

Thesis Ref. No. _____



ISOLATION AND MOLECULAR DETECTION OF NEWCASTLE DISEASE VIRUS
AND VACCINE IMMUNOGENICITY EVALUATION IN AND IT'S SURROUNDING
BISHOFTU, ETHIOPIA

MSc thesis

BY: AYELE TESHOME

PRINCIPAL ADVISOR: HIKA WAKITOLE (Associate Professor)

CO-ADVISOR: MIRTNEH AKALU (BSc., MSc, PhD)

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE DEPARTMENT OF
VETERINARY MICROIOLOGY, IMMUNOLOGY AND PUBLIC HEALTH
MASTERS PROGRAM IN VETERINARY MICROBIOLOGY

JUNE, 2023
BISHOFTU, ETHIOPIA

ISOLATION AND MOLECULAR DETECTION OF NEWCASTLE DISEASE VIRUS
AND VACCINE IMMUNOGENICITY EVALUATION IN AND IT'S SURROUNDING
BISHOFTU, ETHIOPIA



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

Veterinary Microbiology

By: Ayele Teshome

Principal advisor: Hika Waktole (Associate
Professor)

Co-advisor: Mirtneh Akalu (BSc., MSc, PhD)

Department of Veterinary Microbiology, Immunology and Veterinary Pulic Health MSc.

in Veterinary Microbiology

JUNE, 2023
BISHOFTU, ETHIOPIA

Addis Ababa University
College of Veterinary Medicine and Agriculture
Department of Microbiology, Immunology and Veterinary Public Health

As members of the Examining board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: Ayele Teshome Entitled "Isolation and Molecular Detection of Newcastle Disease Virus and Vaccine Immunogenicity Evaluation in and it's Surrounding Bishoftu, Ethiopia," and recommended that it is accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

_____	_____	_____
Chairman	Signature	Date
_____	_____	_____
External Examiner	Signature	Date
_____	_____	_____
Internal Examiner	Signature	Date

Final approval and acceptance of the thesis is contingent upon the submission of its final copy to the CGS/FGC through the departmental graduate committee (DGC) of the candidate's major department. I here by certify that; I have read the revised version of this thesis prepared under my direction and recommend that it is accepted as fulfilling the thesis requirements.

Hika Waktole (Associate Professor) _____

Advisor	Signature	Date
---------	-----------	------

TABLE OF CONTENTS

TABLE OF CONTENTS	i
AUTHOR DECLARATION.....	iii
ACKNOWLEDGEMENTS	iv
LIST OF ABBREVIATIONS.....	v
LIST OF FIGURES.....	vi
LIST OF TABLES	vii
LIST OF APPENDIXES	viii
ABSTRACT	ix
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1. Detection of the agent.....	4
2.2. Virulence.....	5
2.3. Description of Newcastle disease	6
2.4. Epidemiology.....	7
2.4.1. Susceptible hosts	7
2.4.2. Sources of Infection and Route of Transmission	7
2.4.3. Geographical Distribution of ND.....	8
2.5. Pathology	8
2.6. Signs and symptoms	9
2.7. Samples for virus isolation	10
2.8. Viral isolation	10
2.8.1. SPF-Egg Inoculation	10
2.8.2. Duck fibroblast cell inoculation	11
2.8.3. Enzyme Linked Immuno-Sorbent Assay (ELISA)	11
2.8.4. Haemagglutination Test	12
2.8.5. Haemagglutination Inhibition Test (HI).....	12
2.9. Intracerebral pathogenicity index.....	13
2.9.1. Intracerebral pathogenicity index test	13
2.10. Molecular identification.....	13
2.11. Economic impact of Newcastle disease.....	15
2.12. Prevention and control measures	16
2.13. Vaccination	16
2.13.1. Inactivated Vaccines	16

2.13.2.	Live Vaccines	17
2.13.3.	Recombinant vaccine	18
2.14.	Status of ND Vaccine in Ethiopia.....	18
3.	MATERIALS AND METHODS.....	20
3.1.	Study Area and study animals	20
3.2.	Study Design.....	20
3.3.	Sample collection	20
3.4.	Laboratory Investigation.....	21
3.4.1.	Egg Inoculation	21
3.4.2.	Infected embryo allantoic fluid harvesting	21
3.4.3.	Haemagglutination Test	22
3.4.4.	Haemagglutination Inhibition Test (HI).....	22
3.4.5.	Embryo Mean death time determination	23
3.4.6.	Intracerebral pathogenicity index test	23
3.4.7.	Molecular identification	24
3.4.8.	Master Mix preparation.....	25
3.4.9.	Polymerase chain reaction.....	25
3.4.10.	Gel electrophoresis	25
3.5.	Animal management and vaccine efficacy test	26
3.6.	Blood Collection.....	27
3.7.	Experimental animals challenge	27
3.8.	Data management and analysis.....	27
4.	RESULTS	28
4.1.	Embryo means death time determination	28
4.2.	Pathogenicity index test.....	29
4.3.	Vaccine efficacy and immunogenicity evaluation.....	29
4.4.	Experimental animal challenge.....	31
4.5.	Post Mortem diagnosis on experimenal groups	32
4.6.	Polymerase chain Reaction.....	33
5.	DISCUSSION	36
6.	CONCLUSION AND RECOMMENDATIONS	39
7.	REFERENCES	40

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.

Name: Ayele Teshome Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: _____

ACKNOWLEDGEMENTS

I am profoundly grateful to my principal adviser **Hika Waktole** (Associate Professor), granting me permission, encouragement, patience and effort in my work progress to completion.

My heart felt gratitude goes to my co-advisor **Dr. Mirtneh Akalu** (PhD) lead researcher at NVI research and development, who guide me in all duties during my experimental work by advising, guiding and facilitating the laboratory work and unwavering effort and commitment in work progress overseeing this to completion.

I also forward my appreciation to **Dr. Liyuwork Tesfaw** (Research and Development Directorate), **Dr. Yeneneh tesfaye** (lead researcher in virology), **Mr. Kassaye Adamu** (research coordinator), **Mr. Eyob** (experimental animals focal person) and staff members of Research and Development Directorate of NVI, who had facilitated the experimental rooms, purchase of feeds and compassionate support during my Research work.

I am privileged to thanks Dr. Belayeneh Getachew (PhD) for his support during experimental work in chickens specially ICPI test.

I would also give appreciation to the National Veterinary Institute; all my duties will not come true without the supply of all necessary research materials.

Many thanks to my friends **Bogale Gossaye** and **Kenaw Berhanu** who helped me during my research work.

I would like to credit to my family for their encouragement in my thesis work to completion.

Above all, I thank God almighty for seeing me through this work.

