 2013; Zhang et al., 2012) that codes for six structural and two non-structural proteins (Choi *et a*l., 2010). 'Rule of six' should be followed by genome because it should be of polyhexameric length to replicate rapidly. It encodes for six proteins in 3' to 5' direction; these are Nucleoprotein (NP), phosphor-protein (P), Matrix (M), Fusion (F), Hemag-glutinin Neuraminidase (HN), and Large RNA polymerase (L) (Linde *et al*., 2011; Al-habeeb *et al.,* 2013). The proteins W and V are additionally created within the P gene during transcription of mRNA at editing site by insertion of guanines (Qiu *et al.,* 2011; Xiao *et al*., 2012).

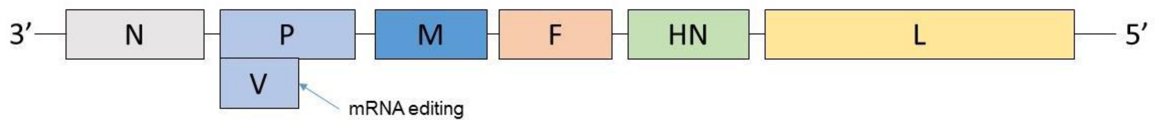


Figure 1. The structure of single stranded, non-segmented, negative-sense RNA viruses genome

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

2.3.3. Virus entry

The envelope proteins are responsible for viral entry into a target cell. The HN protein mediates cell attachment, whereas the F protein is necessary for cell fusion. The HN protein requires sialic acid residues on the surface of the host cell in order to bind. It then also plays a role in viral release from the cell via its neuraminidase activity which removes sialic acid receptors. The fusion protein is a key component of the pathogenesis and virulence of the virus, as the ability of the virus to enter a cell is dependent upon the cleavage of the inactive F0 protein by cellular proteases. After fusion of the virus envelope with the host cell membrane, the viral nucleocapsid is released into the cytoplasm.(Maclachlan and Dubovi, 2010).

2.3.4. Replication Newcastle disease virus

After entry into the cell, transcription of the negative sense viral genome occurs in the cytoplasm. The RNA-dependent RNA polymerase (RdRP) transcribes the leader RNA and each of the viral genes into individual 5' capped and 3' polyadenylated mRNAs, along a transcription gradient. This leads to larger numbers of mRNA transcripts being produced from genes closest to the promoter region when compared with genes closer to the 5' end. The individual mRNAs are then translated into viral proteins (Whelan et al., 2004)

After sufficient numbers of viral proteins are produced, transcription stops and replication begins. Replication produces a full-length antigenome of the negative sense RNA, in

association with the N protein.Each of the N subunits is associated with 6 nucleotides of genomic RNA and hence adheres to the 'rule-of-six' of most paramyxoviruses. This feature explains why the genome sizes of all NDVs are always a multiple of six (Peeters*et al.*, 2000; Lamb and Parks, 2007).

2.3.5. Assembly and Release

Nucleocapsids assemble in the cytoplasm of the host cell with initial attachment of the N protein to the RNA to form a helix followed by integration of the P and L proteins. The nucleocapsids are then transported to the plasma membrane ad are connected to the F and HN surface glycoproteins via the M protein. The viral envelope is formed during the process of budding from the host cell (Lamb and Parks, 2007)

2.4. Classification ND

Even though all strains of NDV are of a single serotype, they can be classified genetically in a number of ways. They have traditionally been classified on the basis of the full length F gene sequence and have been described either in terms of lineages and sub-lineages, or classes and genotypes. The use of multiple classification systems has led to confusion and so efforts have been made to determine the most appropriate universal system. Such a system has been developed based on F gene phylogeny and evolutionary distances. The fusion gene is usually chosen for comparative analysis because it is more likely to show genetic variation than other internal nucleocapsid genes (Al dus *et al.,* 2003; Cattoli *et al.,* 2010*).*

Using this classification system, two classes of NDV are recognized; class I and class II. Class I viruses have a genome of 15,198nucleotide, whereas class II viruses have a genomes of 15,186 nucleotide ("historic" isolates) and 15,192 nucleotide (isolated after 1960) (Czeglédi *et al., 2006*). The majority of the class I viruses have been isolated from wild birds (predominantly waterfowl and shorebirds) and are avirulent, whereas the class II viruses contain isolates from both wild birds and poultry and contain both virulent and avirulent strains. These two classes can then be further classified into genotypes. Class I

viruses contain a single genotype, whereas Class II viruses contain 18 genotypes (Dimitrov *et al.* 2016).

2.5. Epidemiology

2.5.1. Distribution

Newcastle disease was distributed throughout the world. Newcastle disease is epizootics in Central and South America, Asia, and Africa while sporadic epizootics occur in Europe (Naveen *et al*., 2013). In in Europe Newcastle disease was reached in 1981 then spread rapidly throughout the world (Mase *et al.,* 2002). It is endemic in south East Asia and cause high economic loss in commercial poultry farm. Newcastle disease can infect over 240 species of birds. When infected birds are introduced into susceptible flocks all birds will be infected within two to six days (Munir *et al.,* 2012).

2.5.2. Hosts range

NDV has the ability to infect a wide variety of avian species; however the pathogenicity of the virus amongst species is variable. Poultry are most susceptible to ND, with high mortalities seen in layer and broiler farms. Other commercial species such as turkeys and ducks are also known to be susceptible to ND but clinical signs are typically less severe than those seen in chickens. In addition, viruses with virulent fusion protein cleavage sites have been detected in commercial ducks with no apparent clinical signs seen in the infected birds, thus posing a potential risk to other poultry species (Wajid, et al., 2016)

NDV is also recognized as a zoonotic agent. The predominant clinical sign shown by those infected is conjunctivitis; however there have also been occasional reports of ND causing flu-like symptoms. The majority of people reported to be infected with NDV are those with close contact with poultry (e.g. abattoir workers) or laboratory staff. As yet there have been no reports of human to human spread (Alexander and Senne, 2009).

2.5.3. Source of infection and Transmission ND

The majority of ND outbreaks occur as a result of disease spread from infected poultry. Introduction of disease can occur from a number of sources including trade in poultry and poultry products and via the smuggling of live birds or eggs. Once the disease has been established in a flock, spread of the virus is typically by movement of birds, via fomites (feed, equipment) and potentially by windborne dispersal. During an outbreak, bird to bird transmission is usually via the respiratory route with inhalation of droplets, or via the faecal-oral route (Alexander, 1995).

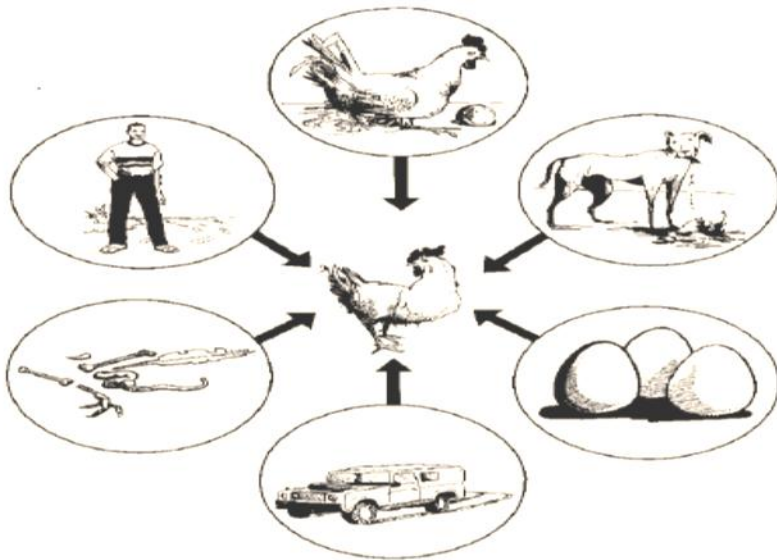


Figure 1 . ND Source of infection

Source:https://www.google.com/search?q=source+of+infection+and+transmition Newcastle+disease+withpicture

2.6. Clinical signs

The clinical signs of ND may vary with the pathotype of the virus. Four main pathotypes were described in the 1970s and the terminology is still in use today. The pathotypes include velogenic (highly virulent), mesogenic (moderately virulent), lentogenic (low

virulence) and avirulent forms which have been derived from the mean death time (MDT) in eggs. The velogenic viruses can then also be further divided into velogenic viscerotropic and velogenic neurotropic forms based on pathological features (Alexander and sene, 1974). In general, velogenic viruses are associated with high mortalities with viscerotropic viruses causing severe depression and diarrhoea. Neurotropic viruses also cause neurological signs such as ataxia, head tremors and paresis, along with respiratory distress. Monogenic viruses usually present with respiratory disease and may also cause mortalities in young birds. Lentogenic viruses induce minimal clinical signs, however when present they are usually respiratory in nature. Avirulent viruses typically do not induce clinical signs at all. There is however overlap between the pathotypes and the age, immune status and the presence of concurrent diseases should be taken into consideration when interpreting the clinical signs (OIE, 2012).

