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ISOLATION AND MOLECULAR IDENTIFICATION OF NEWCASTLE DISEASE VIRUS IN CHICKENS AND EVALUATING THE PROTECTIVE EFFICACY OF NEWCASTLE DISEASE VACCINES IN ADA'A DISTRICT, EAST SHEWA, ETHIOPIA

MSc. Thesis

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A Thesis submitted to College of Veterinary Medicine and Agriculture of Addis Ababa University to fulfill the requirements for the Degree of Masters of Veterinary Science in Veterinary Microbiology

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Isolation and molecular identification of Newcastle Disease Virus in chickens and evaluating the protective efficacy of Newcastle Disease vaccines in Ada'a district, East Shewa, Ethiopia

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AUTHOR DECLARATION

This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture. It is my original work and that all sources of material used for this thesis have been duly acknowledged. The thesis will be deposited at the University / College library to be made available to borrowers under rules of the library.

I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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ABBREVIATIONS

APMV-1	Avian Paramyxovirus 1
bp	Base pair
dpc	Days post challenge
EID_{50}	Embryo infective dose fifty
ELD_{50}	Embryo lethal dose fifty
ELISA	Enzyme linked immunosorbent assay
HA	Haemagglutination
HAU	Haemagglutination unit
HI	Haemagglutination inhibitition
HN	Haemagglutinin Neuraminidase
ICPI	Intracerebral pathogenicity index
loNDV	Low virulent Newcastle Disease Virus
MDA	Maternally Derived Antibody
MDT	Mean Death Time
mRNA	Messenger ribonucleic acid
NA	Neuraminedase
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NP	Nucleoprotein
OIE	World Organization for Animal Health
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
RBC	Red blood cells
RNA	Ribonucleic acid
RNP	Ribonucleo protein
rpm	Revolution per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SPF	Specific Pathogen Free

ssRNA	Single stranded ribonucleic acid
vNDV	Virulent Newcastle Disease Virus

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ABSTRACT

Newcastle disease (ND) is the first most economically important endemic viral disease of chickens that has a potential threat to village chickens and commercial poultry farms in Ethiopia. Reports from NVI Sales and Customer service directorate evidenced that customers are complaining against the effectiveness of lentogenic ND vaccines. Therefore, this study was designed to isolate and molecularly identify Newcastle disease virus and to assess the protective efficacy of ND vaccines against the current circulating viruses. The study was carried out from December 2019 to May 2021 in Ada'a district; Bishoftu town and Dhankaka village. A Cross-sectional study method was used to investigate ND in suspected cases and a total of 52 samples were collected for virus isolation and virus gene detection. ND virus was isolated in specific-pathogen-free (SPF) embryonated chicken eggs and virulence of the isolates were determined *in vitro* and *in vivo.* The current findings confirmed the circulation of virulent strains of ND virus in the study areas. The isolates were further identified by molecular assay, targeting the amplification of avian paramyxovirus-1 (APMV-1). Vaccine protection efficiency was evaluated in chickens hatched from SPF eggs. The chickens were selected randomly and assigned to five experimental groups. Experiment groups were vaccinated by the lentogenic vaccines (HB1, Lasota, and I-2) produced in the National Veterinary Institute (NVI) using $10^{6.5}$Embryo infective dose $(EID)_{50}$ per chicken at day zero (age of 7 days), and boosted at days 14 and 35. Group 1 was vaccinated by HB1 on day zero, revaccinated using Lasota on day 14 and boosted by Lasota on day 35 (HB1-Lasota-Lasota). Similarly, groups 2 - 4 were vaccinated, and boosted as (HB1-I2-I2), (I2-Lasota-Lasota), and (HB1-I2-Lasota) vaccines, respectively while group 5 were unvaccinated and threated as a control group. On day 49 all treatment groups were challenged by 'Dhankaka' ND virus isolate with $10^{6.5}ELD_{50}$ per chicken through occulo-nasal route. The result indicated that chickens in group 1 were induced a protective index of 94%, chickens in groups 2, 3, and 4 were revealed 100% survival while chickens in the unvaccinated group were all dead. Hence, the current ND vaccines produced at NVI induced an immune response against the circulating virulent ND virus using vaccination-boost-boost and challenge protocol in experimental SPF chickens challenge.

Keywords: *Bishoftu, Chicken, Dhankaka, Newcastle disease virus, Vaccine*

1. INTRODUCTION

Newcastle disease (ND) is the first most important viral disease in village chickens and commercial poultry farms of Ethiopia. The poultry population at country level is estimated to be about 48.96 million. According to report of CSA, (2020), indigenous breeds were estimated to be about 81.71%, where as hybrid and exotic breeds share from the total population 10.86% and 7.43% respectively. ND is caused by virulent strains of Newcastle disease virus or avian paramyxoviruses 1 (APMV-1) that belongs to family *Paramyxoviridae*, subfamily *Avulavirinae.* The subfamily divides into three genera; Metaavulavirus, Orthoavulavirus and Paraavulavirus. The taxonomy of the three genera updated to contains 21 species (Walker *et al*., 2021). Newcastle disease virus (NDV) belongs to the genus Orthoavulavirus, is a member of *Avian orthoavulavirus 1* species (Walker *et al*., 2021).

NDV is responsible to cause neurological, respiratory, or enteric disease in birds. Geese and ducks are the least susceptible while chickens are the most susceptible (Wakamatsu *et al*., 2006). The disease is transmitted through direct contact with infected or carriers birds. Transmission can also occur through contact with secretion and excretion of infected birds and contact with contaminated materials. Infected birds are a good source to transmit the disease into susceptible flocks. Hence, all birds will be infected within two to six days (Capua and Alexander, 2009). Mechanical transmission by Fleas, rodent, insect and dog from infected feces is possible (Ullah *et al*., 2004) and experimental studies indicated that transmission through air is also possible (Li *et al*., 2009).

Due to variation in virulence, the clinical signs of Newcastle disease are not pathognomonic. Therefore it is always important to isolate the virus and confirmatory diagnosis to take place. The isolated virus should meet predetermined definition of Newcastle disease that distinguishes it from avirulent enzootic ND viruses (OIE, 2021a). The disease is currently defined as a notifiable if the virus has an Intracerebral pathogenicity index (ICPI) of 0.7 or greater in day-old chicks (*Gallus gallus domesticus*) (OIE, 2021a).

The commercial poultry farms in Ethiopia are currently flourishing specially in urban areas however; major poultry diseases like ND are the limiting factors. For prevention vaccines from the lentogenic strains of the virus, produced at the National Veterinary Institute, Bishoftu, Ethiopia (NVI, 2019) is used.

Seroprevalence of Newcastle disease in Central Ethiopia among the local scavenging chicken as reported by Ashenafi, (2000) was 43.68 % and Tadesse *et al*., (2005) reported 28.57 %, 29.69 %, and 38.33% in Debre Berhan, Sebeta, and Adama, respectively. However, Chaka *et al*., (2012) reported that the prevalence in two districts at different seasons are 6.0 % and 5.9 % in wet and in dry seasons, respectively. Other studies also indicated that the disease is prevalent in different regions of the country (Zeleke *et al*., 2005; Mazengia, 2012).

The evolution of virulent NDV could be resulted due to large phylogenetic and antigenic distances between the current circulating virulent strains and vaccines strain (Miller *et al*., 2007). In Ethiopia the circulation of genotypes II, VI and VII were reported by Fentie, (2013) and sub genotype VIf of class II APMV-1 viruses in village chickens and poultry farms were also reported (Chaka *et al*., 2013; Mulisa *et al*., 2014).

Despite the extensive research after the first Newcastle disease outbreak was reported, ND has challenged the poultry industry for decades. The disease has been affecting commercial and small holder chicken farms and it is still a serious concern to exert a great economic impact in Ethiopia. Effective control of ND involves a combination of efficacious vaccines and strict biosecurity. Hence, ND is one of the disease that requires effective control strategies. Research reports revealed that the scenario of this disease is considered as a potential threat to the poultry industry in the country. Besides, reports from NVI Sales and Customer service directorate evidenced that, customers are complaining against the effectiveness of ND vaccines, that has been produced from the lentogenic strains of NDV by the NVI.

Though, there are different parameters yet to be considered to assess the efficacy of a vaccine, the objectives of this study were:

- To isolate and molecularly identify Newcastle disease virus from the study area
- To evaluate the protective efficacy of Newcastle disease vaccines against the current circulating ND virus

2. LITERATURE REVIEW

2.1. History of Newcastle disease

Newcastle disease (ND) is among the first most important diseases of poultry that causes devastating losses in the poultry industry globally. In Western Isles of Scotland in 1896, as described by Macpherson, (1956) the death of all the chickens is due to ND. The outbreaks to be agreed and recognized first occurred in poultry in 1926, in Java, Indonesia (Kraneveld, 1926). However, the agent was first recognized as filterable in an outbreak occurred in Newcastle-upon-Tyne, England, where the name of the disease was assigned Newcastle disease (Doyle, 1927). It is recognized as a specifically defined disease of poultry with viral etiology that dates from the outbreaks during 1926 in Newcastle-upon-Tyne; however, ND did occur in poultry before this year (Alexander *et al*., 2004).

For the past ninety five years since the first Newcastle disease outbreak was recorded, different researches had been undertaken to overcome the disease but still today it needs to do a lot for its control. Scientists are working to reorganize and reclassify the organism again implies that the disease needs further investigation (Rima *et al*., 2017; Rima *et al*., 2018).

2.2. Etiology

ND is caused by virulent strains of Newcastle disease virus (NDV) synonyms avian paramyxoviruses 1 (APMV-1) that belongs to order Mononegavirales, family *Paramyxoviridae*, subfamily *Avulavirinae,* and genus Orthoavulavirus. The subfamily *Avulavirinae,* comprising three genera; Metaavulavirus, Orthoavulavirus and Paraavulavirus, recently updated into twenty one subtypes designated APMV-1 to APMV-16, APMV-20, APMV-21 and Antarctic penguin virus A (APV-A), APV-B, and APV-C (Walker *et al*., 2021). NDV is a negative-sense, single stranded, non-segmented, enveloped RNA virus (ICTV, 2011). NDV strains differ in genome length. The smallest NDV genomes are 15,186 nucleotides long, but some genomes are longer due to insertions of either 6 nucleotides in the 5’ noncoding region of the NP gene (Huang *et al*., 2004a) or 12 nucleotides in the P gene (Czegledi *et al*., 2006).

2.3. Description of Newcastle disease

The viruses in wild birds and noncommercial poultry are often referred to as APMV-1 to differentiate them from viruses causing virulent infection in poultry, commonly named virulent NDV. A virulent Newcastle disease meets one of the following criteria. Virulence of NDV is dependent on multiple genes; the fusion protein cleavage site, which is the critical site responsible for major changes in virulence (Peeters *et al*., 1999; De Leeuw *et al.*, 2005).

The low virulent viruses (loNDV) have fewer basic amino acids at this site and a leucine instead of a phenylalanine at position 117 (Alexander and Senne, 2008). Reverse genetic techniques shown that the phenylalanine at position 117 and basic amino acids surrounding Q 114 are necessary for virulence (Glickman *et al*., 1988; De Leeuw *et al*., 2003).

Virulent NDV have a multiple basic amino acid at least three arginine (R) or lysine (K) sequence between 112 R/K-R-Q-K/R-R 116 at C terminus of F2 protein and F (phenylalanine) at residue 117, which is the N-terminus of F1 protein. Whereas the viruses of low virulence have a monobasic amino acid sequence in the same region at positions 112 G/E-K/R-Q-G/E 116 and L (leucine) at residue 117 (Kim *et al*., 2008).

'Multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 and 116. Multiple basic amino acids can be demonstrated in the virus either directly or by deduction at the C-terminus of the F2 protein. Phenylalanine at residue 117, which is the N-terminus of the F1 protein. Basic amino acids at 113, 115 and 116 and at position 117 phenylalanine in virulent strains. In this case cleavage of the virus is in a wide range of host tissues and organs can be effected by protease or proteases present. In this agreement to conclude the virus is virulent, amino acid residues are numbered from the N-terminus of the amino acid sequence. Then 113 - 116 corresponds to residues 4 to 1 from the cleavage site, that was deduced from the nucleotide sequence of the F0 gene, (Alexander, 2000; OIE, 2021a).