LIST OF ABBREVIATIONS

APMV-1	Avian Paramyxovirus 1
DFcell	Duke Fibroblast Cell
DIVA	Differentiation Of Infected From Vaccinated Animals
EID50	Embryo Infective Dose Fifty
ELD50	Embryo Lethal Dose Fifty
ELISA	Enzyme Linked Immunosorbent Assay
HA	Haemagglutination
HAU	Haemagglutination Unit
HI	Haemagglutination Inhibition
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	Neuraminidase
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NP	Nucleoprotein
OIE	World Organization For Animal Health
PS	Phosphate buffer Saline
PCR	Polymerase Chain Reaction
PM	Post Mortem
RC	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 Ribonuclic Acid
vNDV	Virulent Newcastle Disease Virus

LIST OF FIGURES

Figure 1: Schematic diagram of NDV genome (A); schematic diagram of NDV	4
Figure 2: World wide Geographical distribution of ND.	8
Figure 3: NCD virus isolation DF1 cell line where, (A) Normal cell culture (B) abnormal cell (CPE on inoculated cell culture, arrow showing aggregation and disruption of the monolayer).27	
Figure 4: Geometric mean HI titer of vaccinated and control group chickens (G-1:group one; G-2: group two; G-3: group three; and G-4: group four).	31
Figure 5: Control groups showing clinical sign of torticollis.....	31
Figure 6: Hemorrhage of proventriculus (A) and Normal (B)	32
Figure 7: Enlarged Spleen (A) and Normal (B)	32
Figure 8: Enlarged liver (A) and Normal (B).....	33
Figure 9: Agarose gel electrophoresis of PCR product (~1100 p) of NDV suspected samples.33	

LIST OF TABLES

Table 1: Strains of Newcastle disease virus used in live vaccines.....	17
Table 2: Type of vaccine and time of vaccination	26
Table 3: Embryo mean death time from allantoic fluid and infected DF1 cell culture samples	29
Table 4: Experimental allantoic fluid samples (NDV isolates) and its corresponding ICPI score	29
Table 5: HI titer group mean, standard deviation and p-value.....	30
Table 6: Description of sample type and gel electrophoresis result.....	34

LIST OF APPENDIXES

Ethical clearance certificate..... 49

ABSTRACT

Newcastle disease (ND) is an economically important endemic viral disease of chickens that has a potential threat to village chickens and commercial poultry farms in Ethiopia. Despite intense ND vaccination, most clients were raising their doubt regarding efficacy of the current National Veterinary Institute (NVI) ND vaccines against the circulating viruses. Therefore, in this research a cross sectional study was designed to isolate and molecularly detect ND virus and to assess the protective efficacy of ND vaccines in and its surrounding Bishoftu from October 2022 to May 2023. A total of 57 samples were processed using SPF embryonated eggs, hatched chickens and DF1cell culture methods to isolate NDV. The pathogenicity of the isolates was evaluated using intracerebral pathogenicity index (ICPI) scoring (60 chickens) and embryo mean death time (MDT) determination. The isolates were further identified by molecular assay, targeting primers of the M-gene (~1100 p) of avian paramyxovirus-1 (APMV-1). The result of PCR revealed that from the total 11 ND suspected samples eight of them were positive. Vaccine efficacy was evaluated in 80 chickens hatched from SPF eggs. Chickens were immunized with lentogenic vaccines of NCH1, NCL and NCTH using three vaccination schedule groups and one control group. Standard dose of $10^{6.5}$ EID₅₀ per chicken was administered at day zero and boosted at days 14 and 35. At the end of experiment period all groups were challenged with isolated NDV ($10^{10.6}$ ELD₅₀ per chicken) through ocular-nasal route. The result revealed that the isolated NDV was characterized as very virulent (velogenic pathotype) based on the mean death time (MDT) and ICPI score. The geometric mean (GM) haemagglutination inhibition (HI) antibody titer was peaked post-second-boost and revealed a significance difference ($P < 0.05$) compared to the control group. Thus, secondary boost produces higher antibody HI titer than the first boost. Besides, the challenge assay revealed that vaccinated chickens were protected against the challenge strain while unvaccinated chicken were deceased 100% within 6 days post challenge. Accordingly, it can be concluded that the current ND vaccine is efficient to protect vaccinated chickens against the circulating NDV in the study area. Thus, the study recommends further investigations of ND outbreaks, training and awareness regarding ND and vaccine management.

Keywords: *Bishoftu, Chicken, Newcastle Disease Virus, Vaccine.*

1. INTRODUCTION

Poultry farming plays an essential role in generating income, ensuring food safety, and reducing scarcity in developing countries (Attia *et al.*, 2022). Likewise, production of chickens, to meet the nation's demand for eggs and meat, is the primary focus of Ethiopia's government in an attempt to sustain food security. The total chicken in the country is estimated to be about 57.01 million of which 78.85%, 12.02%, and 9.11% are indigenous, hybrids, and exotic poultry, respectively (CSA, 2021). Despite large number of chicken, the benefit and contribution to the country is y far away from the existing potentials due to poultry disease like ND.

Newcastle disease (ND) is a highly contagious and frequently severe illness that affects birds, particularly domestic poultry worldwide (Mayers *et al.*, 2017; Napit *et al.*, 2023). ND is caused by a member of the family Paramyxoviridae and all species and age groups of birds are prone to this acute to chronic illness (OIE, 2021). This illness was given the name of the England town where it was originally discovered. The first epidemics of NDs were found in chicken in 1926, which occurred in Java, Indonesia as well as Newcastle-upon-Tyne, United Kingdom. However, there have been previous stories of outbreaks of identical disorders throughout Europe prior to this time (Arman, 2002; Doyle, 1927; Halasz, 1912; Kraneveld, 1926). In Ethiopia, ND remains the first and most common infectious disease, which has prevalence rates of 80% and 43% in commercial poultry farms and rural village chicken, respectively (Mesfin and Bihonegn, 2018).

Newcastle disease viruses (NDVs) are paramyxoviruses that are single-stranded, non-segmented, negative sense, enveloped RNA viruses in the genus Avulavirus. Virulent strains of the avian paramyxoviruses type 1 (APMV-1) of the genus Orthoavulavirus, family Paramyxoviridae, subfamily Avulavirinae, are the primary cause of ND (OIE, 2021; Miller and Koch, 2013). Based on phylogenetic analysis and serological assay, the paramyxoviruses isolated from avian species have been divided into 21 serotypes known as APMV-1 to APMV-21. Each virus is a member of one of three virus genera of metaavulavirus, orthoavulavirus, and paraavulavirus (OIE, 2021; Amarasinghe *et al.*, 2019; ICTV, 2019). Besides, NDV is classified into five pathotypes according to the clinical features of infected chickens, including viscerotropic velogenic, neurotropic velogenic, mesogenic,

lentogenic or respiratory, and asymptomatic. Pathotype groupings are rarely definite (OIE, 2021; Miller and Koch, 2013).