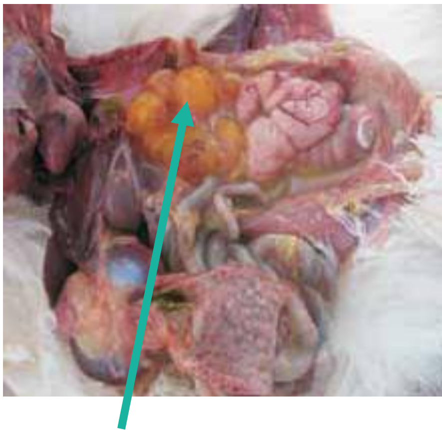
Figure 2. NDV infected chicken showing clinical sign

(A) nervous signs(B) Whitish to greenish diarrhea

Source:https://www.google.com/search?q=pictures+of+newcastle+disease

2.7. Pathology

The gross and histological features of ND vary with the pathotype of virus. Velogenic viruses have a strong tropism for lymphoid tissue and the central nervous system (Cattoli*et al.,* 2011). Infection with viscerotropic velogenic pathotypes typically produces haemorrhagic lesions within the intestinal tract which can be attributed to necrosis of lymphoid tissue within these regions. In particular, fibrinonecrotic lesions are commonly seen in the caecal tonsils and the small intestine. Spleens are often enlarged and mottled and the proventriculus may contain haemorrhagic foci, again usually centered on lymphoid aggregates. These gross findings correspond histologically to necrosis of lymphoid tissue with replacement haemorrhage and fibrin. Immunohistochemical staining and in situ hybridization has shown that the lymphoid tropism observed is usually associated with replication of virus in macrophages. Initially, replication occurs within lymphocytes and macrophages in the eyelid conjunctiva. This progress to the detection of positive staining in mononuclear cells throughout multiple organs, along with positive staining in epithelial cells of the respiratory and gastrointestinal tracts (Wakamatsu *et al*., 2006Cornax*et al*., 2013)



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Petechial haemorrhages in proventricus

Ovarian follicle ovarian follicle hemorrhagic ruptured

Figure 3. Pathological lesion of Newcastle affected bird

Source:https://www.google.com/search?q=pictures+of+newcastle+disease

2.8. Immunity against ND Virus

The goal of vaccination is always sterilizing immunity, however, that has not yet been achieved with NDV vaccines. At best, NDV vaccines induce an immune response that reduces or completely prevents clinical disease and mortality from ND, decreases the amount of vNDV shed into the environment, and increases the amount of virus needed to infect the vaccinated animal (Marangon and Busani, 2006; Miller et al., 2009). Herd immunity beneficial consequence of a successful vaccination program as it provides some protection to suboptimal-vaccinated or unvaccinated birds in an otherwise well vaccinated flock (Marangon and Busani, 2006). However, this outcome is only achieved with ND when greater than 85% of the flock have hemagglutination inhibition (HI) antibody titers greater than 8 after two vaccinations (van Boven et al., 2008).

2.8. Diagnosis

Newcastle disease can be diagnosed based on history, clinical sign and laboratory test. Newcastle disease clinically resembles highly pathogenic avian influenza so during outbreak rapid and accurate diagnosis is important to control and prevent dissemination of disease (Khan *et al*., 2010).

2.8.1. Diagnosis based on clinical sign and lesion

The clinical sign of NCD is depends on age, immune status of the host, tissue tropism and virulence of virus strain. Sudden high mortality in a flock in the absence of premonitory clinical signs occurs when susceptible species are exposed to highly virulent strain. In susceptible flocks the mortality rate in fully can reach 100 % (Quinn et al., 2002). The incubation period of NCD is usually about five days. In chicken's nerve, respiratory and digestive sign may occur. The major clinical sign observed in Newcastle disease are: greenish white diarrhea, with ruffled feathers; depression in the birds and a state of

prostration, a condition known as torticollis (the head turned to one side (Figure 2) and other neurological sign like paralysis of leg and wing. NCD is acute disease can cause death within 2 to 3 days (Hasan *et al.,* 2012).

Necropsy lesions caused by velogenic APMV-1 viruses have mainly been characterized in poultry, especially chickens. Viscerotropic velogenic and neurotropic velogenic strain cause hemorrhagic lesion particularly in mucosa of the proventriculus, small intestine and ceca. In respiratory tract gross lesions are not observed. It is less likely in older birds, haemorrhages of the thymus and bursa of fabriceus may also occur (Alexander, 2003).

2.8.2. Laboratory diagnosis

Laboratory diagnosis for NCD includes virus isolation, serological (enzyme-linked immune sorbent assays (ELISA), immunodiffusion test, agar gel precipitation and molecular test (Reverse transcription polymerase chain reaction (RT-PCR). Isolation of the NCD virus is definitive diagnosis of NCD (Saif *et al*., 2005).

Virus isolation

The appropriate sample for diagnosis in Newcastle disease includes: tissue sample (trachea, lung, spleen, soft palate, colon, bursa and brain) which are important for hisopathology and cloacal swabs, oro-nasal swabs and Serum sample. Collected sample should be transported at pH 7.0-7.4 in isotonic phosphate buffered saline (PBS), containing antimicrobial drugs (Alders and Spradbrow, 2001)

ND Virus is obligate intracellular parasite that requires living cell in order to replicate. Cultured cells, eggs and laboratory animals may be used for virus isolation. To diagnosis APM-1 infection virus isolation in embryonated eggs or cell cultures serves as important for viral isolation (Alders and Spradbrow, 2001).centrifugation of sample from feces or tissue for 10 minute at temperature 25°C to obtain supernatant sample and measure 0.2 ml of sample and inoculate in to allantoic cavity of embryonated SPF fowl egg of 9 -11 days or confluent monolayer of cell culture then incubate for 4-7 days at 35-37°C (OIE,2012).

Serological test

In the absence of vaccination, the presence of specific antibodies against the ND virus is not necessarily that it was suffering from the disease at the time of sampling, but indicates that the bird has been infected by the virus at some time. In practice, a high antibody titre is indicative of a recent infection. NDV may be employed as an antigen in a wide range of serological tests. Although numerous serological tests may be used to detect antibodies in serum they give little information on the infecting NDV strain. Two methods are used to measure antibody titres: the haemagglutination inhibition (HI) test, and the enzyme-linked immunosorbent assay (ELISA).The most commonly used and show accurate result is haemagglutination inhibition (HI) test (Alexander, 2003). For both tests, it is necessary to collect blood samples from the chickens and should be taken from the wing veins (Alders and Spradbrow, 2001).

Molecular technique

Molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) and polymerase chain reaction (PCR) have been used for rapid and sensitive detection for Newcastle disease (Hewson *et al*., 2009).

Reverse transcription polymerase chain reaction (RT-PCR) is used to detect RNA virus which is negative and single stranded RNA virus. There are two different configurations of the RT-PCR assay. In the two step RT-PCR configuration, the cDNA is synthesized in a different tube before performing PCR assay. In contrast a one step RT-PCR firstly synthesises the cDNA. The reverse transcriptase is inactivated and the polymerase is activated simultaneously and the PCR reaction is carried out in a single tube (Pfaffl, 2004). This is rapidly becoming the assay of choice. Several important steps need to be considered in developing an RT-PCR protocol. The first aspect is the RNA extraction. This needs to be an efficient process that can extract RNA from the samples even when it's in low concentrations and eliminate contaminants that will degrade the RNA (Pfaffl, 2004). Another important aspect is the gene being targeted and the choice of primers. This can have a profound effect on the efficacy of the assay. Poorly design primers can

result in mispriming and the amplification of non-specific products or the formation of primer dimer (Abd-Elsalam, 2003).

PCR done based on principle that it has the ability to amplify and differentiate multiplespecific nucleic acids using polymerase enzymes (Bellau *et al.* 2005). However, those techniques can detect only one specific pathogen at a time. PCR can detect virus following following the growth of virus in embryos in the laboratory and clinical specimens. It has the potential to have high sensitivity and is now it is considered as the gold standard for nucleic acid detection (Mackay, 2002). However, PCR requires DNA as a template and the target viruses in this study have RNA as their nucleic acid. Therefore, RNA viruses require a reverse transcription step to produce single stranded complementary DNA (cDNA) through reverse transcriptase using a specific oligonucleotide primer and viral RNA as a template (Turner *et al.,* 2005).

2.9. Control and Prevention Newcastle disease

2.9.1. Management and hygiene procedures

The principal management procedures should include strict biosecurity measures which help in preventing the spread of infective material from house to house and from farm to farm (Markos and Abdela, 2016). Good biosecurity can protect poultry flocks from Newcastle disease. Avoid flocks not be to contact with domesticated poultry of unknown health status, any pet birds (particularly psittacines), and wild or feral birds (particularly cormorants, gulls and pigeons). Biosecurity measures include well ventilated houses, clean water supplies, minimizing travel on and off the facility, and disinfecting vehicles and equipment that enter the farm. Separation of infected from health flocks and proper disposal of died birds. Control of Pests such as insects and mice is also important for control measures of NCD. All in/all out breeding (one age group per farm), with disinfection between groups, is also advisable (CFSPH, 2016).

2.9.2. Vaccine and vaccination

Vaccination is the most important method of controls and prevention of new castle disease. Currently, both inactivated and live vaccines for NCD are available around the world (Shim *et al*, 2011). There is also thermo stable vaccine which was specifically developed to be used in village chicken (Spadbrow, 1992). We can use varieties of route for administration of live vaccine and schedules from hatching till grow-out(Cho et al., 2008. Killed virus oil emulsion vaccines are administered parentally prior to the onset of egg production. Lentogenic virus vaccines are generally recommended in drinking water, by eye drop, by aerosol or intranasally. A vaccine using a heat-tolerant V4 strain has been developed for feeding to village chickens in countries where these constitute a significant proportion of poultry production. Although proper vaccination protects the birds from clinical disease but it does not prevent virus replication and shedding, which results in a source of infection (Chukwudi *et al*, 2012).

2.9.3. Type of vaccine

Most of the commercially available ND vaccines against NDV are live attenuated and

Inactivated vaccine.

Live vaccine

Live vaccines are relatively cheap, sold as freeze dried, easy to administer and can be used for mass vaccination. These have been divided in to mesogenic and lentogenic groups with their preferred mode of administration being eye drop, beak intranasal installation, or dipping for lentogenic vaccines while mesogenic vaccine requires intramuscular injection. Drinking water and aerosol administrations can also be used (Alexander, 2003).

In most countries, Hitchner B1 and La Sota vaccines are used and are derived from the lentogenic strain of NDV. Some mesogenic vaccines may cause disease; particularly in young birds, especially if there is a dual infection with exacerbating organisms. Because of heat liable the live vaccines are also have disadvantage under village management

system where transport and cold storage facilities are often inadequate (Otim et *al*., 2005, Yune and Abdela, 2017)

Inactivated vaccine

Inactivated vaccine can be used situations unsuited for live vaccine and induce high level of protective antibody over long period of time. These vaccines are produced from infective allantoic fluid of virulent NDV treated with B-propiolactone or formalin to kill the virus and then mixed with adjuvant. The vaccine can administered ether subcutaneous or intramuscular injection (Alexander, 2003).