For replication lentogenic viruses are restricted in the sites like the respiratory and intestinal tracts which are rich with trypsin like enzymes, whereas virulent viruses resulting

in a fatal systemic infection as they can replicate in different tissues and organs (Rott, 1979). Characterization of the isolated virus by ICPI test would be required if fail to demonstrate the characteristic pattern of amino acid residues. The virulent virus has an ICPI in day-old chicks *(Gallus gallus)* of ≥ 0.7 (OIE, 2021a).

2.3.1. Newcastle disease virus genome and morphology

The genome is composed of six genes and encodes their corresponding six structural proteins (Figure 1). They are the nucleoprotein (NP), the phosphoprotein (P), the matrix (M) protein, the fusion (F) protein, the hemagglutinin-neuraminidase (HN) protein, and the RNA polymerase (L) (Chambers *et al*., 1986). RNA editing of the P protein produces additional non-structural proteins V and possibly W (Chambers and Samson, 1982; Collins *et al*., 1982; Mebatsion *et al*., 2001).

NDV appears morphologically pleomorphic but usually spherical in shape, although filamentous enveloped particle with projections of F and HN spike glycoproteins that participate in the initiation of virus infectious cycle (Lamb and Kolakofsky, 1996). Virions are 150 nm or more in diameter, consist of a lipid envelope surrounding a nucleocapsid. The envelope is derived directly from the host cell plasma membrane by budding and contains two or three transmembrane glycoproteins. The viral nucleocapsid consists of a single species of viral RNA and associated proteins. It has helical symmetry and is 13–18 nm in diameter with a 5.5–7 nm pitch. Multiploid virions are found, although the vast majority of virions contain a single functional genome. The viral polymerase is packaged in the virion (Rima *et al*., 2019).

To release the packaged newly assembled virus, the M protein functions to uphold its shape and it supports the newly assembled virus in packaging (Battisti *et al*., 2012). The other three proteins are closely associated with the viral genome and are known to do replication related functions. In particular, for virus replication and mRNA biosynthesis the minimum template was required, thus the NP have to precisely covers the entire viral RNA to form the ribonucleoprotein (RNP) (Conzelmann, 2004). The RNA dependent RNA polymerase L protein, functions as the viral replicase and transcriptase during the

infectious cycle, whereas the P protein serves as the cofactor of the polymerase (Dortmans *et al*., 2010).

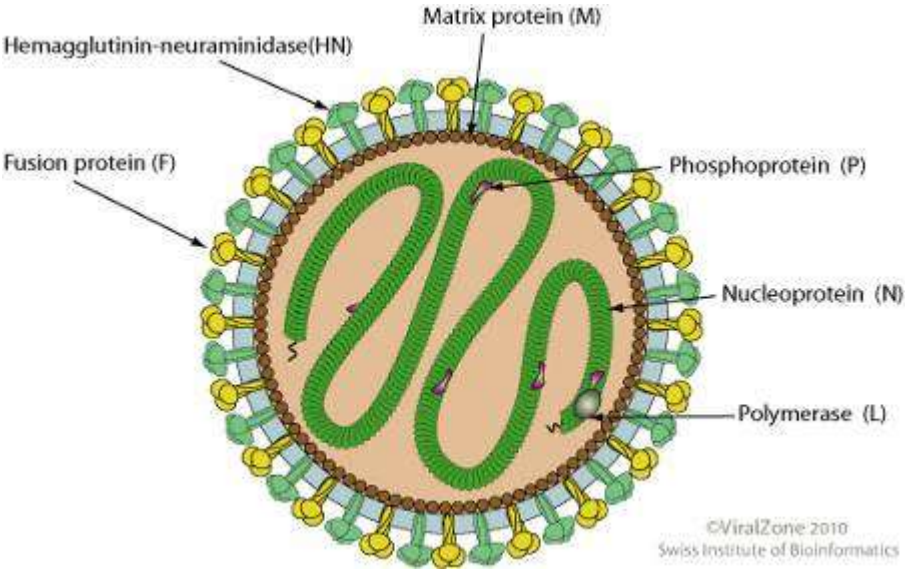

Figure 1. Newcastle disease virus virion structure

(Source: SIB, 2019)

Virions contain a single molecule of linear, negative sense, ssRNA that is not infectious alone but is infectious in the form of the nucleocapsid. The RNA genome size varies substantially: 15,156 nt for Newcastle disease virus (NDV); 14,904–17,262 for other orthoavulaviruses; Genome lengths for all viruses in the subfamily *Avulavirinae* are multiples of 6, which is a requirement for the efficient replication (Calain and Roux, 1993). During gene expression the viral RNA dependent RNA polymerase binds the encapsidated genome at the leader region (Figure 2), then sequentially transcribes each genes by recognizing start and stop signals flanking viral genes. The mRNAs are capped and polyadenylated by the L protein during synthesis. Most express a C protein by leaky scanning and V/W protein is produced through editing of mRNA (ViralZone, 2019).

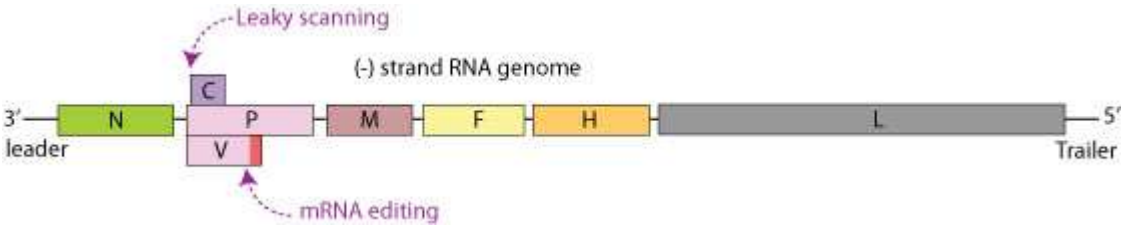

Figure 2. Genome organization of Newcastle Disease virus

(Source: SIB, 2019)

2.3.2. Genome organization and replication

After attachment to cell receptors, virus entry achieved by fusion of the virus envelope with the cell surface membrane. This can occur at neutral pH (ICTV, 2011). Virus replication occurs in the cell cytoplasm and thought to be independent of host nuclear functions. RNA replication occurs through an intermediate, the antigenome, which is a complete exact positive sense copy of the genome (Rima *et al*., 2019). Nucleocapsid assembly occurs in the cytoplasm and tightly linked to RNA synthesis. Nucleocapsids are enveloped by budding at the cell surface plasma membrane at sites containing virus envelope proteins (Rima *et al*., 2019).

2.3.3. Molecular basis for Newcastle disease virus pathogenicity

In the replication of NDV a fusion protein which is functionally important was produced. Thus, for the progeny virus to be infectious, the precursor glycoprotein F0, has to be cleaved to F1 and F2 (Raghavan *et al*., 1998).This post translation cleavage is intermediated by host cell proteases (Nagai *et al*., 1976a). Non infectious virus particles are produced if cleavage fails to take place. Trypsin has the capacity to cleave F0 for all NDV strains therefore, infectivity will restore by *in vitro* treatment of non-infectious virus with trypsin (Nagai *et al*., 1976b).

2.3.4. Classification of Newcastle disease virus

Serological testing have been used to classify paramyxoviruses isolated from avian species (Kim *et al*., 2011) and confirmed by phylogenetic analysis (Alexander *et al*., 2008). There

are two nomenclature systems proposed and are used. The first one, dividing NDV strains into six lineages, and sub lineages was based on phylogenetic analyses performed on partial F gene sequences (Aldous *et al*., 2003). The second, separating NDV strains into two classes and several genotypes. This nomenclature was based on restriction site mapping, genome length, and F gene sequences (Ballagi-Pordany *et al*., 1996; Czegledi *et al*., 2006**)**.

An updated NDV classification and nomenclature system that incorporates phylogenetic topology, genetic distances, branch support, and epidemiological independence was developed (Dimitrov *et al*., 2019). Based on these criteria, NDVs can be categorized as class I or class II viruses. Class I NDVs are occasionally isolated from domestic poultry and wild aquatic birds and are mostly avirulent to chickens. Class II viruses are studied in more detail, and comprise twenty one (I – XXI) genotypes (Dimitrov *et al*., 2019).

The genotypes that are considered ‘‘early’’ (1930– 1960) I, II, III, IV and IX contain 15,186 nucleotides (Czegledi *et al*., 2006). Viruses that emerged ‘‘late’’ (after 1960), V, VI, VII, VIII, and X contain 15,192 nucleotides. Class II contains viruses that have been isolated from poultry species and several wild birds. Most of the viruses within this class are grouped as virulent. They cause to the poultry industry worldwide a significant economic losses (Miller *et al*., 2009). These viruses are continuously evolving and highly diversified (Czegledi *et al*., 2006; Diel *et al*., 2012).

All known class II, genotype I viruses are low virulence, and some are often used as live vaccines except for the vNDV responsible for the 1998–2000 Australian outbreak. Class II, genotype II includes viruses of low virulence that are used as vaccine viruses worldwide, such as LaSota, Hitchener B1 and VG/GA. Genotype III viruses were mostly isolated before 1960 in Japan, but have been isolated sporadically in Taiwan in 1969 and 1985 and in Zimbabwe in 1990 (Yu *et al*., 2001). Genotype IV viruses were the predominant viruses isolated in Europe before 1970 (Czegledi *et al*., 2006).

Genotypes V, VI, VII, and VIII are the predominant genotypes circulating worldwide and contain only virulent viruses. Genotype VII was initially divided in to two sub-genotypes: VIIa, representing viruses that emerged in the 1990s in the Far East and spread to Europe

and Asia and VIIb, representing viruses that emerged in the Far East and spread to South Africa (Aldous *et al*., 2003). The two sub-genotypes of VII are further divided into VIIf, g, and h, which represent African isolates (Snoeck *et al*., 2009) and VIIc, d, and e, which represent isolates from China, Kazakhstan and South Africa (Wang *et al*., 2006; Bogoyavlenskiy *et al*., 2009).

2.4. Pathogenesis

2.4.1. Virus entry and replication

The surfaces of NDV particles contain two important functional glycoproteins: the F and HN proteins. Membrane glycoproteins drive the assembly and budding of enveloped RNA viruses (Simons and Garoff, 1980) and are the key players in determining host range and tissue tropism. The F protein mediates both virus-cell and cell-cell fusion (Hernandez *et al*., 1996). The two glycoproteins found on the surface of paramyxoviruses are involved in the generation of immunity to infection (Chen *et al*., 1971).

Initiation of NDV infection in the host is by receptor recognition. Subsequently on the host cell surface, the virion bind to sialoglycoconjugates. The binding process is followed by a fusion of the viral lipid envelope with cell host membrane (Connaris *et al*., 2002). During the attachment process of NDV to a host cell, HN protein acts as an agent to recognize a sialic acid receptor on the surface of targeted host cells. The HN protein of NDV is a multifunctional protein that possesses both the receptor recognition and Neuraminidase (NA) activities in the release of virus from an infected cell (Connaris *et al*., 2002). Besides, the protein triggers a fusion process of viruses in target cell membranes in conjunction with F protein. It promotes the fusion activity of the F protein, thereby allowing the virus to penetrate the cell surface; and it acts as an NA by removing the sialic acid from progeny virus particles to prevent self-agglutination of progeny virus. Theoretically, F protein requires cooperation with HN protein to perform a membrane fusion during the fusion process so that the virus may penetrate a host cell's surface (Lamb and Kolakofsky, 1996; Porotto *et al*., 2012; Heiden *et al*., 2014).

Interaction of the two viral surface glycoproteins, i.e. HN and F, accomplishes the NDV infection process. In particular, HN protein has been recognized as having a critical contribution during NDV pathogenesis (Huang *et al*., 2004b; Kim *et al*., 2011).

2.4.2. Host range

NDV infects a wide range of domestic and wild bird species resulting in heavy economic losses to the poultry industry. It affects most species of birds, and for susceptible poultry it is highly contagious and usually fatal (Alexander, 1988a). In addition to poultry, more than 230 species from more than one-half of the 50 orders of birds have been shown to be susceptible to natural or experimental infections with avian paramyxovirus type 1(Alexander and Senne, 2008). Wild birds seem to be the reservoir of avirulent strains, whereas poultry are the most important host of the virulent viruses but both hosts exchange viruses (Cardenas *et al*., 2013).