Prevention and control measures of ND involve implementation of culling of diseased birds along with strict biosecurity and rigorous immunization protocols. These approaches have been successful in areas where properly implemented and the most cost effective alternative is the use of aggressive vaccination programs (Nasser, 1998; Mesfin and Bihonegn, 2018). However, worldwide outbreaks have been associated with a number of factors, such as a lack of biosecurity, inadequate vaccinations and programs, antigenic variation, maternal antibodies inhibiting the effectiveness of live vaccines, a short duration of the immune response, and immune suppression (Chume *et al.* 2017; Dimitrov *et al.*, 2017). Besides, outbreaks of the disease still occur worldwide in the poultry industry despite vaccination. Large sums of money are spent on vaccination to evade disease and testing to ensure the disease existence in different countries (Miller and Koch, 2013).

The current vaccines reduce disease symptoms and fatalities, but the virus can still infect and multiply in chickens that have received the vaccine. Once the viruses have multiplied, they are released into the environment through saliva and feces and can infect other birds (Miller and Koch, 2013; OIE, 2021). The National Veterinary Institute (NVI) has received several ND cases from nearby areas for diagnosis. The most common methods for diagnosing the illness include virus isolation, serological assay, and molecular detection. Studies thus far have primarily focused on NDV isolation and molecular detection. Conversely, there are limited studies on vaccine efficacy and immunogenicity in Ethiopia vis-à-vis continuous reports and complaints of disease prevalence after immunization. This implies that ND remains endemic in the Ethiopia chicken population and potential threat for production. Hence, it urges to design effective and safe vaccine as a disease prevention strategy

Therefore, this study is aimed at:

1. Isolation, molecular detection of NDV from outbreak sample to NVI and stored samples originated from Bishoftu and its surroundings.
2. Assessment of the immunogenicity of the current ND vaccine produced yNVI
3. Evaluation of ND vaccine efficacy produced at NVI against a circulatingviruses

2. LITERATURE REVIEW

2.1. Newcastle disease

The production of chicken in Ethiopia is a cost-effective investment that is important to the local economy and has the potential to increase food security and help reduce poverty (Zelege *et al.*, 2005). Production of chicken provides considerable social and cultural rewards in developing nations. It is crucial for providing 35 essential amino acids (Yoriyo *et al.*, 2008). The most serious of the infectious illnesses that endanger poultry productivity is ND. Regardless of differences in sex and age, it is a highly contagious, acute viral disease that affects domestic poultry and other 45 bird species (OIE, 2021). In many third-world nations where agriculture is the main source of national affluence the disease is endemic (Ganar *et al.*, 2014).

Newcastle disease (ND) is a highly contagious illness with symptoms that can vary greatly in nature and severity. The disease is one of the biggest challenges to the worldwide trade of chicken and poultry products. The World Animal Health Organization (OIE) recognizes ND as a disease, ensuring a global risk study possible. It allows for the separation of high- and low-challenge countries (based on the frequency of ND outbreaks) (Ganar *et al.*, 2014).

2.2. Characteristics of the virus

Newcastle disease is caused by a group of closely related viruses that form the avian paramyxovirus virus (APMV-1) serotype. Some serological relationships have been demonstrated between NDV and other Paramyxovirus serotypes. The most significant serotype is considered as APMV-3 serotype. For many years NDV strains and isolates were considered to form a serologically homogenous group and this has been the basis of vaccination procedures employed for prophylaxis in most countries (Al-Gari, 2003).

Violent strains of the Newcastle disease virus (NDV), also recognized as avian paramyxoviruses 1 (APMV1), which is a member of the order Mononegavirales, family Paramyxoviridae, subfamily Avulavirinae, and genus Orthoavulavirus, are the cause of this illness. 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-, 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).

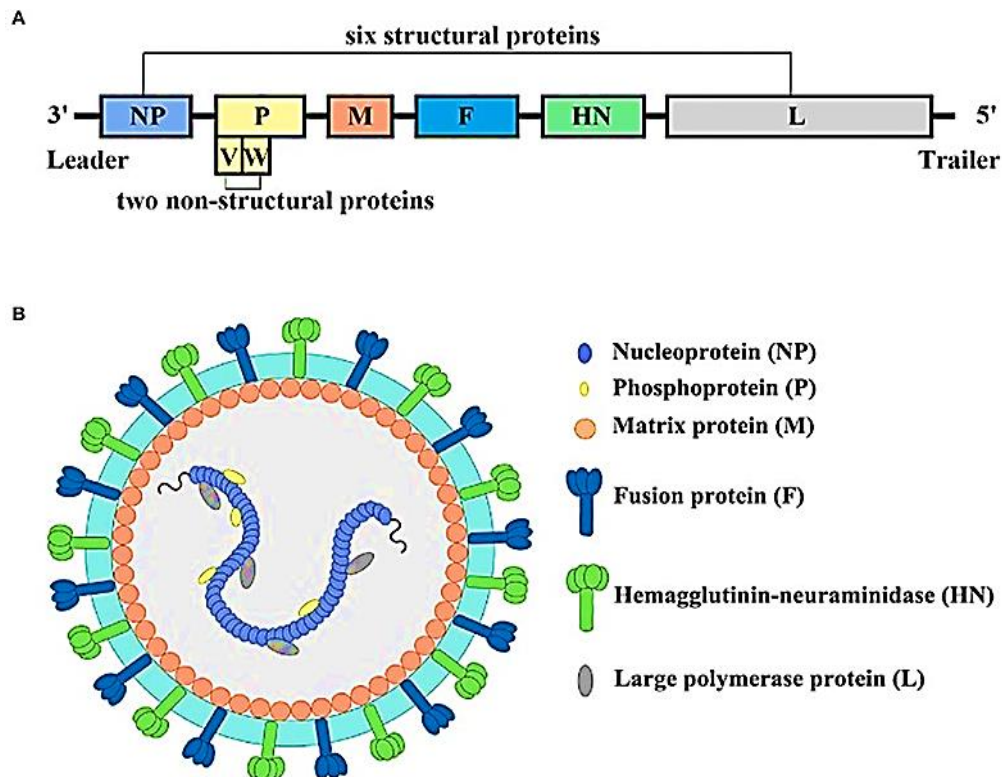


Figure : Schematic diagram of NDV genome (A); schematic diagram of NDV structure (B)

Source: (Bello *et al.*, 2018)

2.3. Virulence

Based on the antigenic characteristics of the hemagglutinin, there are currently 21 different serotypes of avian paramyxoviruses. ND virus is the prototype virus for APMV-1. On the basis of their antigenic relatedness HI tests, ND virus belongs to APMV-1. ND virus can also be classified into five patho-types based on the clinical signs induced in infected chickens. This includes Viscerotropic velogenic Newcastle disease, (VVND) characterized by an acute lethal infection of chickens of all ages, and it causes high mortality of 90-100% among adult birds.