2.9.4. Vaccination program

Vaccination program affect the duration of immunity. One of the most important considerations affecting vaccination programs is the level of maternal immunity in young chickens, which may vary considerably from batch to batch, farm to farm, and among individual chickens. For this reason, one of several strategies is employed. Either the birds are not vaccinated until 2-4 weeks of age when most of them will be susceptible, or 1-day-old birds are vaccinated by conjunctival instillation or by the application of a coarse spray. This will establish active infection in some birds that will persist until maternal immunity has waned. Revaccination is then carried out 2-4 weeks later (OIE, 2012)

2.10. Status of Newcastle disease in Ethiopia

There is no clear record about the introduction of the virus to the country, however, NCD first occurred in and around seaports of the country and spread to the interior of the country along transport routes. The first documented outbreak of NCD in Ethiopia dates back to 1971 and reported from a small poultry farm in Asmera, Eritrea, located closed to a seaport and the province of the country. The first NCD virus reported was a velogenic type, which was classified according to the virulence of the strain and caused about 80% mortality (Kebreab*et.al.* 2001). In the following years the disease spreads fast to other

parts of the country. In 1972, outbreaks had been reported in Addis Ababa and in 1974 at the then Alemaya college of Agriculture poultry farm (Tadelle and Yilma, 2004) and also in 1995; NCD outbreaks in the surrounding areas of Bishoftu, Adama and Addis Ababa killed almost 50% of the local bird. (Mazingia, 2012).

Outbreaks of ND usually happen at the beginning of the main rainy season (end of May and beginning of June). Nevertheless, this seasonal pattern seems to have changed after the 1984 to 1986 villagenization programs were launched, and it has become a problem throughout the year, although it is still more serious at the beginning of the main rainy season (Tadelle and Jobre, 2004). Nasser (1998) has also reported the occurrence of the disease all year round. Ashenafi (2000), on the other hand, reported that few clinical cases of ND during the dryer months of the year even if the seroprevalence of antibodies against NDV remained high throughout the year like reports by other studies (Tadelle and Jobre, 2004). It is possible to say that currently there are no low risk areas for ND remaining in Ethiopia.

Table 1. Summary of occurrences of Newcastle disease in certain areas in Ethiopia.

Study area	**year**	**Prevalence (%)**	Reported by
Central highlands of Ethiopia	1983-1995	58.50	Nasser, 1998
State poultry Farm Ethiopia	1983-1995	-	Nasser, 1998
Rift valley area	2004-2005	-	Zeleke *et al*.2005a
Bahirdar district	2007- 2008	29.60	Mazengia *et al.,* 2010
Farta District	2007-2008	21.70	Mazengia *et al*., 2010
Bishoftu	2004-2005	-	Zeleke *et al.,* 2005b
mecha	2012		Nga *et al*., 2012
farta	2012		Nega *et al.,*2012
East showa	2011-2012		Chaka, *et al.,* 2013
Kersana kondeltity	2014		Belayenh *et al.* 2014
Agarfa &Sinana district ,Bale zone	2014-2015	27.86	Geresu et al., 2016

SOURCE: Mazengia *et al*. 2012

3. MATERIALS AND METHODS

3.1. Study area/location

The Experiment was conducted from September 2017 to April 2018 at National Veterinary Institute, (NVI) Ethiopia which is located on Bishoftu Town. The town is located 45 Km south east of Addis Ababa. The area is located at 9oN latitude and 40° E longitude at an altitude of 1850 meter above sea level with annual rainfall of 866 mm (NMSA, 2010).

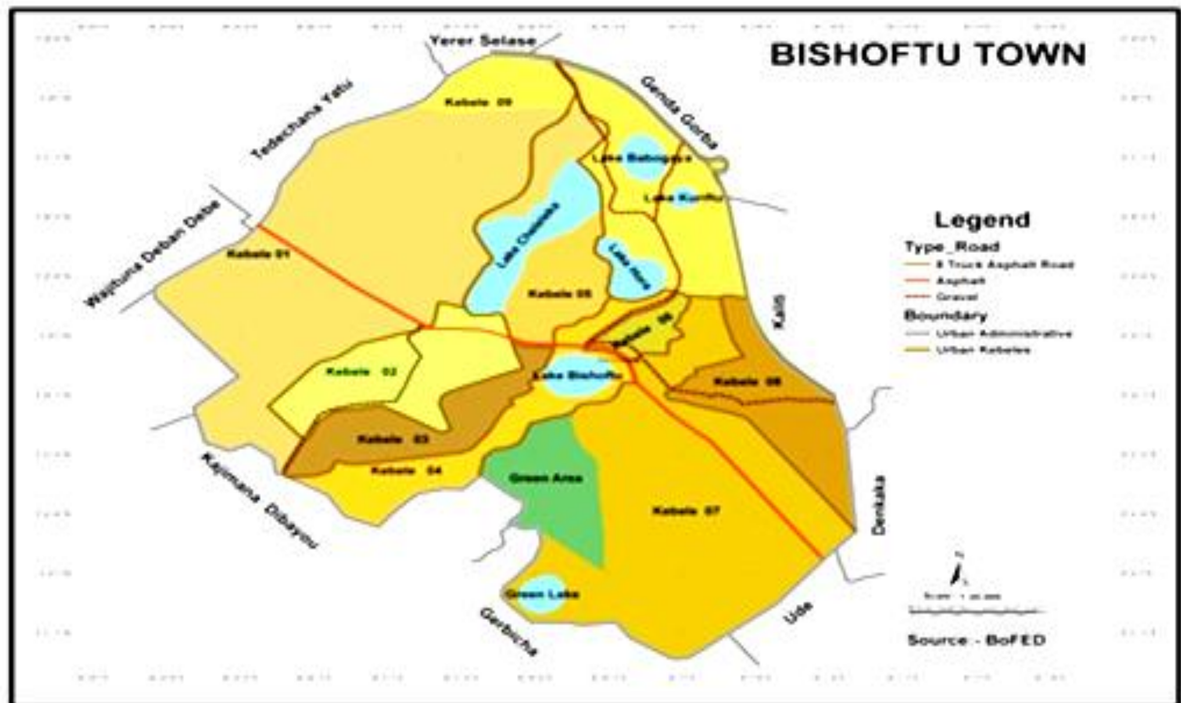


Figure 4. Map of the Study area (Bishoftu Town)

Source: https://www.google.com.et/search map of Bishoftu.

3.2. Study Design

In the present study, experimental study design was employed to adapt NDV in African green monkey kidney (vero) cells and evaluate safety and immunogenicity of its vaccine under laboratory conditions. The study was divided into three major steps. In the first step, adaptation of NDV was performed with inoculation of African green monkey kidney cells (vero) cell line monolayers by serial passages. The presence of NDV Infected Cell Culture Fluid (ICF) of each passage was detected by HA and HI test. Further confirmation of identity was done by molecular detection. Then trial vaccine was prepared from vero cell culture adapted NDV I-2 srain after determination of its infectivity titer using a standard protocol (OIE, 2012).Finally safety test was done in chicken and immunogenicity was evaluated. Seventy (70) apparently healthy and ND antibody low chickens were used at 2 week age for safety test and 4 weeks age for immunogenicity evaluation (OIE, 2012). Experimental chickens were randomly allocated into four isolation units of 20 birds each for trail vaccine, positive control safety test and 10 birds for negative controls. A pre-vaccination serum was collected from chickens before vaccination and their maternal antibodies against NDV were assessed. Four experiments (three treatments and one control) chickens housed in isolation units and vaccinated at age 14th and 28th days of age for safety test and immunogenicity evaluation respectively. Safety of vero adapted ND I-2 inoculating 10 times the single dose (50µl) by eye drop and clinically checked for 3 weeks to determine the presence of local and/or systemic adverse reactions. To evaluate immunogenicity of vero cell adapted ND vaccine, collection of blood at day 0 before and 7th, 14th, 21^{th,} 28$^{th\ and}$ 35th day post vaccination from experimental chickens and compared with the positive control by HI test.

3.3. Study animals and husbandry

The study was conducted on 70 day old unvaccinated apparently healthy chickens purchased from a local hatchery (Alema poultry farms, Bishoftu, Ethiopia).The chickens

were raised together in a disease-free house in the NVI (Bishoftu, Ethiopia) until grouping for the experiment. After experiments, Chickens were separated into appropriate experimental groups and were housed in isolation units. Both male and female chicken were used in the experiment. Chickens in all groups were given the same feed and water ad *libitum* throughout the experiment. The experiments complied with the international guideline on animal experiments and were reviewed by an ethical review commite in Collage of Veterinary Medicine and Agriculture, Addis Ababa University.

3.4. Source of virus strain, cell line and culture media

Lyophilized Lentogenic NDV thermosthable I-2 strain working seed (passage 3) with a titer of a titer $10^{9.2}$ EID50 /Ml and African green monkey kidney cells passage 18 (P18) , Glasgow minimum essential medium (GMEM) supplemented with trypsin and fetal calf serum (10%) lot number 22, Dulbecco's phosphate buffered saline (PBS) and trypsin-EDTA (0.25%) solutions obtained from NVI were used throughout the study.

3.5. Study Methods

3.5.1. Sub-culturing of vero cell adherent monolayer

An established confluent monolayer Vero cell line passage eighteen (p18) was obtained from non-infected cell preparation and quality control laboratory of NVI, Bishoftu Ethiopia. This obtained cell was subcultured to confluent monolayer in 25 cm^2 tissue culture flasks in side biosafety cabinet. The growth media from the flask containing vero cell was removed followed by washing with sterile PBS for 2 times. Then 1 ml of trypsin (0.25%) was added into the flask and mixed with the cell to detach cell from the flask wall. The flask was left in the incubator for 2 minutes. After pippeting the cell to be detached from the flask and to make the single cells, 25 ml of the maintenance media was added in a 25 cm^2 flask from which 8ml of media containing cell was poured into other fresh sterile25cm^2 cell culture flask and incubated 37^oC in the presence of 5% of CO_2 in

CO_2 incubator. Cultured cells were observed carefully under inverted microscope until the formation of semi-confluent monolayer.

3.5.1. Infection of vero cells monolayer

Normal and Confluent monolayers of vero cells were used for infection with NDV I-2 strain. Old growth medium from the flask containing vero cell was removed and cell monolayer was washed with sterile PBS two times. Later, confluent monolayer cells were infected with ND virus by inoculation of 0.25 ml NDV I-2 strain sterile inoculum of a titer $10^{9.2}$ EID50 /ml virus in GMEM Supplemented with trypsin. The virus inoculums was spread uniformly and incubated in humidified CO2 incubator for 1 h to allow adsorption. One flask was kept as un-inoculated control. Eight milliliters of sterile maintenance medium with 2% of serum was added to each flask. The flasks were incubated37 ^{o}C in the presence of 5% of CO_2 in CO_2incubator and monolayers were examined twice daily under inverted microscope for evidence of cytopathic effect (CPE).