NDV strains have a zoonotic importance that cause conjunctivitis. The sign develops within 24 hours of NDV exposure to the eye. The condition has been limited primarily to laboratory workers and vaccination teams exposed to large quantities of virus. Before poultry vaccination was widely practiced, conjunctivitis from NDV infection occurred in crews eviscerating poultry in processing plants. The disease has not been reported in people who rear poultry or consume poultry products (Garcia *et al*., 1997; Swayne and King, 2003).

2.5. Method of transmission of Newcastle disease virus

Newcastle disease virus show marked difference in the organs they affect. Infected birds shed the virus in exhaled air, respiratory discharges, and feces. The virus will shed during incubation period, at clinical stage, and for a varying but limited period during convalescence. The virus may also be present in eggs laid during clinical disease and in all parts of the carcass during acute vNDV infections. Chickens are readily infected by aerosols and by ingesting contaminated water or food. Infected chickens and other domestic and wild birds may be sources of NDV. Movement of infected birds and transfer of virus, especially in infective feces, by the movement of people and

contaminated equipment or litter are the main methods of virus spread between poultry flocks. Inhalation of infectious virus may occur as the result of the presence of either larger droplets or fine aerosols containing virus (Alexander, 1988a).

2.6. Clinical signs

The clinical signs seen in birds infected with NDV vary widely. Morbidity and mortality depend on virulence of the virus strain, degree of vaccinal immunity, environmental conditions, and condition of the flock (Alexander *et al*., 2004). The clinical signs do not present a consistent basis for diagnosis of ND in birds. Signs for NDV usually depend on different factors such as, the strain of virus and it's virulence, host species and it's susceptibility, age of host the more younger are more susceptible, concurrent infection with other organisms, environmental stress and immune status of the host. Infections with ND viruses can lead to a broad range of clinical signs that are varying from asymptomatic enteric infections that have sub clinical enteric infection to a systemic infections causing 100% mortality. NDV strains have been divided into five groups based on clinical signs seen in experimental NDV infections of chickens (Beard and Hanson, 1984; Getabalew *et al*., 2019; OIE, 2021a).

The viscerotropic velogenic form, which is highly pathogenic, haemorrhagic intestinal lesions are frequently seen. The neurotropic velogenic form, causing high mortality which is usually seen following the respiratory and nervous signs, the mesogenic form that presents with respiratory signs and occasional nervous signs but with low mortality, the lentogenic or respiratory form that cause mild or subclinical respiratory infection and the asymptomatic form that usually consists of a subclinical enteric infection. This classification sometimes overlaps (OIE, 2021a).

2.7. Diagnosis techniques of Newcastle disease virus

2.7.1. Conventional Diagnosis

Due to variations in virulence and the lack of pathognomonic clinical signs of Newcastle disease, isolation of the virus from affected birds and identification of the virus as NDV followed by confirmatory diagnosis is required. Finally, the identification of the isolated

virus have to fulfill the predetermined definition of ND that distinguishes it from strains of vaccine viruses, or avirulent enzootic ND viruses. Latter an *in vivo* estimation of pathogenicity for chickens such as the mean death time determination in embryonating eggs, the intracerebral pathogenicity index assay in day old chicks, or the intravenous pathogenicity index assay in 6-week-old chickens (Alexander, 1988b; OIE, 2021a).

2.7.2. Samples

Samples from live birds shall be taken in the early stages of the disease and include cloacal, tracheal or oropharyngeal swabs. Sample from recently dead birds or moribund birds include oro-nasal swabs, lung, and kidney, intestine including contents and caecal, tonsils, spleen, brain, liver and heart tissues collected separately or in pool (OIE, 2021a).

2.7.3. Isolation of Newcastle disease virus

Tissue homogenate or faeces and cloacal swab suspensions are clarified by centrifugation and 0.2 ml of the supernatants are inoculated through the allantoic cavity route. Each sample were propagated into at least five embryonated SPF eggs of 9 –11 days old. If SPF eggs are not available, NDV antibody negative eggs are required. After inoculation, the eggs are incubated at 35–37°C for 4–7 days. Newcastle disease is diagnosed by isolation of NDV in SPF embryonating chicken eggs. All NDV isolates are known to replicate in Embryonated chicken egg and the mean death time (MDT) to kill the embryo varies depending on the virulence of the virus (Alexander and Senne, 2008; OIE, 2021a).

2.7.4. Identification of Newcastle disease virus

Internationally agreed that, identification of an isolate of NDV from birds showing clinical signs is not a confirmatory diagnosis of ND. Due to an extreme variation in virulence of different NDV isolates and the widespread use of live vaccines other confirmatory tests are required (OIE, 2021a). Differentiation of virulent from avirulent strains of class II NDV was successfully and rapidly achieved by using Next Generation Sequencing based on pyrosequencing procedure (De Battisti *et al*., 2013). Cross-reactivity can be detected between APMV-1 and APMV-3 viruses or APMV-7. A panel of reference sera or monoclonal antibodies (MAbs) specific for APMV-1, APMV-3 and APMV-7 can be used

and the risk of mistyping an isolate significantly diminished (Box *et al*., 1988; Alexander, 1990).

2.7.5. Haemagglutination test

NDV has the capacity to agglutinate chicken red blood cells. As it is mentioned above in pathogenesis, HN protein of NDV is a multifunctional protein that possesses both the receptor recognition and NA activities associated with the virus. Thus, the HN protein is responsible for hemagglutinating (Choppin and Compans, 1975) and neuraminidase activities for adsorption of the virus to receptors on host cells (Scheid *et al*., 1972). The HA test is not specific for NDV, because APMV-1, -2, -3, -4, -6, -7, -8, and -9 are common in layers, broiler-breeders and broilers (Warke *et al*., 2008). In addition to APMV-1; serotypes APMV-2, -3, -6, and -7 cause disease in poultry and other viruses will agglutinate red blood cells (Alexander, 2003).

Hence, the HA test is used to detect the presence of titrate agents of NDV, avian influenza virus, infectious bronchitis virus, hemagglutinating adenovirus (Hess, 2000), and psittacine circovirus (PsCV), which agglutinate some avian erythrocytes. Therefore a sample of allantoic fluid testing positive for hemagglutinin will need further testing to confirm the presence of NDV. The existence of NDV in a sample is confirmed by haemagglutination inhibition test (Alexander, 1988b).

2.7.6. Haemagglutination inhibition test

The HI test is used to identify a virus as NDV (Alexander, 1997). Monoclonal antibody (mAb) testing can further be used to characterize NDV. While no single mAb can determine the virulence of NDV, a panel of mAb can be used to characterize differences. The use of mAb assays for rapid characterization of these viruses is not optimal for class I viruses as most of the mAb were developed and optimized to recognize class II viruses and fail to recognize viruses of class I (Alexander *et al*., 1987).

2.7.7. Enzyme linked immunosorbent assay

Several strategies are applied to detect ND antibodies. Enzyme linked immunosorbent assay (ELISA) works on the principle of recognition of anti-NDV antibodies that are produced in another species against chicken antibodies. The antibodies were attached to a plate coated with viral antigen (OIE, 2021a). Using MAbs for detection of ND antibodies, sandwich, indirect and blocking or competitive ELISAs are performed. There are different commercially available ELISA kits and one kit uses a subunit antigen. Both ELISA and HI may quantify antibodies to different antigens. ELISA test; depending on system used can detect antibodies to more than one antigen but, HI test is probably restricted to those directed against the HN protein. ELISAs are reproducible and have high specificity and sensitivity (Brown *et al*., 1990); they have been found to correlate well with the HI test (OIE, 2021a).

2.8. Pathogenicity index

An *in vivo* test used to determine the virulence based on the severity of the clinical signs or death that was produced on chicken's ≥ 10 within the test period. It classify the isolates as velogenic, mesogenec or lentogenic viruses based on the clinical sign results of ICPI scores (OIE, 2021a).

2.8.1. Intracerebral pathogenicity index test

Virulence of the isolate of NDV can be assessed by ICPI test. The ICPI test should be done as: fresh infective allantoic fluid with an HA titre of greater than 2^4 (1/16) is diluted 1/10 in sterile isotonic saline, with no additives and 0.05 ml of the diluted allantoic fluid is injected intracerebrally into each of ten one-day-old chicks hatched from an SPF eggs. The birds are observed every day for eight consecutive days, at each examination the birds are scored from 0 up to 2 (0 if normal, 1 if sick, and 2 if dead). The mean score per bird per observation over the eight-day period is considered as the ICPI. Lentogenic strains will give values close to 0.0 whereas, the most virulent viruses will give indices which is close to the maximum score of 2.0 (Alexander, 1988b).

2.9. Molecular technique

2.9.1. Polymerase chain reaction

The molecular basis for NDV pathogenicity is dependent on the F protein cleavage site (Glickman *et al*., 1988) and amino acid sequence (Le *et al*., 1988) and the ability of specific cellular proteases to cleave the F protein of different pathotypes (Gotoh *et al*., 1992). Fewer basic amino acids are present in the F protein cleavage site of lentogenic NDV isolates than either mesogenic or velogenic strains, which have similar cleavage site sequences (Glickman *et al*., 1988).

Reverse transcription by real-time polymerase chain reaction (RT-PCR) was used to amplify F protein gene sequences of various NDV strains (Jestin and Jestin, 1991). However, when the amplification product was analyzed by agarose gel electrophoresis following restriction enzyme digestion, results were inconsistent. Amplification of a portion of the F protein gene and the cleavage activation site was deduced from nucleotide sequences of the amplification product to classify NDV pathotypes (Collins *et al*., 1993).

A real-time reverse-transcription PCR (RT-PCR) detects avian paramyxovirus- 1 (APMV-1) RNA in clinical samples from birds. The assay uses a single-tube protocol with fluorogenic hydrolysis probes. Oligonucleotide primers and probes designed to detect sequences from a conserved region of the matrix protein (M) gene that recognized a diverse set of APMV-1 isolates, a second primer-probe set to sequences in the fusion protein (F) gene that code for the cleavage site and detect potentially virulent NDV isolates. A third set, against the M gene, was specific for the North American pre-1960 genotype that includes the common vaccine strains used in commercial poultry in the United States (Wise *et al*., 2004).

2.10. Prevention and control of Newcastle disease virus

The prevention and control of ND requires management procedures including strict biosecurity measures which help in preventing the spread of infective material from house to house and from farm to farm (Alexander, 1995b). Good biosecurity can protect poultry flocks from Newcastle disease. Avoiding contact with unknown health status domesticated

poultry, any pet birds, and wild birds particularly gulls, pigeons, and cormorants. Biosecurity measures include well ventilated houses, clean water supplies, and movements on and off the farm should be kept to a minimum, and disinfecting vehicles and equipment that enter the farm. Separation of infected from health flocks and proper disposal of died birds.

On the farms; houses, food stores and water tanks should be bird-proofed, all equipment, especially vehicles, should be disinfected before access to the site, movements between different farms for egg collection, carcass collection, food delivery and the like should be to and from a specified collection and delivery point away from the poultry flocks. Visits by personnel are the most likely method of introduction of ND and, if such visits are unavoidable, regimens of clothing change, equipment disinfection and other basic hygiene controls must be enforced. Control of Pests such as insects and mice is also important for control measures of ND. Similar age group production per farm and all in or all out breeding methods with disinfection between groups is also indispensable to control ND (Alexander, 1995b).

2.11. Vaccines against Newcastle disease

The emergence of virulent NDV isolates is certainly a serious concern in the global poultry industry. Isolation of NDV from different species of wild birds, which excrete the vaccine derived NDV are among the major reservoirs for the emergence of the virulent NDV in poultry (Ayala *et al*., 2016). Through ecological contact interfaces, the lentogenic viruses have been shown to be easily be transmitted from wild birds to domesticated poultry, where they are silently maintained without causing any clinical disease (Brown and Bevins, 2017).

A number of conventional (Alexander, 2003) and genetically engineered (McMillen *et al*., 1994) vaccines had been developed against ND virus. A vaccine used must be potent and efficacious at the time of use. The European community allowed when the seed vaccine to be tested shows an ICPI <0.4 (10^7EID50/ bird), or <0.5 (10^8EID50/bird). Banned ND vaccine seed of ICPI of greater than 0.4 and inactivated ND with an ICPI exceeding 0.7 (CEC, 1993). The OIE Standard Committee recommends that a vaccine must have an ICPI

< 0.7 in order to meet the estimated inter laboratory variability. A safety margin is allowed, and the seed strains used for vaccination must have an ICPI ≤ 0.4 (Alexander, 1995a). These guidelines were adopted by OIE (2021a).