Acute deadly infection of chickens of all ages is a characteristic of the neurotropic velogenic ND virus. Mesogenic ND virus causes respiratory and sometimes nervous signs with low mortality reduced egg production. Deaths are seen only in young birds. Lentogenic ND virus causes mild or impairment respiratory infections in which deaths confined to young chickens, which are commonly, used as live vaccines. It is also called hitchhiker's form. Asymptomatic enteric form causes in apparent intestinal infection (Fikre, 2003). Severity of infection depends on virus virulence and age, immune status, and susceptibility of the host species. Chickens are the most and waterfowl the least susceptible of domestic poultry; however, some differences may be seen if the NDV strain is adapted to a particular species (Miller, 2014).

In molecular pathogenicity of the disease the genome of NDV encodes for six major structural proteins. Viral replication, transcription and translation occur in the cytoplasm of the host cell, while virus particles are assembled in plasma membrane by budding (Zanetti *et al.*, 2003). Important pathogenic marker of NDV exists in F protein (Madadgar *et al.*, 2013), and Disulphide linkage is present between F1 and F2. These proteins enabled the virus to attach to the host cell membrane (Wen *et al.*, 2007). At cleavage site, F0 protein has two pair of basic amino acids that can be cleaved by the host proteases (Pham *et al.*, 2005). Highly virulent NDV has three or more basic amino acids, which are lysine (K) or arginine (R) present at 113 - 116 residues and phenylalanine (F) at position 117 (OIE, 2012). Cleavage of F0 protein is due to the presence of these basic amino acids in virulent NDV (Boostani *et al.*, 2013)

2.4. Description of Newcastle virus

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. One of the following conditions applies to a severe case of Newcastle disease. The crucial region responsible for significant changes in virulence is the fusion protein cleavage site, which is dependent on many genes for NDV virulence (Peeters *et al.*, 1999; De Leeuw *et al.*, 2005). Less basic amino acids are present at this site and leucine rather than phenylalanine is present at position 117 in the low virulent viruses (loNDV) (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. While the monobasic amino acid sequence in the same region is present in low virulence viruses at residues 117 and 112 (leucine), respectively, and positions 112 G/E-K/R-Q-G/E (Kim *et al.*, 2008). At least three arginine or lysine residues between residues 113 and 116 are considered to be multiple basic amino acids. Directly or by deduction, the presence of many basic amino acids in the virus can be established at the C-terminus of the F2 protein, which contains phenylalanine at position 117, the N-terminus of the F1 protein. Virulent strains had phenylalanine at position 117 and the basic amino acids at positions 113, 115, and 116. In this case cleavage of the virus 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 chickens (*Gallus gallus*) of ≥ 0.7 (OIE, 2021a)

2.5. Epidemiology

2.5.1. Susceptible hosts

ND viruses have been reported to infect animals other than birds, ranging from reptiles to man (Lancaster, 1966). Kaleta and Baldauf (1988) concluded that NDV infections have been established in at least 241 species of birds representing 27 of the 50 Orders of the class. It seems probable that all birds are susceptible to infection

but, as stressed by Kaleta and Baldauf, the disease seen with any given virus may vary enormously from one species to another.

2.5.2. *Sources of Infection and Route of Transmission*

Other than from predatory Birds or the practice of feeding untreated swill containing poultry meat to poultry, it appears that spread from bird to bird happens as a result of either inhaling ejected droplet particles or ingesting infectious material like feces. There is surprisingly little experimental evidence that sick birds transmit the virus to susceptible birds in this way, even across short distances, despite the fact that the administration of live vaccines by aerosol makes it evident that infection may be established via the respiratory route. The effectiveness of this transmission channel is influenced by a variety of environmental conditions, including temperature, humidity, and stocking density. It seems most likely that the pigeon variant virus, the "asymptomatic enteric" viruses, and other viruses which fail to induce significant respiratory signs in infected birds, are transmitted primarily in this way (Alexander *et al.*, 1984)

2.5.3. *Geographical Distribution of ND*

Throughout many ways, determining the prevalence of ND throughout the world at any particular time is very challenging. In some nations or regions, sickness is only ever reported when it affects commercial poultry and is overlooked when it affects private or village flocks of hens. Monitoring surveys frequently find symptomless infections with avirulent viruses that have likely been transmitted from ducks or other wild birds, even in nations that have long been acknowledged as being free of ND. There is little doubt, however, that the highly pathogenic form of ND poses a significant threat, either as an enzootic illness or as the root of frequent, recurring epizootics in Africa, Asia, Central America, and parts of South America (Alders & Spradrow, 2001). In other areas such as Europe, the situation appears to be one of sporadic epizootics occurring despite vaccination programmes (Kaleta and Heffels, 1992).

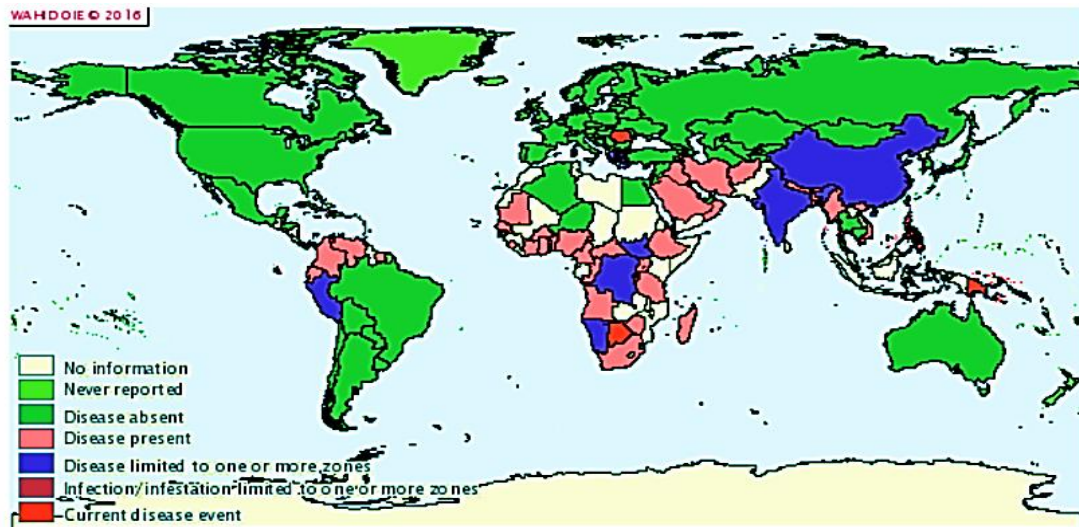


Figure :World wide Geographical distribution of ND.