3.5.1. Virus harvesting

Vero cell adapted ND I-2 virus of each passage was subjected to three freeze-thaw cycles, followed by clarifying centrifugation to remove cell debris and the supernatant was used for further passages. The culture supernatant was harvested by three freeze-thaw cycles, as described by Peilin *et al.* (1997). Production of about 80% CPE was taken to be the harvesting criterion. An infected monolayer was removed from the flasks and transferred to Eppendorf tubes for further processing. The culture medium (viral suspension) was centrifuged at 3000 rpm for 10minutes at $4^{0}C$ to pellet cell debris. The clear supernatant was collected carefully, divided into aliquots labeled as passage 0ne (P1) and stored at-70 $^{0}{}_{C}$as viral stock till for further use.

3.5.2. Adaptation of the virus

Adaptation thermostable NDV I-2 strain was conducted in Vero cell line through serial passage. Harvested P1 supernatant virus was again used to infect to Vero cells using same media and techniques. Virus harvested through this second passage was designated as ND passage2 (P2). Similarly 12 subsequent passages were done and the characteristic

CPE were carefully examined in all passage. The time for the appearance and intensity of CPEs was also recorded in each passage. In the case of the serial blind passages, the undiluted inoculum was used as the seed virus. For active passages, a 1:100 dilution of previous passages was used as the seed material.

3.5.3. 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 hemagglutinating virus in the infective cell culture fluid. The positive haemagglutinating allantoic fluids were confirmed Hyperimmune chicken anti-NDV serum, raised in chickens by repeated inoculation with a NDV vaccine virus.

3.5.4. Reverse-transcription polymerase chain reaction (RT-PCR)

The presence of NDV in tissue culture supernatant was further reconfirmed by RT-PCR. In brief, RNA QigeinRNeasy® mini kit (Invitrogen, USA) and superscriptTM III first-strand synthisis system (Invitrogen, USA) was used for RNA extraction and cDNA synthesis respectively following the protocol provided by the manufacturers. The extracted RNA was subjected to RT-PCR with Qiagentwo Step RT-PCR Kit (Invitrogen,Germany). A primer:-NDV-F--(5′-CTGTACAATCTTGCGCTCA-3′) NDV-R (5′CTGCCACTGCTAAGTTGTG) -3′) were used to amplify a 1100 bp genome fragment containing the fusion (F) protein. Initial denaturation and activation of Taq polymerase at 95ºC for 5 minute and then 35 cycles of PCR with denaturation at 95ºC for 30 second,annealing at 56ºC for 30 second,extension at 72ºC for 30 minute,and final extension at 72ºC for 7 minute. The amplified RT-PCR products were subjected to agarose gel electrophoresis and the resulting cDNA band was visualized in an image documentation system.

3.5.5. Infectivity assay for virus yeild

The titer of vero cell adapted NDV in each serial passage was measured. 1 ml of virus containing fluid (supernatant) was serially passed in ten tubes containing 9 ml of base medium (GMEM) for each passage separately. 100 µl Vero cell was dispensed into all wells of the first eight rows of the Microtiteration plate leaving 11th column. 100µl of GMEM was added in to the first eight wells of 12th column for control. 100µl serially diluted virus was added to all wells of five rows up to 10th column starting from low concentration to higher one. This was the same procedure for all passages to deterimine the titer of each passage. The plates were incubated 37 ^{o}C in the presence of 5% of CO_2 in CO_2incubator for up to seven days and examined twice daily for CPEs. The titer for each virus passages was determined according to Spearmann Karber formula (Spearman, 1908).

3.5.6. Preparation of experimental vaccine

The culture supernatant vero adapted NDV I-2 after passage 12 was harvested by 3 times freeze-thaw cycles, clarified by centrifugation at 3000 rpm for 10 minute. Then sterile supernatant was mixed the gelatin stabilizer with 1% final concentration was added to help preserve infectivity during storage. The formulated vaccine was tested and confirmed free of viable bacterial and fungal contaminants that could be harmful to chickens receiving the vaccine during experiments.Sterility was seen by using sterility media such assoyabean-casein digest medium (SCDM) for aerobic bacteria and fungi incubated at 37^{0}c and 25^{0}c 14 and broth, thioglycollate (FTM) broth for culture of anaerobic bacteria and incubated at37^{0}c for 7 days (OIE, 2012).

3.6. Experimental trail vaccine Safety test

Safety vero cell adapted trail vaccine was seen by administering 20 two week old low antibody chickens with a quantity of the vaccine virus equivalent 10 times the maximum virus titre to be contained in 1 dose of the vaccine ($10^{6.5}$ TICD50). After inoculation, they were observed for 3 weeks to determine the presence of any local and/or systemic

adverse reactions, which may developed after vaccination according to OIE recommendation (OIE, 2012)

3.7. Immunogenicity test

3.7.1. *Experimental layout*

A total 50 chickens (four week old) were divided into 3 groups (G1, G2 and G3) 20 birds for group 1 and 2 each and 10 birds for group 3. Group 1 was vaccinated with freeze dried live cell culture-adapted I-2 NDV vaccine and Group 2 was immunized with commercially available thermostable I-2 vaccine currently produced by NVI as a positive control. The group 3 (G3) was kept as a negative control in the complete random experimental design. Birds were housed separately per treatment group. A single vaccine dose (50μl) (a vial contain $10^{6.5}$TCID50 concentration of virus per birds) was given to the chickens in G1 and G2.

Table 2 Experimental layout of treatment and control groups

Group chicken	Number	Dose (μl)	route	Sera collection
G1	20	50	eye drop	for 6 week starting from 0dpv
G2	20	50	eye drop	for 6 week starting from 0dpv
G3	10	none	none	for 6 week

3.7.2. Blood collection

Experimental chickens were monitored by collecting blood once a week from day 0 to day 35. Serum samples used to evaluate antibody response were obtained from each bird 1 to 2 ml whole blood aseptically wing vein using 3ml disposable syringe before

vaccination (14 day and21 before vaccination) and at 7, 14 and 21,28 and 35 days post vaccination(dpv). The collected blood samples were labeled with individual chicken number and allowed to clot overnight at room temperature to facilitate serum separation. The sera were harvested in to labeled cryovials and stored at − 20 °C until HI was carried out.

3.7.3. Determination of antibody response

Antibody titre against NDV in serum was determined by haemagglutination inhibition HI test described by (OIE, 2004). HI titration was made to determine the right HI concentration via 2-fold serial dilution of 25 µl sera in 25 µl PBS followed by 25 µl loading of viral antigen per well. Then, after 30 min 25 µl of 1 % RBC per well was loaded and kept for 45 min to determine the end point of haemagglutination inhibition. Positive-control and negative-control antigen and antisera obtained from NVI were run with each test. The antibody level for each serum sample was expressed as a log to the base two and recorded. For convenience, the titer was recorded as just the log index. For example, the titer of $\log 2^2$ was recorded as two. The geometric mean titers (GM) were calculated. In this study we used the published cut off value for the protective HI antibody titer (HI titer ≥ $\log 2^4$ i.e. GM≥ 4) for ND vaccination in chickens (OIE, 2012).

3.8. Data management and analysis

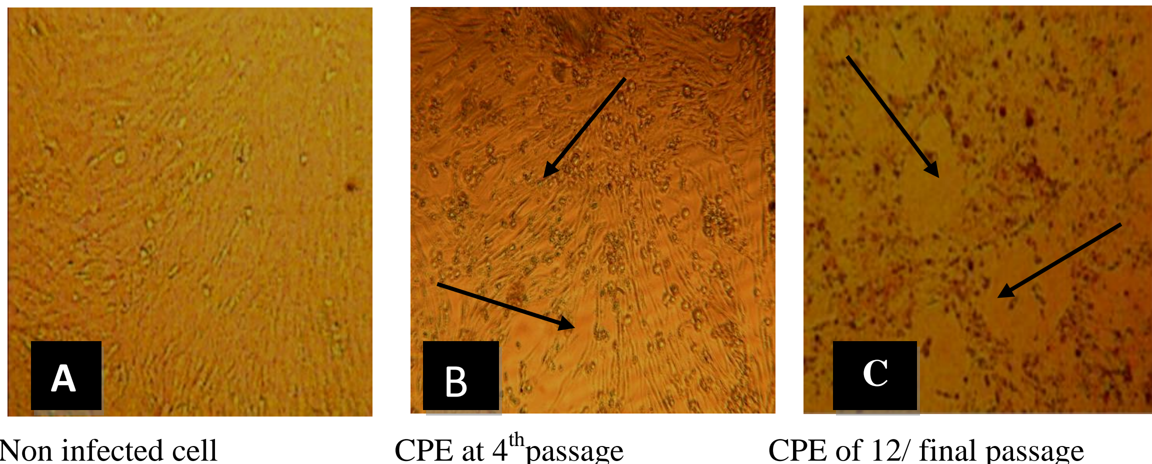
Data obtained from all laboratory investigations and experimental trial will be coded and stored into Microsoft Excel spread sheet 2010.The statistical Data were analyzed by calculating geometric mean titers (**GMTs**), mean ± SD and through one-way analysis of variance (**ANOVA**) followed by Tukey’s test by using STATA 13.0 (STATA COP.) special edition software. HI titres are presented as the geometric mean log2 ± standard deviation for vaccine group. The analyzed data will be interpreted and presented into biological terms. Significance is reported at the level of $P < 0.5$.

4. RESULTS

4.1. Cytopathic effect (CPE)

For adaptation the NDV I-2vaccinal strain, normal and confluent monolayer of Vero cells were established after 48 h of growth in GMEM growth medium and 10% calfserum incubated at 37°C in the presence of 5% of in CO2 incubator (Figure 6A).

After infection, no significant cytopathic changes were noticed in the confluent Vero cell monolayer for up to three consecutive blind passages. Adaptation of the virus in cell line was observed (CPE initiation) from the 4th passage at 72h onwards (Figure 6B), and the extent of the CPE increased with progressive passages up to the 12th/final passage of present study (Figure 6C).



Non infected cell CPE at 4th passage CPE of 12/ final passage

Figure 5. Cytopathic effects of Vero cells after infection with NDV I-2 virus

A: Vero cell control (cell without NDV infection) after 48hour of incubation: CPE of 4th passage after 96hr of incubation and C: CPE 12 passage after 72 hour of incubation (CPE indicated by arrow).