The most popular live vaccines have evolved from field isolates of low virulence the 'lentogenic' viruses Hitchner Bl or La Sota produced in the United States in the late 1940's (Hanson and Brandly, 1955) or similar lentogenic viruses like Ulster 2C, F and V4 are used. In addition some 'asymptomatic enteric' viruses have also been used as live vaccines. A difficulty associated with live vaccines is that the immune response appears to be related to the virulence of the virus strain (Reeve *et al*., 1974). Roakin, Komarov, and Mukteswar strains of more virulent mesogenic virus were also used as a vaccine seed in regions where virulent ND is endemic (Hanson and Brandly, 1955).

Different suitable methods of application mimicking the natural infection such as the use of eye drops and beak dipping are costly; mass application of live vaccine in generated aerosols or sprays or in drinking water which is cheaper and more convenient are applied (Alexander, 1988a). However, sprays and aerosols in particular may result in severe respiratory reaction and, especially with La Sota virus, it will resulted high mortality (OIE, 2021a).

2.11.1. Virulent Newcastle disease virus and Newcastle disease vaccines

The most likely reservoir of vNDV is the vaccinated poultry population. There is evidence that wild birds may represent natural reservoirs of mesogenic viruses (Czegledi *et al*., 2006). Phylogenetically related vNDV of genotype V have been isolated from double-crested cormorants (Phalacrocorax auritus) from 1975 through 2008 and they have been implicated in earlier ND outbreaks (Blaxland, 1951; Heckert *et al*., 1996; Allison *et al*., 2005; Aldous and Alexander, 2008).

Virulent pigeon paramyxovirus-1 (PPMV-1) isolates, which are clinically neurotropic in chickens, were first isolated in 1981 in pigeons (Columba livia), and continue to circulate in feral birds of the Columbidae family worldwide (Kaleta *et al*., 1985). Isolation of viscerotropic vNDV was documented in pigeons (Pearson and McCann, 1975), typical

pigeon isolates are variants from genotype VIb, that cause neurological signs, have specific monoclonal antibody binding patterns, and often do not hemagglutinate chicken red blood cells well (Ujvari *et al*., 2003). Other virulent viruses have been isolated sporadically from imported tropical bird species but the existence of a reservoir in these other species remains to be confirmed. It has been shown that some wild birds were not natural reservoirs of vNDV, but infected at quarantine stations prior to export (Kaleta and Baldauf, 1988).

2.11.2. Immunogenicity of Newcastle disease vaccines

Maternal antibody to NDV had a significant effect on the immune response to the vaccine strains regardless of whether the strains were administered by eye drop or aerosol methods. All live attenuated ND vaccines are known to stimulate both mucosal and systemic immune responses similar to those of the natural infection, because of their ability to replicate in chicken irrespective of the site of administration (Rauw *et al*., 2009). Furthermore, a single administration of 10^5 EID_{50} of live NDV vaccine is enough to rapidly stimulate an immune response capable of inducing a 100% protection against clinical disease, even though the shedding of the challenged virulent virus via the cloacal and oropharyngeal routes may still occur. There are indications that the replication and shedding of the virulent virus can be substantially reduced when much higher doses of the live vaccines are administered (Dimitrov *et al*., 2016; Dimitrov *et al*., 2017).

As this is not an economically viable option because of the high cost of vaccination per bird, improved and cost effective vaccination approaches are required to tackle the problem of virus shedding associated with the conventional ND vaccines. An important factor that determines the effectiveness of vaccination is the tissue tropism of the vaccine. Conventional live attenuated NDV vaccines including the famous LaSota strains are predominantly respirotropic, inducing stronger mucosal immunity in the respiratory airways where initial exposure to the virus might occur. Other vaccines such as the VG/GA strains are more of enterotropic (Perozo *et al*., 2008), stimulating gut mucosal immunity among the vaccinated birds. A natural recombinant virulent NDV strain IBS025/13 which demonstrates a strong tissue tropism in both the respiratory and gastrointestinal systems is isolated (Satharasinghe *et al*., 2016). The mesogenic NDV live vaccines the Komarov and

Mukteswar strains, both of which are suitable as booster vaccines following priming with lentogenic isolates (Senne *et al*., 2004).

The evolution of virulent NDV could be resulted due to large phylogenetic and antigenic distances between the current circulating virulent strains and vaccines strain (Miller *et al*., 2007). Previous studies showed that, the current vaccines in use prevent disease but unable to stop virus shedding (Utterback and Schwartz, 1973; Kapczynski and King, 2005; Miller *et al*., 2009).

2.12. Status of Newcastle disease in Ethiopia

Newcastle disease is the major poultry viral disease in Ethiopia with prevalence throughout the year that varies from 6 percent (Chaka *et al*., 2012) to 38 percent (Tadesse *et al*., 2005) in intensive and scavenging poultry respectively. As reported by Fentie, (2013) the circulation of vNDV genotypes II, VI and VII and others indicated that sub genotype VIf of class II viruses, were circulating in village chickens regardless of the geographical origin of the isolates (Chaka *et al*., 2013; De Almeida *et al*., 2013; Mulisa *et al*., 2014; Damena *et al*., 2016).

Recently, very old NDV isolates, found in Ethiopia from 1976 to 2008 were characterized by partial F gene sequencing and the isolates were classified as genotype VI (Bari *et al*., 2021).

3. MATERIAL AND METHOD

3.1. Study area and study animal

The study was conducted in Ada'a District; Bishoftu town and Dhankaka village and the laboratory activities were performed in National Veterinary Institute, Research and diagnostic laboratory. Bishoftu town is located in Oromia Regional state, East Shewa zone, at 47 km east of Addis Ababa/Finfinne, the capital of Ethiopia. It is locked by lakes, lies at an altitude ranging 1900 to 1995 meters above sea level and $8^0$45'N and $38^0$59'E. It has a short rainy season from March to April and a long rainy season from June to September. The average annual rainfall is 1,150 mm, while the maximum and minimum temperature is 28.6°C and 12.9°C, respectively (NMA, 2019).

Chickens of all age group, without sex and breed limit suspected of Newcastle disease were used as a study animal.

3.2. Study design and sample collection

A cross-sectional study was conducted in Ada'a District of East Shewa zone of Oromia Regional state in the study period (December 2019 to May 2021). Chicken handling and experiments were approved by Ethical clearance certificate Ref. No. VM/ERC/20/02/12/2020 held according to the animal handling and use guidelines set by Animal Research Ethics Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture. From a total of 36 chickens, 52 samples were collected from live, dead or moribund chickens that show clinical signs of ND. Samples of cloacal swab, feces from live chickens, brain, spleen and trachea from euthanized chickens were collected by sterile swabs and post mortem samples were collected using sterile surgical blade, universal bottles and gloves according to the laboratory procedures described by OIE (2021a). Samples were transported in medium of sterile phosphate-buffered saline (PBS) pH 7.2 with antimicrobial additives (penicillin 10,000 units, streptomycin sulfate 10 mg and amphotericin B 25 µg per ml) to the National Veterinary Institute Research and diagnostic laboratory and stored at -80^0C until processing.

Table 1. Sample type and size from different sampling areas

N^{o.}	Sample type per site/area	Number of euthanized chickens	N^o of Samples collected
1	Bishoftu town	21	**29**
	• Cloacal swab	-	2
	• Brain	-	18
	• Spleen	-	3
	• Trachea	-	5
	• Liver	-	1
2	Dhankaka village	15	**23**
	• Cloacal swab	-	2
	• Brain	-	13
	• Spleen	-	3
	• Trachea	-	4
	• Liver	-	1
	Total	**36**	**52**

Specific-pathogen-free (SPF) eggs were purchased from VALO Biomedia (GmbH, Germany) and Newcastle disease vaccines; Lasota vaccine Batch No NCL 03/2020, Hitchner B1 (HB1) vaccine Batch No NCHB1 02/2020 and Thermostable (I-2) vaccine Batch No NCTH 02/2020 were obtained from the National Veterinary Institute of Ethiopia.

3.3. Sample processing

Tissue and swab homogenate was prepared in level II biological safety cabinet. A tissue sample approximately 1 gm was cut by sterile scissors. The cutted samples were kept in sterile petridishes and washed three times by sterile PBS, pH 7.2 supplemented with antimicrobial additives (penicillin 10,000 units, streptomycin 10 mg, and amphotericin B 25 µg per ml). The washed sample were transferred to sterile mortar and pestle, chopped using sterile scissors and forceps then grinded in to small pieces by sterile sand. A 10% tissue homogenate and 10% swab suspension were prepared in sterile PBS, pH 7.2 supplemented with antimicrobial additives, then the homogenate centrifuged at 3000 rpm/min for 10 minutes at 1000g. The supernatant from tissue homogenate or swab

suspension was filtered through 0.22 µm filter pore size. The remaining tissue samples were kept at -80^{0}C (OIE, 2021a).

3.4. Virus isolation

Embryonated SPF eggs were disinfected by 70% ethyl alcohol, in level II biological safety cabinat and the eggs were pierced by sterile egg driller. The filtered supernatant of tissue homogenate, faeces, or swab a volume of 0.2 ml were inoculated, through the allantoic cavity route into five 9-11 days old embryonated SPF chicken eggs. Holes of the inoculated eggs were sealed by melted paraffin wax. The inoculated eggs were incubated at 37^{0}C in humidified egg incubator for 5 days and candled every 24 hours to observe the effect of the inoculated virus. Those embryos dead within 24 hours of incubation were discarded and the remaining followed for virus replication. Embryos deaths after 24 hours of incubation were considered as due to virus replication and can be chilled at +4^{0}C overnight for allantoic fluid collection (OIE, 2021a).

3.5. Allantoic fluid harvesting

To harvest the allantoic fluid, the eggs were disinfected by 70% ethyl alcohol, and kept in level II biological safety cabinet. The eggs were decapped by sterile thumb forceps and allantoic fluid was harvested by using sterile pipette, spatulla and the fluid was collected in sterile reagent and universal bottles. The collected allantoic fluid was checked for sterility by inoculating in to tryptose soya broth and thioglycollate medium. The sterile allantoic fluid were tested for haemagglutination of chicken red blood cells (OIE, 2021a).

3.6. Haemagglutination test

A 10% Chicken RBC was prepared from chickens blood. The blood was collected in 1:1 ratio with alsever's solution, centrifuged at 2500 rpm/min for 10 min to separate alsever's solution and other debries then washed with PBS (pH 7.2) and centrifuged at 2500 rpm/min for three times. Using 1% RBC the haemagglutination (HA) test were done for agglutination of chicken red blood cells according to OIE protocol (OIE, 2021a). These allows to prove the presence of any hemagglutinating agents in the harvested allantoic fluid. Those tests positive for HA were confirmed by Haemagglutination inhibition test.

3.7. Haemagglutination inhibition test

The harvested allantoic fluids that were tested positive by HA were subjected to Haemagglutination inhibition (HI) test. The test was based on the inhibition of haemagglutination by monoclonal specific anti sera against NDV. If the collected allantoic fluid have the suspected ND virus, nucleic acids of the viruses encode hemagglutinin proteins expressed on the surface of the virus bind to or clump erythrocytes creating a lattice, which settle irregularly in the bottom of the microtiter well. Specific antibodies inhibit such agglutination. In the absence of ND virus, the erythrocytes do not clump; instead they form a compact button (Florman, 1947, OIE, 2021a).

Serum samples were inactivated at 58^0C in water bath and two fold serial dilution of HB1 vaccine virus titer were prepared up to dilution 12 in micro titration plate and 2^{10} HA titer of the virus were obtained. According to NVI test protocol 8 Haemagglutination unit (HAU) of the antigen were prepared for the HI test. A 25 µl PBS were added per each well of the micro titration plate, then 25 µl of the serum samples were added in the first well of the V-bottom micro titration plate from A to H and two fold serial dilution were done up to well No 12 horizontally. A 25 µl of 8HAU antigen/HB1 was added per each well and incubated at room temperature for 15 minutes. Finally, 50 µl of 1% chicken RBC were added per each well and incubated at room temperature for 30 minutes. The agglutination were judged by tilting the plates. The wells RBCs stream at the same rate as the control wells (positive serum, virus and PBS controls) were considered to show inhibition. A cut-off value $\geq \text{Log}_2{}^3$ were used as a standard positive (OIE, 2021a).