Source: World Animal Health Information Data base (WAHID Interface, 2015)

2.6. Pathology

Any kind of ND can be diagnosed by the absence of microscopic or gross abnormalities. Birds that susceptible to virulent ND typically have fevered, dehydrated carcasses. The virus that is causing the lesions varies. The intestinal tract frequently develops hemorrhagic lesions as a result of virulent panzootic ND viruses. These can vary greatly in size and are best visible if the gut is opened (McFerran and McCracken, 1988). A= hemorrhage within the crescent-shaped lymphoid patch in the lower eyelid is a characteristic early feature of NDV, cecal tonsil necrosis and B=focal hemorrhage, C= hemorrhagic foci in the proventriculus correspond to necrosis of underlying lymphoid tissue, and D= mottled spleen indicates multifocal necrosis. On the other hand, velogenic neurotropic NDV is defined by E=Birds are frequently right and alert, but they also suffer hemiparesis, and F= histologically, velogenic, brain lesion are velogenic ND is characterized by significant brain lesions. Additionally, the lamina propria of the cecal tonsil and G, which stand for numerous infected cells in the spleen, respectively, reflect the histopathologic lesion of Velogenic Viscerotropic NDV (Cattoli, 2010).

2.7. Signs and symptoms

According to Al-Hae et al. (2013), the clinical symptoms displayed by birds with NDV infection rely on a variety of variables, including the virus strain's virulence, host range, the species and age of the host, immunological status, and environmental

conditions. The clinical symptoms do not offer a reliable foundation for the diagnosis of ND in birds. The symptoms of NDV are often determined by a number of variables, including the virus strain and its virulence, the host species and its susceptibility, the age of the host (younger people are more susceptible), concurrent infection with other organisms, environmental stress, and the host's immune system. Based on the clinical symptoms observed in experimental NDV infections of chickens, five families of NDV strains have been identified (Beard and Hanson, 1984; Getabalew et al., 2019; OIE, 2021a). Intestinal hemorrhagic lesions of the highly pathogenic viscerotropic velogenic kind are frequently observed. The respiratory form (lentogenic or respiratory) causes a mild or subclinical respiratory infection, the neurotropic velogenic form, with high mortality, which is typically seen after the respiratory and nervous signs, the mesogenic form, with respiratory signs and occasional nervous signs but low mortality, and the asymptomatic form, which typically consists of a subclinical enteric infection. There may be some overlap between these categories (OIE, 2021a).

Loss of appetite, unusual thirst, frailty, decreased egg production; air sacculitis, tracheitis, and conjunctivitis are the most common symptoms in chickens. Sneezing, gasping for air, nasal discharge, and coughing are respiratory symptoms, but clear digestive symptoms include greenish watery diarrhea. Wings and/or legs may become paralyzed, the head and neck may twist, or the entire body may become paralyzed (haiyat et al., 1994). (Hadipour et al., 2011) Layers exhibit a decline in egg production and soft, malformed egg shells. Death occurs suddenly under acute and severe conditions, such as neurotropic velogenic strain, and birds don't exhibit any clinical symptoms before passing away.

2.8. Diagnosis of Newcastle Disease

Spleen, lung, kidney, intestine, heart, brain, and liver may be taken independently or as a group from recently deceased birds (postmortem) as well as from live hens (antemortem samples), oronasal swap, cloacal swap, and serum (OIE, 2021).

2.9. Viral isolation

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 was propagated into at least five embryonated SPF eggs of 9 –11 days old. Eggs lacking the NDV antibodies must be used if SPF eggs are unavailable. The eggs are incubated at 35–37°C for 4–7 days following injection. The NDV gene is isolated from SPF embryonated chicken eggs to diagnose Newcastle disease. According to Alexander and Senne (2008) and OIE (2021), all NDV isolates are known to replicate in chicken egg embryos, and the mean death time (MDT) required to kill the embryo differs depending on the virus's virulence.

Rapid and accurate diagnosis of ND outbreak is important because it clinically resembles highly pathogenic avian influenza (Khan *et al.*, 2010). Clinical diagnosis based on history, signs and lesions may establish a strong index of suspicion but the laboratory confirmation must be done, and these include:

2.9.1. SPF-Egg Inoculation

Isolation of Newcastle Disease Virus Tissue homogenates or fecal and cloacal swab suspensions are clarified by centrifugation and 0.2 ml of the supernatant is inoculated via the allantoic cavity. Each sample was propagated to at least 5 embryonated eggs with 9-11 day old developmental SPF eggs. If not, NDV antibody-negative eggs are required. After inoculation, eggs are incubated at 35-37°C for 4-7 days. Newcastle disease is diagnosed by isolating NDV from SPF chicken embryo eggs. All NDV isolates are known to replicate in embryonated chicken eggs, and the mean death time (MDT) before the embryo dies varies with viral virulence (Alexander and Senne, 2008; OIE, 2021). Necropsies are performed twice daily during the incubation period of infected eggs. Eggs containing dead embryos as they arose, embryos that died within the first 24 hours of virus inoculation were discarded, and at the end of the culture period all remaining eggs were chilled overnight to +4 °C and exsanguinated in urine. The luminal fluid was collected (OIE). 2012).

2.9.2. Duck fibroblast cell inoculation

Tissue samples for virus isolation will be processed in the virus isolation laboratory by chopping into small pieces and grinding with sterile sand with a mortar and pestle. Brain, lung, trachea, spleen, kidney, liver, heart, and intestine tissue and

archival specimens from various outbreaks will be pooled to increase viral concentrations. Tissue sample suspensions (10% (w/v)) will be mixed with sterile phosphate-buffered saline (PS) containing penicillin (100 IU/ml) and streptomycin (1000 µg/ml). The suspension was filled into sterile Falcon tubes and centrifuged at 3000 rpm, +4°C for 20 minutes. The supernatant will be harvested and filtered in 0.45 µl, and then 0.1 ml of sample will be inoculated into confluent duck fibroblasts cultured in 24-well plates and maintained in DMEM containing 2.5 µl of serum, daily for 1 week. The cytopathic effect (CPE) will be measured using the properties of NDV on cell lines.

2.9.3. *Enzyme Linked Immuno-Sorent Assay (ELISA)*

This method is used to measure antibody titers and requires taking a blood sample from the chicken. Catching village chickens for this purpose can cause problems. A blood sample is taken from a wing vein. A detailed description of this method can be found in Alders and Spradrow (2001). Roosters are generally more difficult to sample than hens. Blood can be drawn directly into a syringe or into a tube by inserting a needle into a vein. In other cases the sample is placed approximately horizontally to allow clotting and separation of the serum sample which should be straw colored. Serum samples should be kept refrigerated until they can be frozen in the laboratory.

Identification of Newcastle disease virus there is international consensus that the identification of her NDV isolate from birds exhibiting clinical signs is not a definitive diagnosis of ND. Due to the extreme differences in virulence of different her NDV isolates and the widespread use of live vaccines, further confirmatory studies are needed (OIE, 2021a). Distinction of pathogenic from nonpathogenic strains of NDV class II was successfully and rapidly achieved using next-generation sequencing based pyrosequencing methods (De attisti et al., 2013). Cross-reactivity between APMV-1 and APMV-3 or APMV-7 viruses can be detected. A panel of reference sera or monoclonal antibodies (MAs) specific for APMV-1, APMV-3, and APMV-7 can be used, greatly reducing the risk of typographical errors in isolates (ox et al., 1988) ; Alexander, 1990).