4.1. **Heamagulitination and haemagulitination inhibition test**

After the identification of adapted virus through characteristic CPEs, the virus from each passage was subjected to slide heamagulitination (HA) test (figure 8). Infectious cell culture fluid show CPE were also positive for HA.Haemagglutinating activity of these samples were inhibited by NDV-specific hyperimmune serum in HI test, which is confirmatory for NDV (Alexander, 2009).

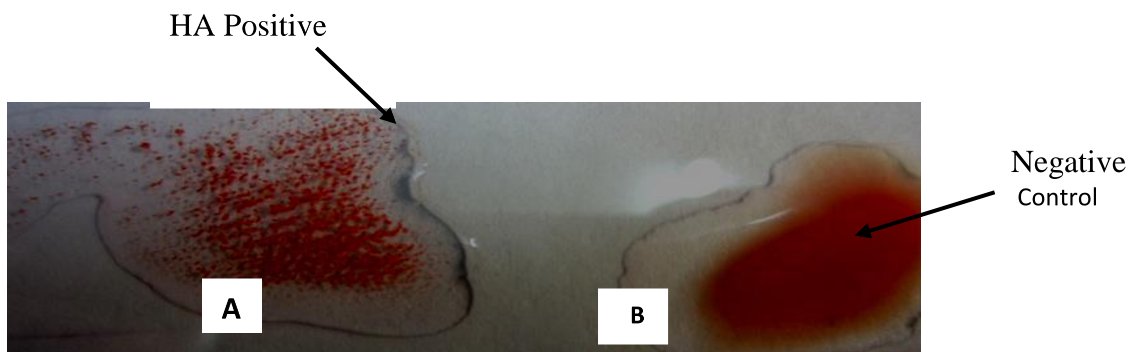


Figure 6. Slide Hemagglutination (HA) of cRBC for vero cell adapted NDV

4.2. RT-PCR for Cell Culture Adapted NDV I-2 Strain

Among HA positive cell culture supernatant fluid sample of vero cell adapted Vaccine Strain three sample passages levels (5,8 and 12) were done and all resulted in generation of an amplicon at the correct expected size of the F gene(1100bp) as ascertained by agarose gel electrophoresis (Fig. 9)

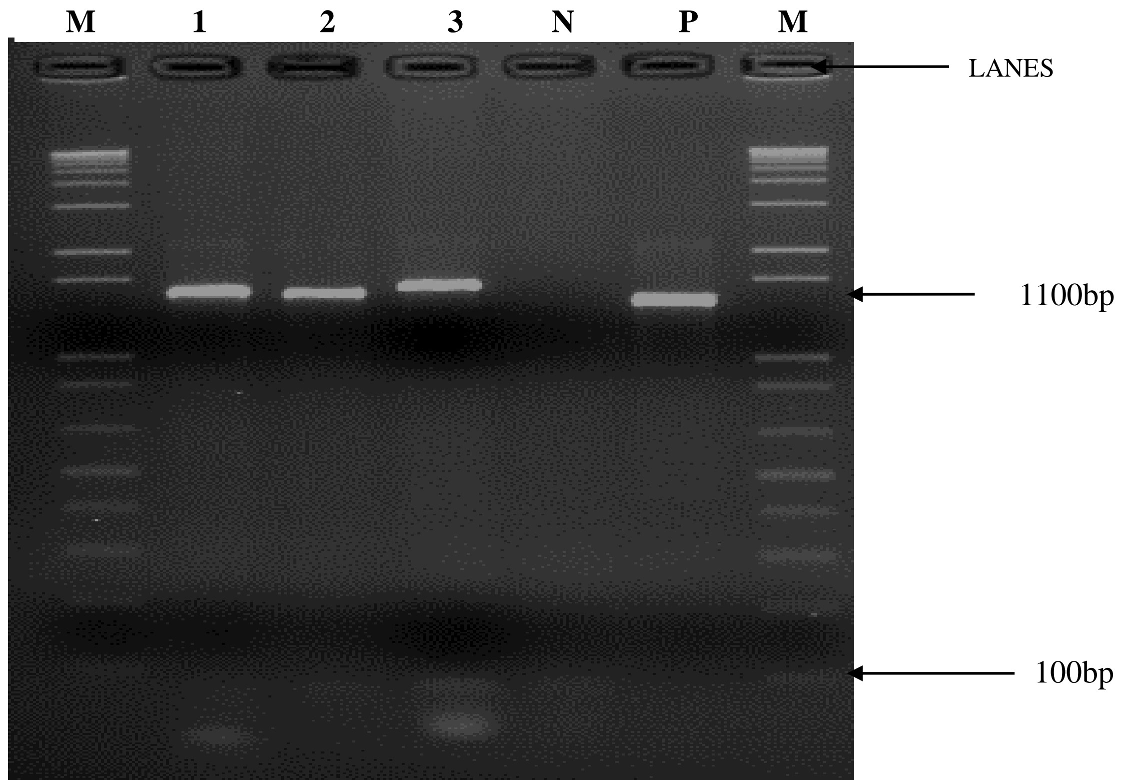


Figure 7.Agarose gel electorophpresis (1.5%) of 1100-bp fragment of the F gene

M- (Lane 1 and 7) Molecular Ladder started 100 BP (Invitrogine) 1 KP plus

1 – NDV I-2 vaccine vero cell adapted passage 12 – Positive.

2 – NDV I-2 vaccine Vero cell adapted Passage 8 – Positive

3– NDVI-2 vaccine Vero cell adapted Passage 5 – Positive

NC – Negative control without template – Negative.

PC – Positive control – Positive

4.3. Tissue culture infective dose 50 (TCID50) assay

Table 1 represents data of $TCID_{50}$ assay vero cell adapted NDV I-2 strain from passage 5 to passage 12. From these data the $TCID_{50}$ titers active passages increased from $10^{3.4}$/ml to $10^{7.2}$/ml. This data indicates that NDV adapted on vero cell with gradual increase of infectivity titer.

Table 3. $TCID_{50}$ % (log10/ml) vero cell adapted ND I-2passage 5 to passage 12.

passage No	$TCID_{50}$
5	3.4
6	4.2
7	5.1
8	5.7
9	6.7
10	6.8
11	7.0
12	7.2

4.4. Safety test

During 21 day observation periods, NDV I-2 trail vaccine Safety was evaluated by observation of by the absence of clinical signs and death in chicken. During observation period, No abnormal clinical signs or mortalities were observed in the group of 14-day old chickens which receiving ten doses at intra ocular routes of vaccination.

4.5. Antibody response evaluation

4.5.1. Maternal antibody antibody titer before vaccination

The mean maternal antibody (geometric mean ± SD) titer of the 70 study chickens at day 7 age was 3.3 ± 0.5 and reduced over time during 14 days to 1.8 ± 0.6 at time of vaccination.

4.5.2. *Seroconversion Status of the Monitored chicken after Vaccination*

For the period of 5 weeks antibody titer of experimental birds categorized under three treatment groups and one control group were analyzed. The geometric mean antibody titre results (log2 ± standard deviation) on days 7, 14, 21, 28 and 35post vaccination in vaccinated and negative control birds after single vaccination are presented in Table 4.

Table 4 Geometric mean HI antibody titre ($\log_2$ ± standard deviation) vaccinated and non-vaccinated groups single vaccination.

Antibody mean titer/experiment day					
Group	7dpv	14dpv	21dpv	28 dpv	35 dpv
G1	2.8 ±0.4	4 ± 0.4	6.7 ± 0.5	6.3± 0.1	6.2± 0.1
G2	2.6 ± 0.6	4± 1.4	6.5 ± 1.4	6.3± 0.3	6.2± 0.3
G3	1.8 ± 0.5	1.8 ± 1.2	1.8 ± 1.0	1.8± 0.9	1.8 ± 1.0

dbv= day before vaccination , dpv= day post vaccinationG1= Ttrial vaccine, G2= positive control G= Negative control

5. DISCUSION

Production of Newcastle disease vaccine need high amounts of specific pathogen-free eggs, high labor-intensity, time consuming and require large area for the incubation of egg. Besides the process is slow and difficult to scale-up, so large strategic stocks must be kept to respond in cases of epidemics (Souza, *et al.,* 2009). An alternative to this method it has been reported that Newcastle disease virus strains are able to replicate in cell culture such as vero cell, chicken fibroblast cell and DF-cell (Ravindraa, 2008, Arifin*et al*., 2011).Therefore, the above mentioned problem emphasizes the importance of maintaining research on adaptation and propagation NDV in cell culture system. In this study adaptation of vaccine srain of newcastle disease I-2 virus in vero cell line and evaluation its trail vaccine safety and immunogenecity in chicken after adaptation by comparing with conventional commercially available live vaccine produced by using SPF embryonated eggs.

In present study, after three consecutive blind passages were conducted, Cytopathic effect began in Vero cell monolayer after 72 hours of incubation following infection during 4th passage (CPE initiation) and the extent of the CPE increased with progressive passages up to the 12th/final passage of this study. CPE was characterized by of syncytium (irregularly shaped cells), gaint cells, clustering of infected cells, death of cells (plaque) floating in media when observed under inverted microscope, Similar patterns of CPE of NDV in vero cell line are close agreement with the findings Ahamed *et al.,* (2004) who demonstrated that at fourth and fifth passages, CPE was rapid and consistent and 5 serial passages of NDV on Vero cell line confirm successful adaptation. Observed CPE characterized by granularity in cytoplasm rounding of infected cells, development of micro plaque clustering of infected cells, intracytoplasmic bridge cell connecting those clusters, vacuolization in the cell system and the formation of syncytia.

The adapted virus was identified and confirmed by heamagglutination and heamagglutination inhibition test. Further, The PCR analysis is successfully amplified the

virus F gene and revealed that the vero cell were infected with NDV virus by producing the expected band size on agarose gel electrophoresis analysis.

One of the most important considerations affecting vaccination programmes is the level of maternal immunity in young chickens, which may vary considerably from farm to farm, batch to batch, and among individual chickens.OIE, 2012). In this study, 7 old, chicks included in this study have HI antibody titer above 3 $\log_2$ during evaluation. Such high maternal antibody titer in chicks is deleterious to vaccination (OIE, 2004). Thus, we waited until it declined to 1.8 $\log_2$titer at 21days to overcome the risk of its interference with the vaccine.