3.8. Embryo mean death time determination

To determine the MDT for embryo death, allantoic fluids were selected. Then 9 – 11 days old SPF embryonating chicken eggs were disinfected by 70% ethyl alcohol and tenfold serial dilutions of the allantoic fluid of the virus isolates were prepared in level II biological safety cabinet. The eggs were pierced by sterilized egg driller and 0.1 ml of the dilutions between 10^{-5} and 10^{-9} was inoculated in to 5 eggs per dilution in to the allantoic cavity as described by Hanson and Brandly, (1955) and the holes are sealed by melted paraffin wax then incubated at 37^0C in humidified egg incubator. The highest dilution at

which all embryos die was considered as mean lethal dose (MLD) and the MDT/MLD was calculated. The virulence of the isolated viruses was determined *in vitro* into low virulent or lentogenic, moderately virulent or mesogenic, and highly virulent or velogenic pathotypes, based on chicken embryo mortality at >90 hours, 60-90 hours, or, <60 hours respectively (Hanson and Brandly, 1955).

3.9. Molecular identification

3.9.1. RNA extraction

RNA extraction was performed from allantoic fluid harvests confirmed positive by HA and HI test and extracted according the procedure described by the manufacturer (QIAGEN, Germany) RNeasy Mini Kit. Allantoic fluids were thawed and vortex to mix. A volume 350 µl of the homogenized allantic fluid harvests were transferred to eppendrof tube and 350 µl of RLT buffer was added and then homogenized by vortex then centrifuged at 13,000 rpm/min for 3 minute. After centrifugation 350 µl of 70% ethanol was added to the homogenized lysate and mixed by pipetting. A volume of 700 µl of the sample was transferred to RNeasy spin column, centrifuge for 30 sec at 13,000 rpm/min, and the flow-through liquid were discarded.

Buffer RW1 a volume of 700 µl was added to RNeasy spin column then centrifuged for 30 sec. at 13,000 rpm/min and discarded the flow-through liquid. Then, 500 µl RPE buffer was added to RNeasy spin column, centrifuged for 30 sec at 13,000 rpm/min and discarded the flow-through liquid. Added again 500 µl RPE buffer to RNeasy spin column, centrifuged for 30 sec at 13,000 rpm/min and discarded the flow-through liquid and place RNeasy spin column in a new clean tube. Secondly added 500 µl RPE buffer to RNeasy spin column and centrifuged for 2 min at 13,000 rpm/min and discarded the flow-through liquid. Then place RNeasy spin column in a new 2ml clean tube, centrifuged for 1 min at 13,000 rpm/min and discarded the flow-through liquid. Finally, place the spin column in a new 1.5ml tube and 30-50 µl of RNase free water was added on to the membrane of the spin column, centrifuged for 1 min at 13,000 rpm/min and collected RNA at the bottom of the tube.

3.9.2. Master mix preparation

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

3.9.3. Polymerase chain reaction

Reverse transcriptase polymerase chain reaction

In amplification room, the template RNA a volume of 5 µl were added to the Master Mix in biological safety cabinate. Amplification of the M-gene were performed in a 29 µl reaction volume. One step reverse transcriptase polymerase chain reaction (RT-PCR) were conducted by synthesis of cDNA. A reaction temperature of 50^0C for 30 minutes for one cycle, initial denaturation at 95^0C for 15 minutes for one cycle then 1^{st} denaturation at 94^0C for 30 sec, annealing at 55^0C for 30 sec, and elongation at 72^0C for 30 sec was used. A total of 40 cycles, final elongation at 72^0C for 5 minutes for one cycle was applied and stored the PCR product at 4^0C performed for the amplification of the M-gene of APMV-1 in Applied Biosystems 2720 Thermal Cycler.

3.9.4. Gel electrophoresis

A 2 % agarose gel were prepared, and 4 µl gel red with loading dye was added. Then 10 µl of each PCR product and 10 µl of molecular marker (ladder) started at 100 bp plus loaded in the gel. Electrophoresis was conducted in 1X Tris-Borate-EDTA (TBE) buffer in agarose gel electrophoresis System (BIO - RAD - USA, Thermo EC - 2060) for one hour and 20 minutes at 120V electric current supply. A DNA ladder started with 100-bp increments was used as a molecular weight marker. Thereafter, gels were visualized under

UV light using UV transilluminator (Uvitec UK, Cambrige CB4 - 1QB - UK) and photographed by a digital camera (Uvitec UK).

3.10. Intracerebral pathogenicity index assay

The intracerebral pathogenicity index (ICPI) assay were done for a total of five selected allantoic fluid harvests propagated in ten days old SPF eggs for five passages. The allantoic fluid harvests were selected based on HA titer > 2^4 and RT-PCR test positive results to determine their virulence *in vivo* for challenging of the experimental chickens. The chickens hatched were grouped randomly in to six experimental groups each having ten chickens. Experiment group 1 was left un-inoculated, while experiment group 2 - 6 was inoculated with the isolated viruses.

The test was done using the selected allantoic fluid harvestes by inoculating in the brain of one day old chicks hatched from SPF eggs in Research and Diagnostic Laboratory of NVI. The test was done according to the protocol of OIE, (2021a). Fresh infective allantoic fluid with an HA titer > 2^4 were diluted 1/10 in sterile isotonic saline without antibiotics in level II biological safety cabinat. Then the diluted allantoic fluid was kept on ice and taken to the animal experimentation room to inoculate the chickens. The site of inoculation was disinfected by 70% ethyl alcohol, then 0.05 ml was drawn by a sterile graduated insulin syringe guage 30G x 8mm and inoculated into the brain of each one day-old ten SPF chickens (age > 24 and < 40 hours). The chickens were given feed and public tap water *ad libitum*. Each chicken were examined at 24-hour intervals for eight days. The result was scored as zero if normal, one if sick and two if dead. The index was determined by the mean score per bird per observation over the 8-day period. The most virulent viruses give ICPI values approaching the maximum score of 2.0, while lentogenic viruses were give a values close to, 0.0.

3.11. Vaccine Efficacy study

Three types of Newcastle disease vaccines produced from the lentogenic strains; Lasota, HB1 and Thermostable (I-2) were evaluated for their protective efficacy in the National Veterinary Institute, Research and Diagnostic Laboratory at Animal Experiment facility from October 2020 to May 2021.

3.12. Blood Collection

Blood was collected from each experimental chicken using sterile disposable syringe guage 22Gx1¼" (3ml). Each chicken were handled, the site of blood collection were disinfected by 70% ethyl alcohol and blood were collected from the wing vein. A volume of 1-2 ml blood was collected from each chicken, the syringes were layed in horizontal position 3-4 hour at room temperature. Finally the serum was decanted in to sterile cryovial and stored at -20^0C until HI test conducted to evaluate the antibody titer. The chickens were supplied multivitamins after each blood collection.

3.13. Experimental animals and management

Experimental chicken rooms were cleaned and disinfected by 10% sodium hypochlorite then kept free for two weeks before experimental chickens were housed. A total of 85 chickens hatched from SPF eggs in NVI were assigned randomly in to five experimental groups each having seventeen chickens (OIE, 2021a).

Experiment group 1, 2, 3 and 4 were vaccinated (Table 2), according to vaccination schedules recommended by NVI (NVI, 2019) and group 5 were unvaccinated and treated as a control group. The four groups, each with 17 chickens vaccinated through the intraocular route: experiment group 1 vaccinated at day zero (age of 7 day) by Newcastle HB1, booster at days 14 and 35 by Newcastle Lasota vaccine; experiment group 2 vaccinated at day zero by Newcastle HB1 and booster at days 14 and 35 by Newcastle Thermostable (I-2) vaccine; experiment group 3 vaccinated at day zero by Newcastle Thermostable (I-2) vaccine and booster at days 14 and 35 by Newcastle Lasota vaccine;

experiment group 4 vaccinated at day zero by Newcastle HB1 vaccine and booster at day 14 by Newcastle Thermostable (I-2) and at day 35 booster by Newcastle Lasota vaccine.

Table 2. **Immunization schedule of experimental chickens**

Experiment groups	**Vaccines used and age of vaccination**			**Route of vaccination**	**Dose**
	1st Vaccination	Booster			
	Age 7 day (Day 0)	Age 21 day (Day 14)	Age 42 day (Day 35)		
Group 1	HB1	Lasota	Lasota	Intraocular	$10^{6.5}$ EID_{50}
Group 2	HB1	I-2	I-2	Intraocular	$10^{6.5}$ EID_{50}
Group 3	I-2	Lasota	Lasota	Intraocular	$10^{6.5}$ EID_{50}
Group 4	HB1	I-2	Lasota	Intraocular	$10^{6.5}$ EID_{50}
Group 5 (Control)	Not vaccinated	Not vaccinated	Not vaccinated	----	-----

HB1 Hitchner B1,
I-2 Thermostable,
EID_{50} Embryo infective dose 50

3.14. Titration of the allantoic fluid harvests

Tenfold serial dilutions for selected allantoic fluid harvests were prepared in sterile PBS, pH 7.2 in class II biological safety cabinat. Steriized 1 ml pipette, rubber suction bulb, vortex, dilution tubes were used. The embryonated chicken eggs were disinfected by 70% ethyl alcohol, drilled on the inoculation site and starting from dilution -5 to -9; 0.1 ml volume of each dilution was inoculated in to five 9 - 11 day old embryonated SPF eggs. The inoculated eggs incubated at 37^0C in humidified egg incubator for 5 – 7 days. Those embryos died after 24 hours of incubation were discarded and embryos that die after 24 hours of incubation chilled at $+4^0$C. Maximum incubation with a minimum embryo death were used.

The chilled embryos at $+4^0$C were tested for hemagglutination of chicken RBC. Finally the concentration of the virus in the allantoic fluids to produce fifty percent death in

embryonated eggs (ELD_{50}) were calculated according to Reed and Muench, (1938) and OIE (2021a).

3.15. Experimental chickens challenge

On day 49 (age of 56^{th} days), 14^{th} day after second booster the vaccinated and unvaccinated chickens from groups 1, 2, 3, 4 and 5 were challenged by inoculating the locally isolated ND virus. Each chicken were challenged through the oculo-nasal route by inoculating 0.2 ml of $10^{6.5}$ ELD_{50} of the challenge ND virus. Then, according to the definition adopted by OIE, (2021b) for incubation period of ND the chickens were followed for 21 days post challenge (dpc) by supplying feed and clean public tap water *ad libitum*. Recording of the clinical signs, mortalities was performed and samples for postmortem examination were collected from chickens showing clinical signs and from the control groups. Laboratory confirmation done for the collected samples to examine any of the clinical signs and mortalities observed were due to the challenge virus or not. Chickens protection from the virulent challenge virus were determined by absence of clinical signs or absence of death with in 21 dpc.

3.16. Data management and analysis

Data obtained from all experiment were recorded in excel spread sheet. Statistical data analysis were conducted by using STATA software/IC version12.0. The mean HI titer and standard deviation of the groups was compared by Generalized Linear Model with 95% confidence interval and 5% alpha value. *P-value* < 0.05 was considered to analyze the results obtained if there was a significant difference between test groups. ANOVA were used to compare means between and within experimental groups' differences of HI titer and ND vaccines used.

4. RESULTS

4.1. Virus isolation

Newcastle disease virus were isolated from 14 allantoic fluid harvests (26.92%) and 38 (73.08%) samples have shown no viral growth in embryonated SPF chicken eggs and were negative. The allantoic fluids at passage three confirmed positive by HA test were further confirmed by HI test using specific anti-sera for Newcastle disease virus and they were positive. Those allantoic fluid harvests HA and HI test positive were propagated for further two passages in embryonated SPF chicken eggs.

4.2. Embryo mean death time determination

Allantoic fluid harvests at passage five were used for embryo mean death time determination. According to Hanson and Brandly, (1955) embryo mean death time < 60 hours had grouped the isolated viruses into highly virulent pathotypes. All five embryos for selected allantoic fluids died in less than 60 hours, at 47 hour and at 46 hour and they were assigned as highly virulent. Embryos were reacted for dilutions at 10^{-7} for samples designated as 'Bishoftu' and 'Dhankaka' as it is shown in table 3.