2.9.4. Haemagglutination Test

This test is performed to determine whether ND virus is present in the allantoic fluid of eggs inoculated with specimen supernatant. Fifty microliters of allantoic fluid will be removed from each egg and each drop was placed in a separate well of a "V" shaped microtiter plate. Twenty-five microliters of 1% poultry red blood cells will be added to each well of allantoic fluid and mixed gently. This will be kept at room temperature for 45 minutes and the results were read (OIE, 2012).

2.9.5. Haemagglutination Inhiition Test (HI)

The HI test is based on the principle that hemagglutinin on the viral envelope can cause chicken red blood cells to agglutinate, which can be inhibited y specific antibodies. The HI titer is the reciprocal of the highest serum dilution that completely inhibits haemagglutination and is usually most easily expressed as the base logarithm. Although it is difficult to standardize tests across laboratories, HI titers are an indicator of a bird's immune status. A diagnosis of infection can be made on this basis if vaccination has not been performed, but it is not possible to determine exactly when the infection occurred. Serial samples taken at different time points can provide information as to whether titers are rising (indicative of recent infection) or falling (Maas et al., 1998).

2.10. Intracerebral pathogenicity index

Pathogenicity index test used to determine the virulence based on the severity of the clinical signs or death that was showed on experimental chicken's It classifies the isolates as velogenic, mesogenec or lentogenic viruses based on the clinical sign results of ICPI scores (OIE, 2021a).

2.10.1. Intracerebral pathogenicity index test

The virulence of NDV isolates can be assessed y ICPI testing. ICPI tests should be run as follows: Fresh infectious allantoic fluid with an HA titer of ≥ 24 (1/16) was diluted 1/10 with sterile isotonic saline without additives, and 0.05 ml of diluted allantoic fluid was added to SPF. Each 10-day-old chick hatched from the egg is injected intracererally. . Birds are observed twice a day for eight consecutive days. At each examination, birds are scored from 0 to 2 (0 for healthy, 1 for sick, 2 for

dead). The mean value per bird per observation over the 8 days is considered the ICPI. Long-latent strains have values close to 0.0, while the most virulent viruses have indices close to the maximum value of 2.0 (Alexander, 1988).

2.11. Molecular identification

Identification of Newcastle disease virus there is international consensus that the identification of NDV isolates from clinically symptomatic birds is not a definitive diagnosis of ND. Due to the extreme differences in virulence of different His NDV isolates and the widespread use of live vaccines, further confirmatory studies are needed (OIE, 2021a). Distinction of pathogenic from non-pathogenic strains of NDV class II was successfully and rapidly achieved using next-generation sequencing based pyrosequencing methods (De attisti et al., 2013). Cross-reactivity between APMV-1 virus and HE APMV-3 virus, or APMV-7 virus can be detected. A panel of reference sera or monoclonal antibodies (MAAs) specific for APMV-1, APMV-3, and APMV-7 can be used, greatly reducing the risk of typographical errors in isolates (ox et al., 1988); Alexander, 1990).

Classical polymerase chain reaction (PCR)/reverse transcription-polymerase chain reaction (RT-PCR) is usually used. Infection of poultry y highly virulent NDV can be notified to the OIE if the causative APMV-1 strain meets one of the following toxicity criteria:

The virus has an ICPI greater than or equal to 0.7 in postnatal day 1 chickens, or multiple basic amino acids have been detected at the C-terminus of the F2 protein and a phenylalanine at residue 117, the N-terminus of the F1 protein. . The term "basic amino acids" refers to at least three arginine or lysine residues (113 and 116). If the characteristic pattern of amino acid residues described above cannot be detected, the isolated virus should be characterized by her ICPI test. As a result, directly or indirectly. Amino acid sequencing of the F0 protein cleavage site confirms viral virulence, but not its absence (Alexander, 2004). Comparative sequence analysis of the HN and F protein genes from virulent and avirulent NDV isolates revealed that pathogenicity is influenced by the length of the HN protein and the amino acid sequence/composition of the proteolytic cleavage site of the F0 protein precursor encoded y the F gene (Rome et al., 2003). The cleavage of the precursor protein is a prerequisite for viral infection, as virus particles are activated

only if F0 is enzymatically cleaved into functional F1 and F2 peptides, which enables the viral and host cell membranes to fuse.

Based on the data obtained by sequence analysis, NDV strains that are virulent for chickens contain at least three basic residues lysine and arginine between positions 113 and 116 at the C terminus of the F2 protein (cleavage site motif 112R/K-R-Q-R/K-R-F117) and phenylalanine (F) at the N terminus of the F1 protein (residue 117). As a result, the precursor protein of virulent isolates is more susceptible to cleavage by the ubiquitous proteases present in different tissues of the host (Collins *et al.*, 1993). Strains of low virulence have fewer basic amino acid residues at the C terminus of the F2 protein and leucine position 117 (cleavage site motif 112G/E-K/R-QG/E-R-L117), and trypsin-like proteases are required to cleave their precursor. In conclusion, NDV toxicity is highly dependent on cleavage of the fusion glycoprotein precursor (F0) by host cell proteases (Aldous *et al.*, 2001). Another important conclusion from sequence analysis is that mesogenic and velogenic viruses cannot be distinguished by the nucleotide sequence of the F0 proteolytic cleavage site, and the same holds true for asymptomatic enteric and latent viruses (Seal *et al.*, 1995).

2.12. Economic impact of Newcastle disease

Protein is an essential part of a balanced human diet. The two main sources of protein are animals and plants. In developing countries, the human diet lacks animal protein. About 66% of the population follows a low-protein diet (Maqool, 2002). Only 102.7g of protein per person per day. A person consumes 69.61g of protein per day. The main animal protein sources are mutton, beef, chicken, eggs and milk (Maqool and akhsh, 2007). White meat contains the same essential nutrients as red meat, but white meat has the advantage of being lower in cholesterol and saturated fat. In most developing countries, meat is a very important source of protein in people's diets, as it is an affordable and high-quality protein source (Thomazelli *et al.*, 2012).

In developing countries, chicken is the cheapest source of animal protein. Egg availability is increasing by approximately 4% each year (Numan *et al.*, 2005). Poultry production began as a cottage industry in many developing countries of the world. Meat production from farm and poultry accounts for 25.8%. ND and avian

influenza are major problems in animal husbandry due to dangerous infections (Ge *et al.*, 2012).