The worth of any vaccine is represented principally by calculating the antibody titers produced in the birds against a given infection. An HI titer of $\geq \log_2{}^4$ following vaccination has been considered protective against virulent ND virus. HI titers lower than $\log 2^3$ have been associated with lower levels of protection (Ghaniei and Mohammadzadeh N, 2012; OIE, 2012).The result of present study showed that after vaccination the mean antibody titres for two vaccines increased significantly after the 2nd week of vaccination and reach peak at day 21 day post vaccination. Mean antibody titre variation between NDV I-2 trial and positive control was not significant ($P > 0.05$). But antibody produced in both group HI titres$\geq$4 $\log_2$ throughout the study until day 35.which considered adequate antibody level to protect chickens from overt clinical disease (OIE, 2012).

6. CONCLUSION AND RECOMMENDATIONS

In the present study, NDV I- 2 strains was successfully adapted on vero cell line with gradual increasing of infective titer from $10^{3.4}$to $10^{7.2}$TCID50/ml.Thevero adapted prepared trail vaccine was safe in vaccinated chicken. In addition to this, geometric mean antibody titre results (log_2 ± standard deviation) of experimental chickens in trail vaccine and positive control groups indicated that HI titer antibodies are induced after seven days, increased regularly thereafter. After day 14 post vaccination, majority of the monitored chickens developed HI antibody titres which were >4 log_2. The mean. HI titer antibody reached peak at day 21 day post vaccination and remained until the follow up ends at day 35.The protectiveness of such peak titre of antibodies is not confirmed through viral challenge in this study though OIE suggests that such peak antibodies are protective against clinical diseases.Fromthe obtained results of the present study, the following recommendations are forwarded:

- The protectiveness of the antibody titre induced by Vero cell adapted ND vaccine against NDV should be evaluated via challenge.
- The effect of serial passage of the virus in cell line on Molecular structure of the virus should be studied.
- To optimize Newcastle disease vaccine production, factors that influence such as serum concentration, multiplicity of infection on virus yield should be studied.

7. REFERENCES

Ahamed, T., K. M. Hossain, M. M. Billah, K. M. D. Islam, M. M. Ahsan, and M. E. Islam, (2004): Adaptation of Newcastle disease virus (NDV) onVero cell line. Int. J. Poult. Sci. 3:153–156.

Alders R, Spradbrow P (2001): Controlling Newcastle disease in village chickens: a field manual. Australian Centre for International Agricultural Research (ACIAR

Aldous EW, Mynn JK, Banks J, Alexander DJ. (2003): A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol; 32:237-255

Alexander D.J and Senne D.A. (2008b): Newcastle Disease and Other Avian Paramyxoviruses. *In*: A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens, Dufour-Zavala L. (Editor in Chief) Swayne D.E., Glisson J.R., Jackwood M.W., Pearson J.E., Reed W.M, Woolcock P.R., 4th ed., American Association of Avian Pathologists, Athens, GA, 135–141.

Alexander DJ (1991)**:** Newcastle disease. In: Rweyemamu, M.M., Palya, V., Win, T. and Sylla, D., Ed. Newcastle Disease Vaccines for Rural Africa. DebreZeit, Ethiopia, Pan African Veterinary Vaccine Centre, 7:45.

Alexander DJ (2003)**:-**Newcastle disease, other Paramyxoviridae and Pneumovirus Infections.In; Diseases of Poultry.11th edn.Iowa State press, Ames. pp: 63-100.

Al-Habeeb MA, Mohamed MHA, Sharawi S (2013): Detection and characterization of Newcastle disease virus in clinical samples using real time RT-PCR and melting curve analysis based on matrix and fusion genes amplification. Veterinary World 6(5):239-243.

Arifin, M. A., M. Maizirwan, H. S. Siti, I. A. K. Mohamed, and S. H. Sharifah, (2011): Optimization of Newcastle disease virus production in T-flask. Afr. J. Biotechnol. 10:18816–18823.

Bellau-Pujol S, Vabret A, Legrand L, Dina J, Gouarin S, et al. (2005): Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses.JVirol Methods. 126: 53-63

Boostani AR, Pourbakhsh SA, Momayez R, Charkhkar S (2013): olecular characterization and phylogenetic study of Newcastle disease virus isolates from the 2010 to 2011 outbreaks in Shiraz, Iran. Afr. J. Microbiol. Res. 7(8):657-660.

Briand F-X, Henry A, Massin P, Jestin V.(2012): Complete Genome Sequence of a Novel Avian Paramyxovirus. J Virol ;86:7710.

Cao Y, Gu M, Zhang X, Liu W, Liu X (2013): Complete Genome Sequences of Two Newcastle Disease Virus Strains of Genotype VIII. *J. Genome Announcements***1** (1):01.

Catroxo MHB, Martins AMCRPF, Petrella S, Curi NA, Melo NA (2011): Research of viral agent in free-living pigeon feces (*Columba livia*) in the City of Sao Paulo, SP, Brazil, for transmission electron microscopy, *Int. J. Morphol*. **29**(2):628-635.

Cattoli G, Fusaro A, Monne I et al.(2010): Emergence of a new genetic lineage of Newcastle disease virus in West and Central Africa—Implications for diagnosis and control. Vet Microbiol;142:168-176.

Cattoli G, Susta L, Terregino C, Brown C.(2011): Newcastle disease: a review of field recognition and current methods of laboratory detection. Journal of veterinary diagnostic investigation 23:637-656.

Center for food security and public health (CFSPH) (2016): IOWA State University College of Veterinary Medicine.Newcastle Disease Avian Paramyxovirus-1 Infection, Goos.Paramyxovirus infection, Ranikhet disease.

Chaka H, Goutard F, Bisschop SPR, Thompson PN (2012)**:** Seroprevalence of Newcastle disease and other infectious diseases in backyard chickens at markets in Eastern Shewa zone, Ethiopia. *PoultSci***91**:862–869..

Cho S, Kwon H, Kim T, Kim JH, Yoo H*, et al.* (2008): Characterization of a Recombinant Newcastle Disease Virus Vaccine Strain. Clin Vaccine Immunol. 15: 1572-1579.

Choi KS, Lee EK, Jeon WJ, Kwon JH (2010): Antigenic and immunogenic investigation of the virulence motif of the Newcastle disease virus fusion protein. *J. Vet. Sci*. **11**(3):205-211.

Chukwudi OE, Chukwuemeka ED, Mary U (2012): Newcastle disease virus shedding among healthy commercial chickens and its epidemiological importance. Pakistan Vet J. 32: 354-356.

Cornax I, Diel DG, Rue CA et al.(2013): Newcastle disease virus fusion and haemagglutinin-neuraminidase proteins contribute to its macrophage host range. *J Gen Virol* ;**94**:1189-1194.

CSA, (2016): Agricultural sample survey 2014/15, volumeII.report on livestock and livestock characteristics private peasant holdings, 578 Statistical Bulletin 578

Czeglédi A, Ujvári D, Somogyi E et al (2006): Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res ;120:36-48.

De Leeuw OS, Koch G, HerzogL, Ravens horst N, Peeters BP (2005): Virulence of Differentiation of pathogenicity of avian paramyxovirus serotypes 1. Offi J EurUni*AvianPathol*. **31**: 493-499.

Dimitrov KM, Ramey AM, Qiu X, Bahl J, Afonso CL(2016): Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infection, genetics and andevolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases ;39:22-34. .

Doyle TM Rweyemamu, M.M., Palaya,V., Win, T., and Sylla, D (1927)**:** Newcastle disease Vaccines for Rural Africa, Pan African Veterinary Vaccine Center, DebreZeit, Ethiopia, pp. 7-45.

Fentie T, Heidari A, Kassa T, Capua I, Cattoli G, Sahle M (2013)**:**Molecular characterization of Newcastle disease viruses isolated from rural chicken in northwest Ethiopia reveals the circulation of three distinct genotypes in the country. Trop Anim Health Prod 10:11250–11263

Foster, N.M. and C.H. Thompson (1957)**:** Effect of temperature on Newcastle disease virus. *J. Vet. Med*., **52**: 119-121.

Hasan AR, Ali MH, Siddique MP, Rahman MM, Islam MA (2012): Clinical and laboratory diagnoses of newcastle and infectious bursal diseases of chickens. Bangladesh J Vet Med. 8: 131-140.

Hewson K, Noormohammadi AH, Devlin JM, Mardani K, Ignjatovic J (2009): Rapid detection and non-subjective characterisation of infectious bronchitis virus isolates using high-resolution melt curve analysis and a mathematical model. Archives Virol. 154: 649

Hussain I, Rasool MH (2005)**:** Adaptation of indigenous very virulent infectious bursal disease virus on Vero cell line. Pak. *Vet. J*. **25**(3):103-106.

Maclachlan NJ, Dubovi EJ, editors (2010): Paramyxoviridae. Fenner's Veterinary Virology.4th edn. Academic Press, San Diego, :299-325.

J. M. DiNapoli, L. Yang, A. Suguitan Jr.(2007) **:** "Immunization of primates with a newcastle disease virus-vectored vaccine via the respiratory tract induces a high titer of serum neutralizing antibodies against highly pathogenic avian influenza virus,"*Journal of Virology*, vol. **81,** no. 21, pp. 11560–11568,

Karber, G.,(1931)**:** 50% end-point calculation. Arch. Exp. Pathol. Pharmak. 162: 480-483.

Kebreab, G/mariam, Mohamed, Nasser and BerhanuBeyene, (2001): Vaccination of Layers Against Newcastle Disease Using Inactivated Oil Adjuvant and Live Vaccines: vol.5, No.1, Journal of The Ethiopian Veterinary Association:Vol.5,No1 Published by the Ethiopian veterinary association (EVA)Addis Abeba ,Ethiopia, pp 1-7

Khan TA, Rue CA, Rehmani SF, Ahmad A, Wasilenko JL, et al. (2010): Phylogenetic and Biological Characterization of Newcastle Disease Virus Isolates from Pakistan. *J ClinMicrobiol*.**48:** 1892-1894.

Kraneveld FC (1926): Newcastle disease and other Paramyxovirus Infections. In: Calnek, B.W., et al., (edn.) Diseases of Poultry, 9th Edition.Ames, Iowa State University .Press, pp. 496-519.

L. J. Reed and H. Muench (1938): "A simple method of estimating fit per cent endpoints," *American Journal of Epidemiology*, vol. 27, no. 3, pp. 493–497, 1938.

Lamb RA, Parks GD (2007): Paramyxoviridae the viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, et al., editors. Field's Virology.5th edn. Lippincott Williams & Wilkins, Philadelphia, 2007:1449-1496.