Table 3. Embryo mean death time for samples from 'Bishoftu' and Dhankaka

	Number of Embryo death and time									
	24 hour		**48 hour**		**72 hour**		**96 hour**		**120 hour**	
Dilutions	**Bishoftu**	**Dhankaka**	**Bishoftu**	**Dhankaka**	**Bishoftu**	**Dhankaka**	**Bishoftu**	**Dhankaka**	**Bishoftu**	**Dhankaka**
10^{-5}	0	0	5	4	0	1	0	0	0	0
10^{-6}	0	0	4	2	1	3	0	0	0	0
10^{-7}	0	0	5	5	0	0	0	0	0	0
10^{-8}	0	0	2	3	1	2	1	0	1	0
10^{-9}	0	0	0	0	0	1	0	0	0	0

4.3. Molecular identification

4.3.1. Polymerase Chain Reaction

Reverse transcriptase polymerase chain reaction

One step RT-PCR were conducted by synthesis of cDNA. The result confirms 14 allantoic fluid harvests were positive around 121 bp for APMV-1 primer designed to amplify the matrix of ND virus (Figure 3).

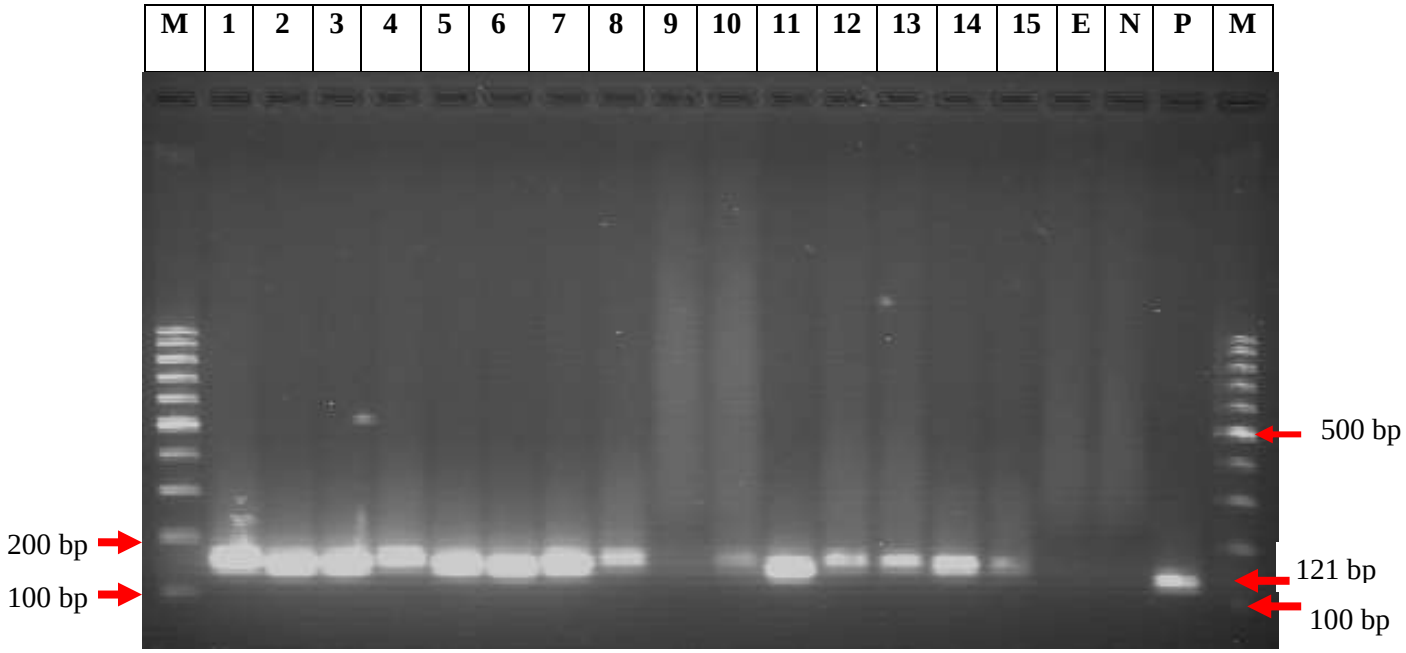

Figure 3. Gel picture at passage 3 of allantoic fluids harvests using primer APMV 1

Key:

Lane		**Result**
M	Molecular marker 100 plus	
1	Brain sample	Positive
2	Spleen	Positive
3	Cloacal swab	Positive
4	Cloacal swab	Positive
5	Brain	Positive
6	Trachea	Positive

7	Cloacal swab	Positive
8	Trachea	Positive
9	Spleen	Negative
10	Liver	Positive
11	Trachea	Positive
12	Cloacal swab	Positive
13	Trachea	Positive
14	Brain	Positive
15	Liver	Weak positive
E	Extraction control	
N	Negative control	
P	Positive control	

4.4. Pathogenicity index assay

The ICPI test were conducted in chickens at age of 30 hours. Allantoic fluid harvests at fifth passage propagated in embryonated SPF chicken eggs were selected by HA titer > 2^4 and amplified positive by RT-PCR were used. The five selected allantoic fluid harvests (Table 4) score ICPI of 0.4 and 0.05, confirmed as lentogenic, ICPI score 1.25 confirmed as mesogenic, ICPI score of 1.61, and 1.85 confirmed as velogenic. ND viruses grouped as lentogenic having an ICPI up to 0.7, mesogenic with an ICPI 0.7 to 1.4 and velogenic with an ICPI higher than 1.4 revealed that the isolated viruses belong from avirulent to very virulent (Alexander, 2000; Afonso, 2021; OIE, 2021).

Table 4. ICPI score for selected allantoic fluids at passage five

Sample code	Sample source	ICPI score
Bishoftu-1	Brain	1.61
Bishoftu-2	Spleen	0.4
Bishoftu-5	Brain	0.05
Dhankaka-7	Cloacal swab	1.85
Dhankaka-14	Brain	1.25

4.5. Vaccine efficacy test

The maternally derived antibody of the SPF chickens were measured at age of 7, 21, 42 and 56 days. The HI geometric mean titer (GMT) ± standard deviation (SD) were 16 ± 0.00 at age of 7 days, 16 ± 4.89 at age of 21 days, 4 ± 0.00 at age of 42 days and 4 ± 0.00 at age of 56 days.

4.6. The immune response of lentogenic Newcastle disease vaccines

The experimental chicken groups 1, 2, 3 and 4 were vaccinated and boosted as HB1-Lasota-Lasota, HB1-I2-I2, I2-Lasota-Lasota and HB1-I2-Lasota vaccines respectively at current recommended dose of $10^{6.5}EID_{50}$.The immune response for groups 1, 2, 3 and 4 experimental chickens at 14^{th} day of the primary vaccination resulted mean HI titer ± SD > $Log_2 3$. After 21 days of the 1^{st} booster vaccination (day 35) the immune response of the experimental chicken groups were also increased from the level that was measured at day 14. The immune response of the chickens at 7^{th} day of the second booster vaccination (day 42) were also increased from the level measured after the first booster vaccination. At 14^{th} day of the 2^{nd} booster vaccination (day 49) the mean HI titer ± SD of the experimental chicken groups was below the level measured at 7^{th} day after the second booster vaccination and above the level measured after 21 days of the first booster vaccination. The overall mean HI titer ± SD of experimental chicken groups 1, 2, 3 and 4 indicated that the lentogenic vaccines used for immunizing the experimental SPF chickens induce a protective immune response (Table 5).

Table 5. Mean HI titer and SD of Newcastle disease vaccines

Groups Mean HI titer on days			
Experiment group 1 (*HB1- Lasota- Lasota)*	**Mean**	**SD**	***P-value***
HI Titer at day 0	16.47	4.44	
HI Titer at day 14	84.70	39.09	
HI Titer at day 35	138.35	102.28	
HI Titer at day 42	244.70	143.52	
HI Titer at day 49	192	137.63	
HI titer of the group	112.70	126.87	0.000
Experiment group 2 (*HB1 - I-2 - I-2)*			
HI Titer at day 0	15.05	2.65	
HI Titer at day 14	90.35	37.92	
HI Titer at day 35	114.82	66.02	
HI Titer at day 42	214.58	129.86	
HI Titer at day 49	141.17	72.49	
HI titer of the group	96.00	99.26	0.011
Experiment Group 3 (*I-2 – Lasota - Lasota)*			
HI Titer at day 0	16	0	
HI Titer at day 14	73.41	33.49	
HI Titer at day 35	89.41	55.73	
HI Titer at day 42	271.05	173.11	
HI Titer at day 49	143.05	80.09	
HI titer of the group	98.82	121.15	0.009
Experiment Group 4 (*HB1- I-2 – Lasota)*			
HI Titer at day 0	14.58	5.82	
HI Titer at day 14	43.29	28.71	
HI Titer at day 35	91.29	72.39	
HI Titer at day 42	116.70	63.94	
HI Titer at day 49	112.94	48.15	
HI titer of the group	63.13	64.41	0.513

4.7. Experimental chickens challenge

Before challenge the virus concentration for selected allantoic fluids were determined. Titration of the challenge virus with an ICPI score of 1.61 and 1.85 were $10^{9.1}$/ml and $10^{9.9}$/ml respectively. The challenge virus with ICPI score of 1.85 and $10^{9.9}$ titer per ml was selected for challenge of the experimental chickens. Clinical signs were not observed in any of the chickens before challenge.

4.7.1. Vaccinated and challenged experimental groups

Only 1 chicken in group 1 showed clinical sign of torticollis (Figure 4). The chicken was unable to feed since day 9 after challenge. On day 14th, sixth day to the onset of clinical signs the chicken was euthanized for post mortem examination (lesion in internal organs). The remaining 16 chickens (94 %) in this group showed no clinical sign until 21 dpc. Experimental group 2, group 3 and group 4 chickens showed no clinical signs until 21 dpc and 100 % survived (Figure 5).

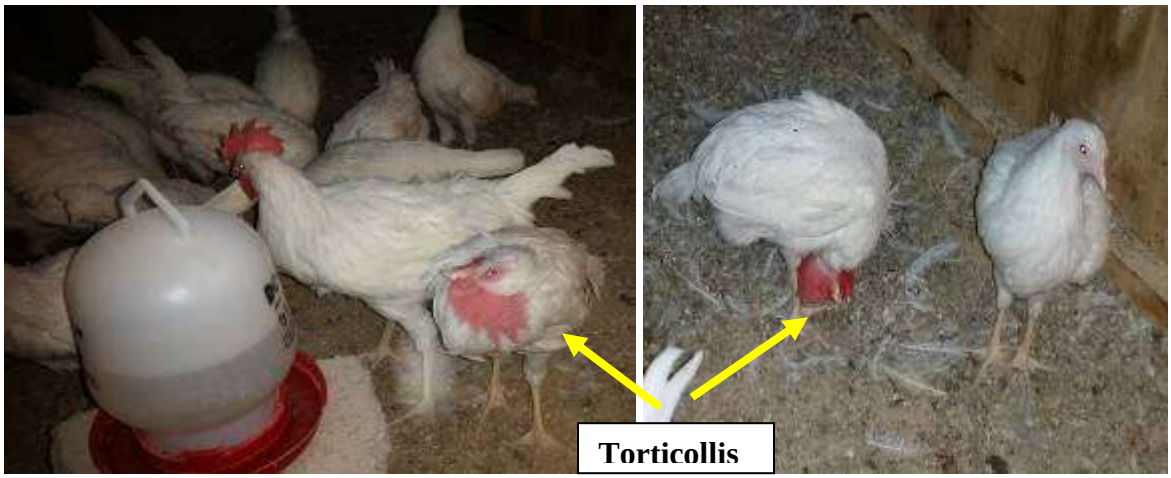

Figure 4. Vaccinated and challenged chicken showing clinical sign of torticollis

Figure 5. ND vaccine immunized and challenged chickens at 21 dpc from group 3

4.7.2. Unvaccinated and challenged experimental group

From unvaccinated or control group, four chickens show clinical signs of depression, ruffled feather and loss of appetite since 36 hours post challenge. In addition, sneezing, and anorexia were observed since 3rd dpc. At 4th dpc, 2 chickens died (Figure 6), on 5th dpc, 11 chicken died (Figure 7) and on 6th dpc, 4 chickens died.

Figure 6. Clinical sign of NDV challenged chicken at 4 dpc

Figure 7. Image of unvaccinated chickens at 5th dpc

4.7.3. Post Mortem Examination

The enlarged spleen (Figure 8) and brain from vaccinated and challenged experimental group 1 sick chicken with clinical sign of torticollis were tested by RT-PCR. The result of the spleen and brain samples from vaccinated and challenged chicken were positive at around 121 bp for APMV-1 by amplifying ND virus M-gene (Figure 11). In addition three chickens unable to feed from unvaccinated or control group were euthanized for post mortem examination. Enlarged liver (Figure 9) in 1 chicken, hemorrhage of the proventriculus in 2 chickens (Figure 10) and hemorrhage of trachea were observed. Brain, spleen and trachea samples from the unvaccinated group confirmed ND positive at around 121 bp for APMV-1 by amplifying ND virus M-gene (Figure 11).