In several developing countries, ND is endemic, with the greatest impact on villages whose livelihoods depend on poultry farming (Mohamed *et al.*, 2011). The disease costs millions of dollars annually in developing countries (Waheed *et al.*, 2013). The situation is similar in Ethiopia. The total number of chickens in the country is estimated at about 57.01 million (CSA, 2021), but the benefits of poultry farming in this country are far from its existing potential. This is due to inadequate feed supplies, inadequate production systems, high prevalence of diseases, scarcity of animal genetic resources and poor marketing (Alemu, 2010). Among the diseases affecting poultry, Newcastle disease is one of the poultry diseases causing heavy losses in Ethiopia. The disease has a local name, 'Fengel', which literally means 'collapse' (Ashanafi, 2000).

2.13. Prevention and control measures

Vaccines are used to control and prevent ND. Many inactivated and live ND vaccines, including recombinant vector vaccines, are now available worldwide (Shim *et al.*, 2011; Xiao *et al.*, 2013). Chickens and turkeys are vaccinated against Newcastle disease. Live-attenuated NDV vaccines are administered by different routes and according to different hatch-to-growth schedule (Cho *et al.*, 2008). Killed virus oil emulsion vaccines are administered parentally before egg laying begins. Appropriate vaccination protects birds from clinical disease but does not prevent viruses. Good biosecurity measures are also important to prevent Newcastle disease in poultry flocks. Commercial flocks must not come into contact with poultry, wild birds or ornamental birds. Workers should avoid contact with birds outside the facility. Biosecurity measures include bird-proof housing, food and water supplies, minimizing access to facilities, and disinfecting vehicles and equipment entering the farm. Pests such as insects and rodents should also be controlled. If possible, employees should shower and wear appropriate clothing before entering the poultry farm.

2.14. Vaccination

2.14.1. *Inactivated Vaccines*

Inactivated vaccines are made by growing the ND virus in eggs and then treating the infectious allantoic fluid with an inactivating agent such as formalin or eta-propiolactone. An adjuvant such as mineral oil is then usually added to make the inactivated virus more immunogenic. Because the vaccine cannot multiply or spread, it must be injected individually into each bird that needs vaccination. It is usually injected behind the thigh muscle, but sometimes the pectoral muscle is also used, 0.3 or 0.5 ml per bird. Inactivated vaccines generate very high levels of antibodies against NDV and provide excellent protection against virulent viruses (ell *et al.*, 1990).

2.14.2. *Live Vaccines*

Live vaccines differ from inactivated vaccines in that they can replicate within the host. These have both advantages and disadvantages. This has the advantage of not having to vaccinate every bird individually. Vaccine viruses can be transmitted from one bird to another. However, this has the disadvantage that it is an infection with a live virus and clinical manifestations may occur due to the inherent toxicity of the vaccine virus or the exacerbation of other microorganisms that may be present, particularly in the respiratory tract (Westury *et al.*, 1984), and the presence or absence of co-infection with other pathogens. Conventional long-latency vaccines (Table 1).

Table 1: Strains of Newcastle disease virus used in live vaccines

Strain type	Strain Description
F	Lentogenic. Usually used in young chickens ut suitable for use as a vaccine in chickens of all ages.
1	Lentogenic. Slightly more virulent than F, used as a vaccine in chickens of all ages
LaSota	Lentogenic. Often causes post vaccination respiratory signs, used as a ooster vaccine in flocks vaccinated with F or 1
V4	Avirulent. Used in chickens of all ages
V4-HR	Avirulent. Heat Resistant V4, thermostable, used in chickens of all ages
I-2	Avirulent. Thermostable, used in chickens of all ages
Mukteswar	Mesogenic. An invasive strain, used as a ooster vaccine. Can cause adverse reactions (respiratory distress, loss of weight or drop in egg production and even death) if used in partially immune chickens. Usually administered y injection.

2.14.3. Recombinant vaccine

Vaccine used biotechnology; recombinant DNA technology has resulted in the development of novel NDV vaccines. Recombinant NDV using viral vectors such as turkey herpes virus or fowl pox viruses in which the HN or F-genes or other expressed have recently developed hence currently some countries applied practically this biotechnological vaccines in use for poultry vaccination (OIE, 2021). One class consists of vector vaccines, which have a suitable carrier virus that express one or more immunogenic NDV proteins (usually F and/or HN), thereby inducing an immune response against both NDV and the vector virus itself.

Other approaches the development of subunit vaccines used on the large scale expression of NDV proteins (usually F and/or HN) and also the use of DNA vaccines, i.e. plasmid DNA encoding relevant immunogenic NDV proteins (Loke et al., 2005). The desired profile for NDV vaccines include:

- 1) prevention of transmission;
- 2) differentiation of infected from vaccinated animals (DIVA);
- 3) induction of protection with a single dose;
- 4) maternal

antibody override; 5) mass vaccination; 6) cross-protection against variant strains, 7) Increased safety and minimal side effects. Some of the recombinant vaccines described above match or exceed the efficacy of conventional vaccines in protecting against antibody-induced or virulent challenge strains and are therefore promising for future use. In addition, it has a number of advantages compared to the conventional His NDV live vaccine, including: . i) improved safety of vaccinated birds due to lack of residual virulence, ii) implementation of DIVA principles, iii) closer immunogenicity correspondence with outbreak strains, iv) optimally controlled Potential for mass vaccination in ovo, v) sustained immunity from a single vaccination over time (Palya *et al.*, 2014)

2.15. Current Status of ND Vaccines in Ethiopia

The National Veterinary Institute (NVI) is one of his centers of excellence in ND vaccine production in Ethiopia and is the only laboratory responsible for the production of over 20 livestock disease vaccines. Therefore, the NDV vaccine is one of center-produced, killed and live ND vaccines (Table 2). Vaccines are available, but only intensive poultry farms vaccinate poultry on a regular basis, whereas poultry in large-scale production systems are not routinely vaccinated due to lack of knowledge and are traditionally vaccinated of heat-labile vaccines are difficult to transport and store at ambient temperatures of 24°C and 36°C (Alders and Spradrow, 2002).

Table 2: ND vaccine produced at NVI with their recommended application program

Type of vaccine	Day of vaccination	Rout of administration
H1	Day 7	Intraocular
Lasota (thermostable)	Day 21	Intraocular
Lasota (thermostable)	Day 42	y drinking water
Inactivated	At point of egg lay	Intraocular

3. MATERIALS AND METHODS

3.1. Study Area Description

The study was conducted in Bishoftu town and laboratory assays were conducted in research and development laboratory at National Veterinary Institute (NVI), Ethiopia. Bishoftu is located in Oromia Regional state, East Shewa zone, at 47 km south-east of Addis Ababa, the capital of Ethiopia. It is surrounded by lakes, lies at an altitude about 1920 meters above sea level and 8°45'N and 38°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 (CSA, 2007).