Lamb RA, Parks GD. Paramyxoviridae (2007): The viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, et al., editors. *Field's Virology*.5th edn. Lippincott Williams & Wilkins, Philadelphia,:1449-1496.

Lancaster JE (1966)**:** Newcastle Disease: A Review 1926-1964. Monograph No. 3 . Ottawa: Canadian Department of Agriculture..

Linde AM, Munir M, Zohari S, Stahl K, Baule C, Renstrom L, Berg M (2011): Complete genome characterisation of a Newcastle disease virus isolated during an outbreak in Sweden in 1997. *J. Virus Genes***41**:165-173.

Mackay IM, Arden KE, Nitsche A (2002): Real-time PCR in virology. NuclAci Res. 30: 1292-1305.

Madadgar O, Karimi V, Nazaktabar A, Kazemimanesh M, Ghafari MM, Dezfouli SM A, Hojjati P (2013): A study of Newcastle disease virus obtained from exotic caged birds in Tehran between 2009 and 2010. *Avian Pathol*.**42**(1):27-31.

Markos T, Abdela N (2016): Epidemiology and Economic Importance of Pullorum Disease in Poultry: A Review. Global Veterinaria 17: 228-237.

Mase M, Imai K, Sanada Y, Sanada N, Yuasa N, et al. (2002): Phylogenetic analysis of newcastle disease virus genotypes isolated in Japan. J ClinMicrobiol. 40: 3826-3830.

Mayo MA (2002)**:** A summary of taxonomic changes recently approved by ICTV. Arch Virol 147:1655–1656

Mazengia H (2012)**:** Review on major viral diseases of chickens reported in Ethiopia. J Infect DiseImmunol 4(1):1–9

Miller PJ, Estevez C, Yu Q, Suarez DL, King DJ (2009): Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild type and recombinant viruses. Avian Dis. 53:39-49.

Munir M, Zohari S, Abbas M, Berg M (2012): Sequencing and analysis of the complete genome of Newcastle disease virus isolated from a commercial poultry farm in 2010. Archive Virol. 157: 765-768

Nasser M (1998) **:** Oral Newcastle disease vaccination trials and studies of Newcastle disease in Ethiopia, M.Sc Thesis, FreieUniversität.

Naveen KA, Singh SD, Kataria JM, Barathidasan R, Dhama K (2013): Detection and differentiation of pigeon paramyxovirus serotype-1 (PPMV-1) isolates by RT-PCR and restriction enzyme analysis. Trop Anim Health Prod. 10: 01-06.

O. De Leeuw and B. Peeters,(19990: “Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae,” *Journal of General Virology*, vol**. 80,** no. 1, pp. 131–136,

OIE (2004): Newcastle disease. In: OIE manual for diagnostic tests and vaccines for terrestrial animals, vol. 1. 5th ed. p. 270–83.

Otim MO, Mukiibi GM, Christensen H, Bisgaard M (2005): Aflatoxicosis, infectious bursal disease and immune response to Newcastle disease vaccination in rural chicken. *Avian Pathol*.**34**: 319-323.

P. V. Ravindra, A. K. Tiwari, B. Ratta (2008)**:** “Induction of apoptosis in Vero cells by Newcastle disease virus requires viral replication, de-novo protein synthesis and caspase activation,” Virus Research, vol. 133, no. 2, pp. 285–290.

Pedersen, J. C. (2011): Procedure for Determining Mean Death Time for Newcastle Disease Virus Isolates, SOP-AV2016, National Veterinary Services Laboratories testing protocol,Ames, Iowa, USA

Pham H M, Konnai S, Usui T, Chang KS, Murata S, Mase M, Ohashi K, Onuma M (2005): *Archive Virol.* **150**:2429-2438.

Qiu X, Sun Q, Wu S, Dong L, Hu S, Meng C, Wu Y, Liu X (2011): Entire genome sequence analysis of genotype IX Newcastle disease viruses reveals their early-genotype phylogenetic position and recent-genotype genome size. *Virol. J*. **8**:01-11.

Quinn PJ, Markey BK, Carter ME, Donnelly WJ, Leonard FC (2002) : Veterinary Microbiology and Microbial Disease. 4thed., Black well publishing company: Blackwell Science, London, UK, pp: 137-143. rapid detection and differentiation of airborne chicken Newcastle disease virus in chicken houses. *J Virol Methods***. 158:** 1-5.

Ravindra, P. V., K. T. Ashok, R. Barkha, C. Uttara, K. P. Sudesh, and R. S. Chauhan, (2009): Newcastle disease virus-induced cytopathic effect in infected cells is caused by apoptosis. Virus. Res. 141:13–20.

Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, et al. (2005): Diseases of poultry, 11th edn, pp: 66-78.

Shim JB, So HH, Won HH, Mo I (2011): Characterization of avian paramyxovirus type-1 from migratory wild birds in chickens. *Avian Pathol*.**40**: 565-572.

Souza MC, Freirea MS, Schulze EA, Gaspar LP, Castilho LR (2009)**:** Production of yellow fever virus in microcarrier-based Vero cell cultures. Vaccine, 27: 6420-6423.

Spadbrow (1992): Newcastle disease in village chicken. Control with thermostable oral vaccine. Proceeding of an international workshop, Kuala lumpar, Malaysia. ACIAR, Canberra.

SpradbrowPB (1999)**:** Epidemiology of Newcastle Disease and the Economic of its control. In Proceedings of international workshop on Poverty eradication and promotion of gender equality, held at Livestock Research Institute 26–26 March1999. Denmark: Tune land boskole;:165-173

Tadelle Dessie, and Yilma Jobre (2004): A Review of The Importance and Control of New castle Disease in Ethiopia, Ethiopian Veterinary Journal, , vol.8, No 1, Ethip.vet.J. Is the official Scientific Organ of The Ethiopian Veterinary Association, Addis Abeba, Ethiopia: 1683-6324, pp 71-79

Turner P, McLennan A, Bates A, White M (2005): Molecular Biology, 3rd edn. Bios Instant Notes, edited by Hames BD, Taylor and Francis, Leeds, UK.

Van Boven M, Bouma A, Fabri THF, Katsma E, Hartog L, Koch G.(2008): Herd immunity to Newcastle disease virus in poultry by vaccination. Avian Pathol.37:1–5.

Waheed U, Siddique M, Arshad M, Ali M. Saeed A (2013): Preparation of newcastle disease vaccine from VG/GA strain and its evaluation in commercial broiler chicks. *Pak. J. Zool*. **45**(2):339-344.

Wajid A, Rehmani SF, Wasim M *et al*. (2016): Complete Genome Sequence of a Virulent Newcastle Disease Virus Strain Isolated from a Clinically Healthy Duck (Anasplatyrhynchosdomesticus) in Pakistan. Genome Announcements; 4**.**

Wambura, P. N. (2006): Comparative propagation of Newcastle disease virus (strains I-2 and V4) on chicken embryotracheal explants. Vet. Res. Community. 30:673–677

Wambura, P. N., J.Meers, and P. B. Spradbrow, 2006: Thermostability profile of Newcastle disease virus (strain I–2) following serial passages without heat selection. Trop. Anim. Health. Produc. 38:527–531.

Wen M, Chen, ZT, Zhang DX, Yang JL, Zhou BJ (2007): Cloning and sequence analysis of F gene of Newcastle disease virus isolated from Guizhou province, China. *J. Food Agric.Environ.* **10**(3&4):484-486.

Whelan SP, Barr JN, Wertz GW (2004): Transcription and replication of non-segmented negative-strand RNA viruses. Curr Top MicrobiolImmunol ;283:61-119.

Whelan SP, Barr JN, Wertz GW. (2004): Transcription and replication of non-segmented negative-strand RNA viruses.*Curr Top MicrobiolImmunol*;283:61-119.

Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King JK, Kapczynski DR, Spackman E (2004): Development of a Real-Time Reverse-Transcription PCR for Detection of Newcastle Disease Virus RNA in Clinical Samples. J. Clin. Microbiol. 42(1):329-338.

Xiao S, Nayak B, Samuel A et al.(2012): Generation by reverse genetics of an effective, stable, live-attenuated newcastle disease virus vaccine based on a currently circulating, highly virulent indonesian strain. PloS one;7:e52751.

Yune N, Abdela N (2017): Update on Epidemiology, Diagnosis and Control Technique of Newcastle Disease. *J Vet SciTechnol***8**: 429.

Zanetti F, Rodriguez M, King DJ, Capua I, Carrillo E, Seal BS, Berinstein A (2003): Matrix protein gene sequence analysis of avian paramyxovirus 1 isolates obtained from pigeons. Virus Genes 26:199-206.

8. ANNEXES

Annex1. Formulation of growth and maintenance Cell Culture Medium

Base cell culture medium preparation

Ingredients	Amounts
GMEM (MEM)(powdered)	12.5 g
NaHCO3	2.75 g
H2O (bi distilled)	1000ml

Dilute and adjust t pH to 7.35 to 7.4 by adding NaHCO3 solution.

Sterilized by filtration with a filter pad 0.22 μl pore size in to sterile bottles, place at +4oC

Preparation of maintenance cell culture medium

Base GMEM medium 800ml

Tryptose phosphate broth 100ml

Gamma irradiated Calf serum 20ml

Antibiotic 1ml/lit

Annex 2. Preparation Trypsin Solution (0.25%).

Ingredient	Amount
NaCl	8.0 g
KCl	0.4 g
Glucose	1.0 ml
Phenol Red	(0.5% solution) 1.0 ml
Trypsin	(1:250) 2.5 g
NaHCO3	0.35 g
Purified H20	1 liter

Adjust pH to 7.4 with NaHCO3 solution. Sterilized by filtration

Annex3. Growth and maintenance media

- Use 9-11 day old embryos. The technique described here is for 3-5 embryos.
- Place all media, solution and trypsin in 37°c water bath.
- Spray eggs with Bio guard disinfectant (70% ethyl alcohol) place in hood. Using sterile
- technique, open shell and remove embryo with blunt ended curved forceps.
- Place embryos in Petri dish and cut off heads. Removal of limbs and viscera.
- Transfer bodies to new Petri dish or beaker containing PBS with antibiotics.
- Wash with PBS 3-4 times to remove red blood cells and extra debris
- In the beaker, the bodies can be fragmented by carefully chopping them with sterile scissors.
- Pour tissue fragments into trypsinization
- flask containing magnetic stirring bar.
- Add about 50 ml pre-warmed (37oC) trypsin solution (0.25%) and put on stir plate at slow speed into 37 oC incubator for 10-15 minutes.
- Pour off supernatant into centrifuge tube with calf serum.
- Add 50 ml Trypsin solution and stir slowly in 37 oC incubator for 8 minutes. This may be repeated 1 more time for a total of 2 trypsinization.
- Centrifuge 10 min. at 1500 rpm. Note the amount of Pelleted cells obtained.
- Pour off Trypsin solution and resuspend cells complete cell culture medium (10% calf
  serum and tryptose phosphate broth contained GMEM or MEM.
- The cells may be counted or diluted 1:200and dispensed with in tissue culture flask and incubate at Co2 connected 37 oC incubator.