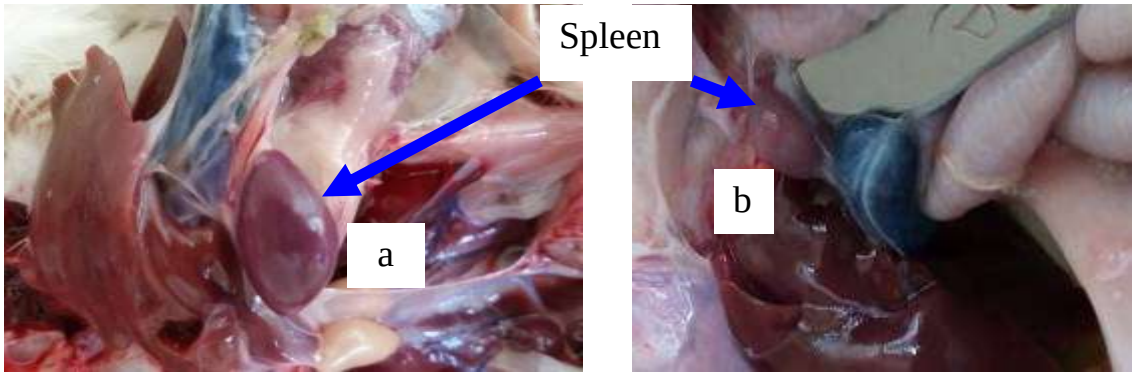

Figure 8. Enlarged spleen from vaccinated and challenged sick chicken (a) and normal (b)

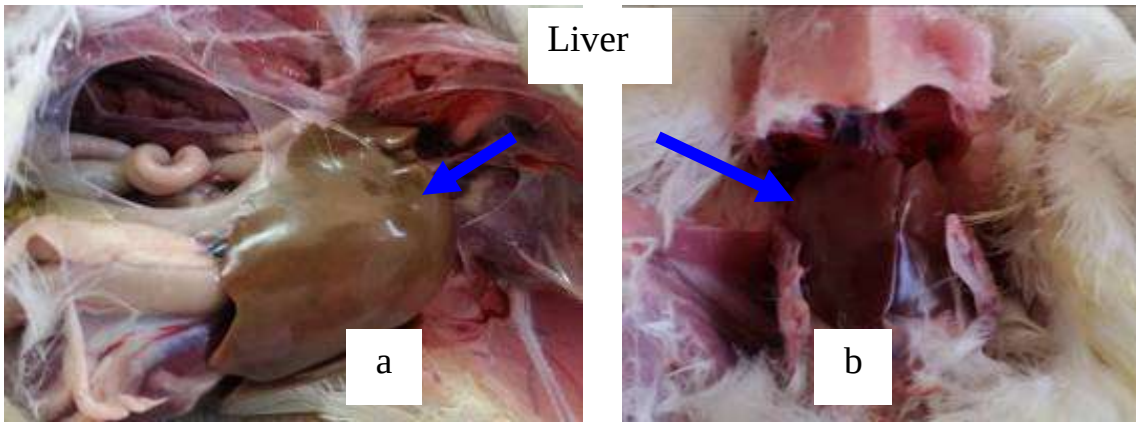

Figure 9. Enlarged liver from unvaccinated and challenged sick chicken (a) and normal (b)

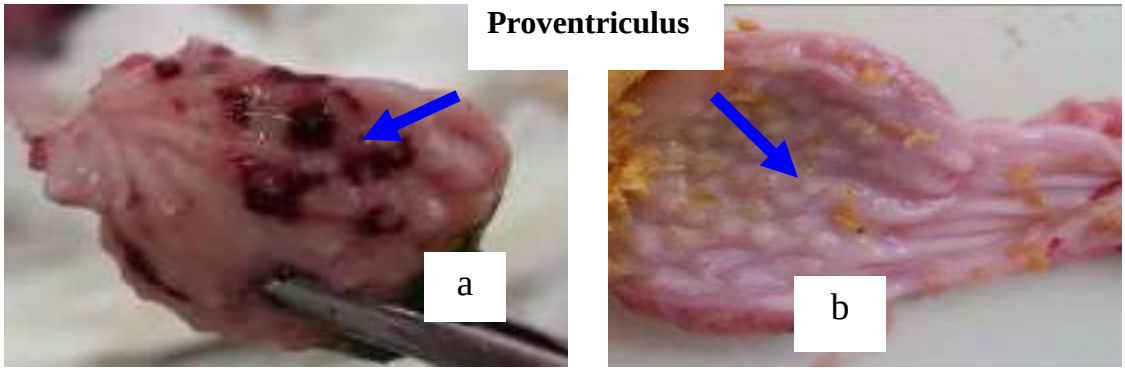

Figure 10. Petechial hemorrhage of proventriculus from unvaccinated and challenged sick chicken (a) and normal (b)

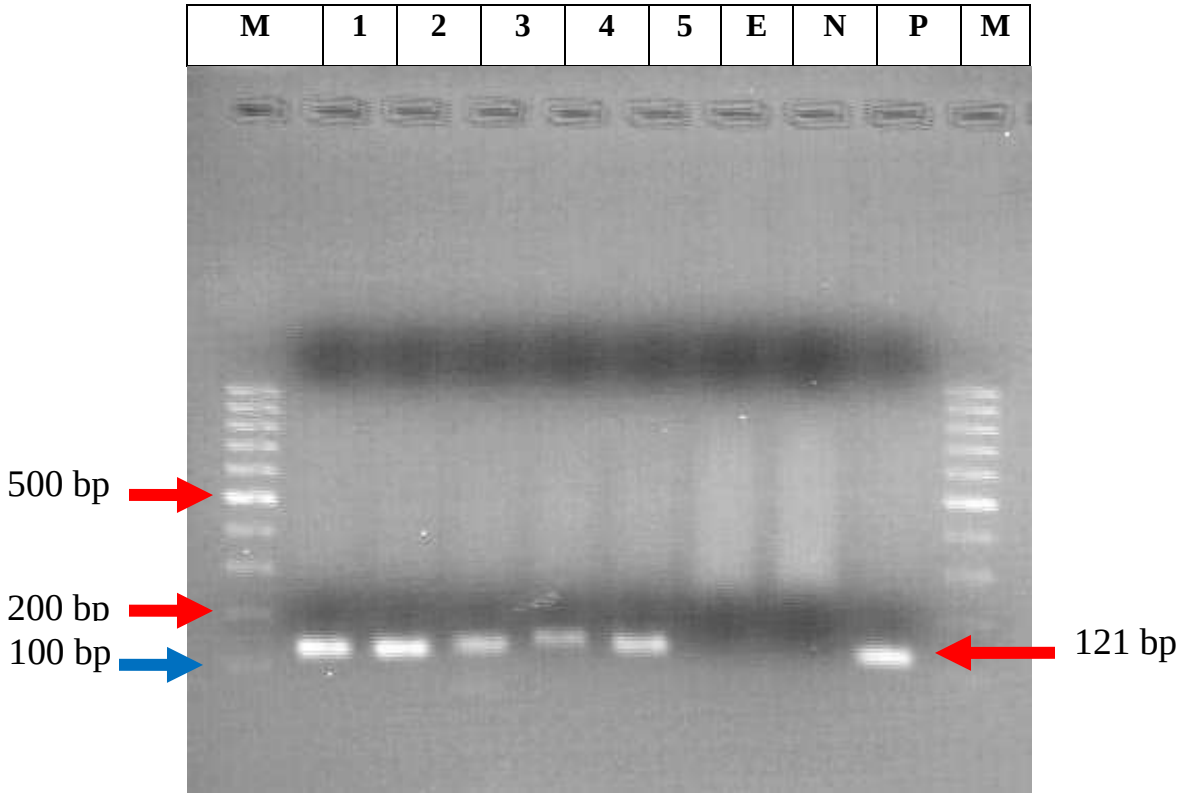

Figure 11. Gel picture of tissue suspension from unvaccinated and vaccinated sick chickens after challenge

Key:

Lane	
M	Molecular marker 100 plus
1	Pooled sample positive around 121 bp
2	Pooled sample positive around 121 bp
3	Pooled sample positive around 121 bp
4	Spleen sample from vaccinated and sick chicken positive around 121 bp
5	Brain sample from vaccinated and sick chicken positive around 121 bp
E	Extraction control
N	Negative control
P	Positive control positive around 121 bp

5. DISCUSSION

Newcastle disease is one of the most economically important diseases of chickens that causes serious losses in intensive and scavenging poultry in Ethiopia (Chaka *et al*., 2012). This has been evidenced by the present study, where NDV was isolated and molecularly detected from suspected cases. Laboratory analysis showed, NDV was isolated from 14 (26.92 %) allantoic fluid harvests in the study areas.

The embryo MDT for the selected allantoic fluids revealed that all embryos died at 46 and 47 hours duration at a concentration of dilution 10^{-7} for sample designated as 'Dhankaka' and 'Bishoftu' respectively. It was in agreement with Hanson and Brandly, (1955) that embryo death <60 hours was grouped as velogenic pathotypes. The ICPI assay of the current NDV isolates conducted *in vivo* showed that ICPI score of 1.85 for allantoic fluids designated as 'Dhankaka' and 1.61 for sample designated as 'Bishoftu'. This was in agreement with previous reports that describing virulent ND viruses are circulating in chicken population in the study areas (Chaka *et al*., 2013; Fentie *et al*., 2013).

Moreover, from this study it was observed tha ICPI score of 0.4 and 0.05 for 'Bishoftu' and 1.25 for 'Dhankaka' designated allantoic fluids indicated that low virulent or lentogenic and moderately virulent or mesogenic NDV strains were also circulating in chicken population in the study areas.

Immune response evaluation of ND vaccines indicates that maternally derived antibody (MDA) gradually decline to a minimum level. In this study, the MDA was detected at age of 7 and 21 days with the HI titer ranging from 1:32 to 1:16, respectively. However, at age of day 42 the mean HI titer decreased to ≤ 2 Log_2 value that can not induce protection against ND. These findings were in consistent with Jalil *et al*., (2009) who reported the detection of MDA up to 28^{th} day, Banu *et al*., (2009) reported detection of MDA with in 27^{th} day, and Geletu, (2018) reported MDA detection on the 31^{st} day. These all studies reported detection of MDA above 21^{st} and below 42^{nd} days.

The HI titer in geometric mean of Log_2 ± standard deviation of each vaccine used in experimental groups was considered to compare the immune response for ND vaccines. Primary immunization of the experimental chickens with the lentogenic ND vaccines revealed that the MDA was increased from it was detected at age of 7 days. After the first booster vaccination it was observed that booster vaccination by Lasota vaccine gives a higher mean antibody titer as compared to Thermostable (I-2) vaccine (Table 5). The current findings were in agreement with the previous reports of Westbury *et al*., (1984) that describing Lasota was more immunogenic than other lentogenic ND vaccines.

In both Lasota and I-2 first booster vaccinations, the *p-value* (< 0.05) indicates there was significance difference in HI titer due to booster vaccination. The results are in agreement with previous reports indicating booster vaccination after primary vaccination increase the immune response (Banu *et al*., 2009).

The mean HI titer after the second booster vaccination at 7^{th} day by Lasota vaccine, for experimental group 1, group 3, group 4 chickens and mean HI titer by I-2 vaccine for experimental group 2 chickens was greater than the mean HI titer measured at 14^{th} day. It was observed that mean HI titer were peak after 2^{nd} booster vaccination up to 7^{th} day. The mean HI titer after the second booster vaccination showed a significane difference with *P-value* (< 0.05). Secondary booster vaccination yields more higher antibody HI titer than the first booster which were in agreement with (Shuaib *et al*., 2003).

In comparison of the mean HI titer ± SD of the experimental groups 1, 2, 3 and 4 revealed that chickens in each group produce a protective immune response against the ND vaccines. The findings of this study supports primary vaccination by HB1, first booster vaccination by Lasota and 2^{nd} booster vaccination by Lasota yields relatively high mean HI antibody titer ± SD (Table 5). Primary vaccination by I-2, first booster by Lasota and 2^{nd} booster vaccination by Lasota yields relatively lower mean HI.