Annex4. Procedure for Inoculating Preformed Monolayer's

- Place all media and solution in 37°c water bath.

- Swirl T.C flask to resuspend as many RBC's and debris as possible and then decant and discard growth medium.
- Wash monolayer gently with 2-3times of pre warmed PBS and discard.
- Add 1 ml sample inoculums to the small T C flask (25 cm2)or 2 ml for the larger size
- Rock each plate gently to distribute inoculums evenly over the cell monolayer.
- Incubate inoculated cultures in 37°c incubator for 45 minutes to 1 hour to allow virus to adsorb.
- Rock tray once or twice during incubation if possible.
- Add 20 ml maintenance medium to each small T C flask (25 cm2).

  NOTE: Maintenance media 2% calf serum.

  Incubate at 37°c. Check plates daily for cytopathogenic effect (CPE) and condition of cells.

  To harvest samples, freeze-thaw 2-3 times and collects

Annex5. Haemagglutination test

i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.

ii) 0.025 ml of the virus suspension (i.e. infective or inactivated allantoic fluid) is placed in the first well.

For accurate determination of the HA content, this should be done from a close range of an initial

series of dilutions, i.e. 1/3, 1/5, 1/7, etc.

iii) Twofold dilutions of 0.025 ml volumes of the virus suspension are made across the plate.

iv) A further 0.025 ml of PBS is dispensed to each well

v) 0.025 ml of 1% (v/v) chicken RBCs is dispensed to each well.

vi) The solution is mixed by tapping the plate gently. The RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving

complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

Annex6. Haemagglutination inhibition test

i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.
ii) 0.025 ml of serum is placed into the first well of the plate.
iii) Twofold dilutions of 0.025 ml volumes of the serum are made across the plate.
iv) 4 HAU virus/antigen in 0.025 ml is added to each well and the plate is left for a minimum of 30 minutes at room temperature, i.e. about 20°C, or 60 minutes at 4°C.
v) 0.025 ml of 1% (v/v) chicken RBCs is added to each well and, after gentle mixing, the RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for about 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.
vi)The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (positive serum, virus/antigen and PBS controls) should be considered to show inhibition.
vii) The validity of results should be assessed against a negative control serum, which should not give a titre>1/4 (>22 or >log2 2 when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

Annex7. Test for potency - estimation of virus content

Materials and equipment

- VERO cell suspension
- GMEM
- bijoux bottles with Screw caps or silicone rubber stoppers
- Tissue culture plates, 96-well, flat bottom

- Inverted microscope.
- CO2 Incubator

Preliminary steps

Vero cells from one flask are trypsinised and suspended in complete culture medium at 300,000cells/ml.
Place into test tubes or bijoux bottles 4.5 ml of GMEM without serum for preparing the serial dilutions.

Chronological instructions

1. Reconstitute freeze-dried vaccine in 1 ml of cell growth medium. A Liquid viral antigen is used as it is.

2. Set up dilution bottles in rack and mark them in order.

3. Prepare 10-fold dilution series from 10-1 to 10-6 thus: pipette 0.5 ml of the undiluted liquid vaccine or the reconstituted vaccine into the first dilution bottle, marked 10-1 without touching the diluent meniscus. The pipette is discarded. With a fresh pipette mix the contents of this bottle thoroughly and transfer 0.5 ml to the second bottle, marked 10-2, again without touching the meniscus of the diluent. Repeat the mix and transfer sequence until the 10-8 dilution, each time with a fresh pipette. This results in a set of 8 bottles containing serial dilutions of the vaccine from 10-1 through 10-8. (Separate sterile pipettes must be used for each dilution of the vaccine. After the required volume of mixture is transferred to the next tube, change the pipette).
4. Dispense 0.1 ml culture medium into each of 6 first wells of column 12.

5. Dispense 0.1ml of vaccine dilution 10-6 into each of 10 wells of column 1 to 10 in Row F and continue for every dilution step towards Row A (dilution 10-1).

6. Add 0.1ml of Vero cell suspension (30,000 cells per well, equivalent to 100 µl of cell suspension) to each of the six first well in column 12.

7. Add 0.1 ml of the cell suspension (30,000 cells per well, equivalent to 100 µl of cell suspension). to each row of column 1 to 10 starting from row F to row A.

8. Column 11 should therefore be empty.

9. One row of wells serves as a control for uninfected cells to which

10. Add 0.1ml of GMEM into each of the first 6 wells of column 12 (control of uninfected cells).

11. Seal and incubate plates in 5% CO2 atmosphere at 37oC for 10-15 days.

12. Using an inverted microscope, fitted with a specimen guide microplate template and 2.5x and 10x objectives, examine the monolayers for evidence of CPE, initially, on day 4-5 and, thereafter, on every second day until day 10-11.

13. Record wells in which the cells are showing CPE. Cells in column 12 should be compact and devoid of any evidence of CPE.

14. Calculate the virus titre using the Spearman-Karber formula (Titre/ml, titre/dose).

Calculations and Interpretations of the result(s)

The calculation of virus titer relies on the principle of quantal dose response relationship. For a stimulus - subject system as virus titration, measurement of response is to record whether or not the subject manifests the expected reaction. The quantal assay so usedmeasures an "all - or - none" response, e.g. CPE as a manifestation of infection. To measure such a quantal response, the most frequently used system is the multiple serial dilution assays. In a multiple serial dilution assay, each dilution is tested in replicates (at least five). The end-point is the dilution of a substance at which a specified number of members of a test group shows a defined effect. The most frequently used and statistically useful end-point is 50%. It is the *Median Effective Dose*, which in virus infectivity titration in cell cultures is the *Tissue Culture Infective Dose (TCID50).* Thus the median effective dose is the dilution of the test population which will demonstrate response in 50% of the population. i.e CPE in 50% of a large number of inoculated cultures.

Procedure using the Spearman-Karber Formula

The test sample is diluted in a geometric series, that is, with a constant ratio between successive dilutions, and a constant volume (usually 0.1ml) of each dilution is inoculated into each of at least five replicate cell cultures. The most commonly used dilution factor is 10-fold.

For the Spearman-Karber formula to be applicable it is necessary to use constant number of test monolayers per dilution (ni), a constant dilution factor and a range of dilutions wide enough to bracket both the dilutions at and below which 100% of ni subjects (i.e. cell culture monolayers) tested will respond and the dilutions at and above which 100% of ni subjects test will be negative.

If one or more of these conditions is not met, it is sometimes assumed that, for a constant dilution factor, the next higher or lower dilution to the last one tested would have produced the desired result. The "fabrication" of data in this way is without any theoretical basis, but if applied with suitable caution it may do little harm. However, it is preferable to repeat the titration with more appropriate range of dilutions, and this is essential if there are serious shortcomings in the data.

Annex 8. Calculation of the geometric mean titre (GMT)

The mean values of data such as HI titres that are expressed as logs are calculated and expressed as geometric means rather than arithmetic means. In this way the effects of results that are much larger or smaller than most are reduced

The general formula for calculating the GMT is: GMT = n√x1x2x3………….xn

where x = value of the observation, n = number of observations.

To calculate the GMT

1. Calculate the log2 of each of the titres in the group.

2. Add the indexes and divide by the total number of samples.
3. This is the geometric mean titre

Annex9. Procedure Preparation of 4 HA units of ND virus antigen suspension

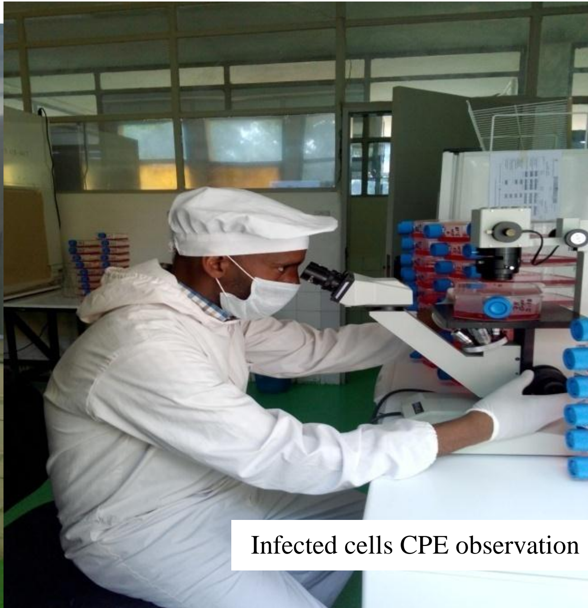
1. Using the quantitative HA test (see section 4.3.2), titrate the ND virus antigen suspension and calculate the HA titre.
2. Divide the HA titre by four to calculate the dilution factor.
3. Calculate the volume of diluted antigen suspension required. Allow 2.5 mL for eachmicrotitre plate.
4. Measure the volume of antigen suspension required and dilute in PBS, using The dilution factor calculated above.

Annex 10. Procedure to preparation of positive sera

1. Vaccinate several 6 to 9 week old chickens with I-2 ND vaccine via eye drop or nasal drop.
2. Vaccinate the chickens again two weeks later.
3. Kill the chickens three weeks after the second vaccination and collect as much blood as possible.
4. Allow the blood to clot.
5. Separate the serum from the clot and pool the serum samples.
6. Centrifuge the serum and store in 1 or 2 mL aliquots at –20°C.
7. Test the serum by HI against reference positive serum to determine the HItitre. Use freshly titrated and diluted antigen. The titre of the serum shouldbe within the range 2^4 to 2^8. (It is best to use serum with a titre of 24 or 25since less serum will be required to produce the laboratory standard serum tomatch national or international standards.)
8. Repeat several times to confirm the HI titer

Annex11. List of picture taken during laboratory work

Infected cell incubation


Infected cells CPE observation

HA and HI test

Sub culturing of vero cell and virus inoculation

Trysinization of vero cell