Primary vaccination by HB1, 1^{st} booster by I-2 and second booster by Lasota in group 4 yields the least mean HI titer ± SD of the groups (Table 5). It indicates that primary vaccination by HB1 produce low mean HI titer ± SD of the groups and the first booster vaccination by I-2 and second booster by Lasota vaccine resulted relatively low immune

response of the groups. These indicates there may be chickens individual immune response difference lowers the group mean HI titer. In comparison of the results obtained from the groups, Lasota was more potent than HB1 and the result were in agreement with previous reports (Borland and allen, 1980; Banu *et al*., 2009; Aliyu *et al*., 2016).

When comparing mean HI titer ± SD of experimental group 1 to that of group 2 revealed that Lasota was more immunogenic than I-2. Therefore, HI titers of the groups concluded that Lasota was more immunogenic than HB1 and I-2. However, the mean HI titer indicated that primary vaccination by I-2 was as effective as HB1 vaccine. In addition, I-2 have an added advantage of its capacity to heat resistance and suitable to reduce mortality in chickens immunizing in rural areas. In comparison of individual groups, except group 4 with *P-value* (>0.05), chickens revealed a significance difference with *P-value* (< 0.05).

Generally, to evaluate the efficacy of ND vaccines, it is important to consider some points including method and performance of vaccination, maternal antibodies, choice of strains, and vaccine titer. However, in the current study maternal antibodies, choice of strains, and vaccine titer were considered. Hence, the efficacy of the current ND vaccines was evaluated against the circulating NDV in the study areas. The findings of the present study showed all the groups vaccinated by HB1, Lasota and Thermostable vaccines yields mean HI titer that can protect chickens from virulent ND.

However, in 1 chicken from group 1 'HB1-Lasota-Lasota' vaccinated chickens, 9 dpc clinical sign of torticollis were observed and confirmed ND positive by RT-PCR. The remaining 16 chickens (94%) did not show any clinical sign. This variation could be the difference in individual chicken immune response to the vaccine as reports of (Westbury *et al*., 1984).

6. CONCLUSION AND RECOMMENDATIONS

In this study the circulating ND viruses in the study areas were isolated and confirmed to be virulent strain *in vitro* by embryo MDT determination and *in vivo* by ICPI tests. Moreover, the efficacy of the lentogenic vaccines produced in NVI was evaluated at the current recommended dose of $10^{6.5}EID_{50}$ per chicken. The protective efficacy of the vaccines against the current circulating virulent ND virus using vaccination-boost-boost and challenge protocol in experimental SPF chickens challenge was protective.

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

- Detailed investigation of the current circulating virulent ND viruses based on gene sequencing and sequence analysis is recommended to understand the evolutionary relationship of the former and the current circulating isolates .
- Continuous surveillance and vaccination of the backyard chickens were important to overcome infection of chicken population from ND.
- Strict biosecurity of ND for an endemic country like Ethiopia, management and hygiene are mandatory as vaccination without biosecurity may not control ND.

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8. ANNEXES

Annex 1. Ethical Clearance

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

Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/20/02/12/2020

Name of Applicant: Bogale Gossaye (BSc in Vet. Lab Tech, MVSc fellow)

Address: College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: *Isolation and molecular identification of Newcastle disease virus in chickens and evaluating the protective efficacy of Newcastle disease vaccine in Bishoftu, East Shewa, Ethiopia*

Date of application:	**23/12/2019**
Nature of the project:	**experimental (invasive)**
Target animal species:	**chickens**
Number of animals involved:	**80**
Study area:	NVI-**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/01/12/020, 03/01/2020**

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

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

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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Annex 2. Materials Used

- Automatic pipettes
- Centrifuge
- Disposable syringe guage 22Gx1¼" (3 ml)
- Egg candler
- Egg driller
- Egg incubator
- Ethyl alcohol 70%
- Forceps
- Gloves
- Insulin syringe
- Level II Laminar air flow hood
- Mortar and pestle
- Paper plaster
- Paraffin wax
- Pencil
- Pipette
- Plate sealer
- Reagent bottles
- Rubber suction bulb
- Scalpel handle
- Scissors
- Surgical blade
- Universal bottles
- V-bottom microtitration plate
- Vortex

Annex 3. Alsever's solution

Reagents	**Amount**
Citric acid $C(OH)(COOH)(CH_2.COOH)_2.H_2O$	0.055g
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	0.8g
D-Glucose ($C_6H_{12}O_6$)	2.05g
Sodium chloride (NaCl)	0.42g
Distilled water	to make up to 100ml

Method of preparation

1. Weigh out reagents into a conical flask
2. Add distilled water and make up to 100 ml
3. Dispense in to sterile bottles
4. Sterilize by autoclaving at 1160C for 10 minutes. Use slow exhaust.
5. Allow to cool, then tighten the lid and label the bottle
6. Store in refrigerator

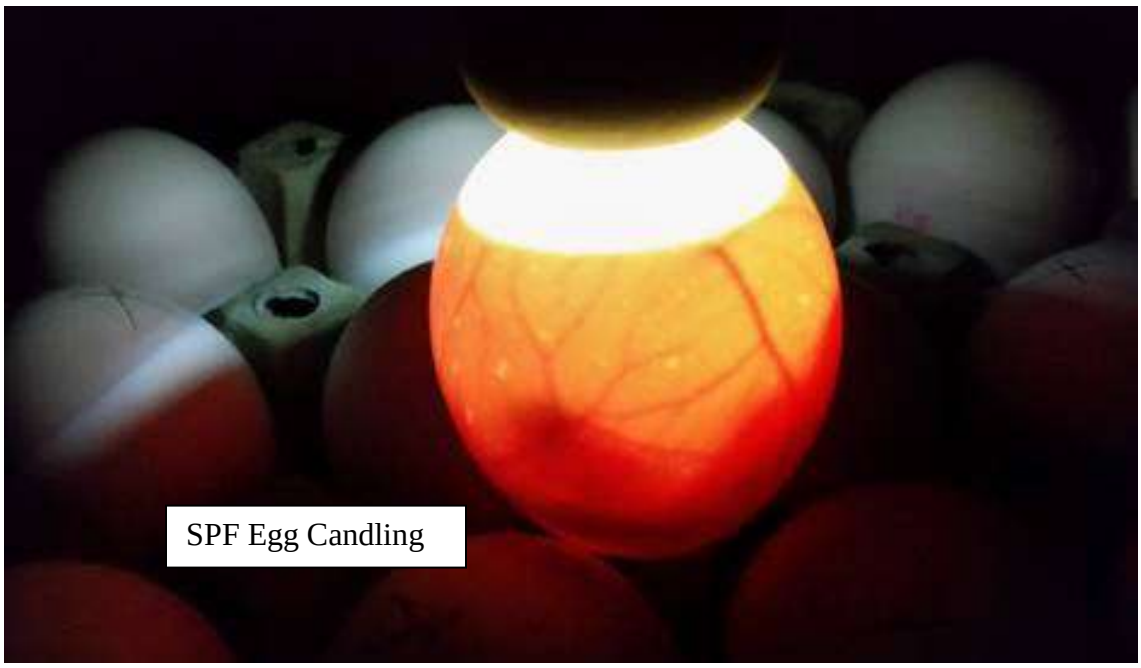
Annex 4. Dulbecco's phosphate buffer saline (PBS)

Ingredients	**Amount**
NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.2 g
$Na_2HPO_4.2H_2O$	1.44 g
Make up with distilled water	1000 ml

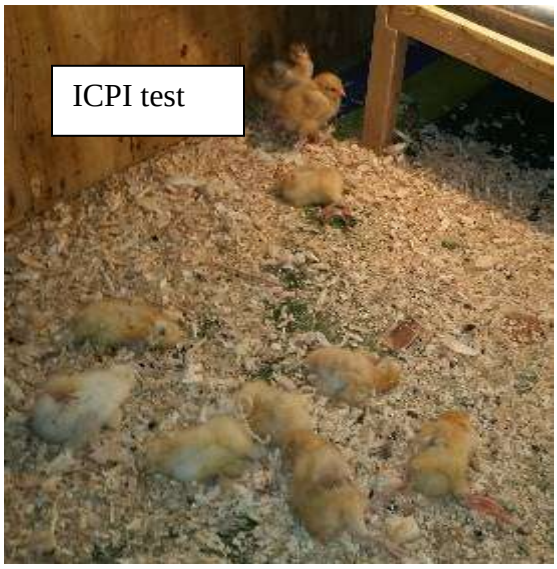
Preparation

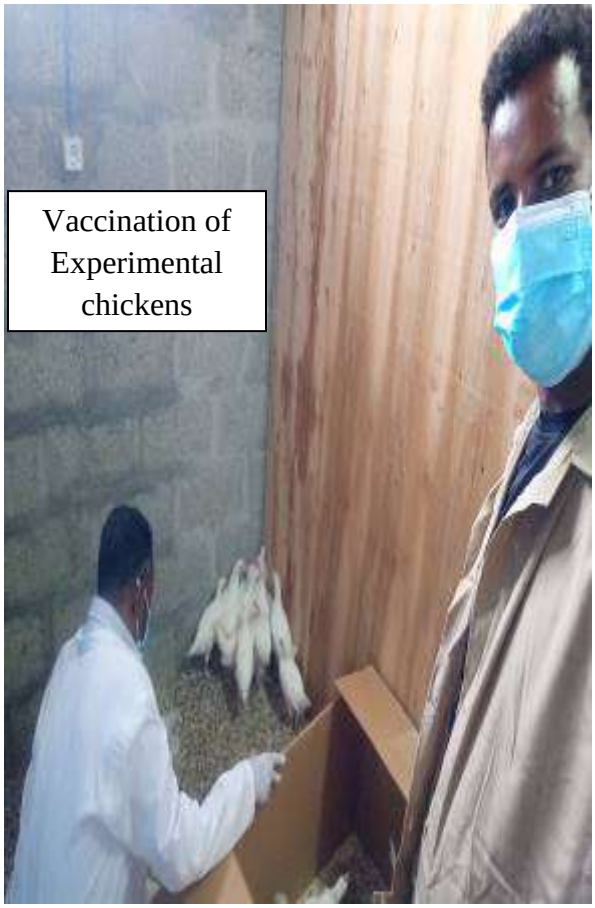
- Dissolve, then make up volumetrically to desired volume
- Mark liquid level before autoclaving
- Adjust the pH to 7.2 at 20ºC or 7.4 at 36.5ºC with sterile 1M NaOH
- Dispense into 200ml bottles
- Sterilize by autoclaving at 121ºC at 15 min.
- Store the solution bottles at +4ºC.

Annex 5. Image of SPF egg candling at 9^{th} day of incubation



Annex 6. Images at Laboratory and animal experimentation work





Vaccination of Experimental chickens

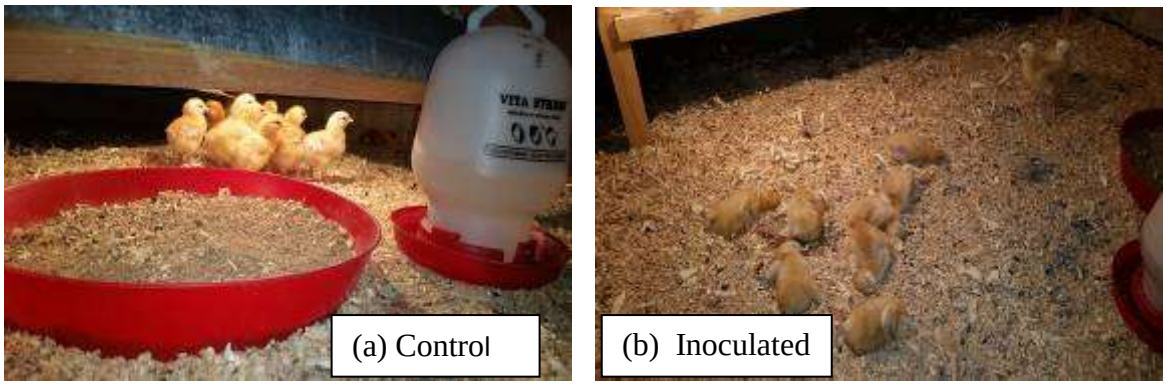
Sampled

Challenged

Annex 7. Clinical signs of chickens infected by Newcastle disease

Annex 8. ICPI test in day old chicken

Annex 9. ICPI test result (a) control and (b) Inoculated by isolated ND virus



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