3.2. Study Animals and Design

In this study for ND virus isolation chickens of all age group, without sex and reed limit suspected of Newcastle disease were used as a study animal. Isolation and detection of NDV from suspected outbreak cases was conducted using a cross-sectional study design during the study period from October 2022 to May 2023. Pursuant to the ND outbreak cases that were reported to NVI, a field investigation was carried out in the outbreak area, clinical data was recorded, and adequate samples were collected from chickens exhibiting symptoms indicative of ND infection. Vaccine efficacy and immunogenicity experiment was conducted in chickens hatched from SPF eggs. An experimental design was used and all laboratory assays were performed in a laboratory safety cabinet level II.

3.3. Sample collection

Organ-specific representative tissue samples were collected from active ND cases. A total of 57 ND suspected samples (42 stored samples at NVI virology laboratory and 15 active cases) were collected for isolation and molecular characterization. Following necropsy, affected tissues rain, lung, trachea, liver, spleen, kidney, and intestine were sampled. Sterile phosphate-buffered saline (PS) medium supplemented with antimicrobials (penicillin 10,000 units, streptomycin sulfate 10 mg, and amphotericin 25 µg per ml) adjusted at pH 7.2 ± 0.2 was used to transport samples. Collected samples were transported to the laboratory in an icebox and kept

there at a temperature of -80°C for further assay. Specific pathogen free eggs and DF1 cell cultures were used for isolation of the virus. HA positive samples both from inoculated DF1 cell culture and inoculated embryo were selected to hemagglutination inhibition test (HIT) to check presence of NDV in the harvest and pooled DF1 cell culture samples. Finally, positive samples with HIT were further characterized using molecular techniques of one step Reverse Transcription-Polymerase Chain Reaction (RT-PCR) to detect the viral RNA. QIAGEN One-step RT-PCR Kit (25) was used.

3.4. Laboratory Investigation

3.4.1. Egg Inoculation

Five embryonated eggs were inoculated with each sample up to four passages in order to test for the presence of the NDV in a total of 57 suspected samples. Five embryonated eggs were kept as a control for each inoculation. The laboratory protocol for viral isolation involved candle-testing incubated eggs at day 10 of incubation; dead or infertile embryos were excluded, and eggs with viable embryos were selected and marked at the air space side for injection. Identified and marked eggs were pierced by a sterile egg driller after being cleaned with 70% ethanol. The filtered supernatant of tissue homogenate, or swap a volume of 0.2 ml was inoculated through the allantoic cavity route for each sample (five 10 days old embryonated SPF chicken eggs) Hanson and Grandly (1955).

Melted paraffin wax was used to seal the hole of the inoculated eggs. Infected eggs were incubated for four days at 37°C and 65% humidity level in a humidified egg incubator environment while being candled twice daily to monitor the effects of the inoculated virus.

Those embryos dead within 24 hours of incubation were discarded (rejected) which were believed mechanically injured during inoculation and the remaining followed for virus replication under controlled temperature and humidity. Embryos deceased after 24 hours of incubation were considered as due to virus replication and chilled at +4°C overnight for allantoic fluid collection (OIE, 2021).

3.4.2. *Infected embryo allantoic fluid harvesting*

The allantoic fluid was harvested from infected embryo after eggs were disinfected with 70% ethyl alcohol. Briefly, the top part of the egg shells were decapped by sterile thumb forceps and allantoic fluid was harvested by using sterile pipette, spatula and the fluid was transferred into sterile universal bottles. The collected allantoic fluid was checked for purity and subjected for further assay (OIE, 2021).

3.4.3. *Haemagglutination Test*

This test was conducted to confirm the presence of ND virus in the allantoic fluid of eggs and chicken fibroblast culture cell inoculated with supernatant fluid which got from samples. Fowl RC was collected in 1:1 ratio with Alsever's solution, centrifuged at 3000 x g for 10 min, washed with PS (pH 7.2 ± 0.2) and centrifuged at 3000 x g for 10 min, three times to prepare 10% fowl RC. Finally, 1% RC was prepared in 1:9 ratios with sterile PS. 25 µl of allantoic fluid from each egg were taken and dropped in a separate well of "V" shaped microtiter plate. 25 µl of 1% fowl RC was added to each well with allantoic fluid and mixed gently and checked for agglutination (OIE, 2021). These allow proving the presence of any hemagglutinating agents in the harvested allantoic fluid or CPE positive DF1 cell culture pooled samples. Those tests positive for HA were further confirmed by HI assay.

3.4.4. *Haemagglutination Inhibition Test (HI)*

The HI test is based on the standard principle that the haemagglutinin on the viral envelope can ring about the agglutination of chicken red blood cells and this can be inhibited by specific antibodies. The HI titer is the reciprocal of the highest dilution of serum which completely inhibits haemagglutination and most conveniently expressed as the logarithm to the base. Briefly, the collected allantoic fluid sample that were tested positive by HA assay was subjected to HI test to detect specific anti sera against NDV.

Serum samples were inactivated at 58°C in water bath and two fold serial dilution of NCTH vaccine titer were prepared up to dilution 12 well in micro titration plate and the HA titer of the virus (2^8). From the result, 8 haemagglutination units (HAU) were prepared for the HI of the test. 25 µl PS were added into 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 conducted. 25 µl of 8 HAU antigen/ NCTH vaccine was added per each well and incubated at room temperature for 15 minutes. Finally, 50 µl of 1% chicken RC was added into each well and incubated at room temperature for 30 minutes. The agglutination was judged by tilting the plates. The wells RCs stream at the same rate as the control wells (positive serum, virus and PS controls) were considered to show inhibition. The cut off value $\geq \text{Log}2^3$ were used as a standard positive (OIE, 2021).

3.4.5. Embryo Mean death time determination

Embryo mean death time (MDT) was determined in both the allantoic fluids and infected DF1 cell line pooled samples. Subsequently, 10 days old SPF embryonated chicken eggs were disinfected with 70% ethyl alcohol and tenfold serial dilutions of the infected allantoic fluid and DF1 cell pooled samples of the virus isolates were prepared. The eggs were pierced by sterilized egg driller and 0.1 ml of the dilutions between 10^{-5} and 10^{-9} was inoculated into 5 eggs per dilution into the allantoic cavity as described by Hanson and Brandly (1955) and the holes were sealed with melted paraffin wax and incubated at 37°C in humidified egg incubator. The highest dilution, 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 virulence lentogenic, moderately virulent or mesogenic, and highly virulent or velogenic pathotypes, based on chicken embryo mortality at > 90 hours, 60-90 hours, or less than 60 hours, respectively Hanson and Brandly (1955).

3.4.6. Intracerebral pathogenicity index test

The intracerebral pathogenicity index (ICPI) assay was conducted for a total of five selected allantoic fluid samples propagated into four passages (Blind). Fluid samples were selected based on HA titer ($> 2^4$) and RT-PCR test positive samples were used to determine the virulence *in-vivo* during challenge of the experimental day old chickens. 60 chickens were randomly assigned into 6 experimental groups each having ten chickens. Experiment group six was left un-inoculated, while experiment groups 1 to 5 were inoculated with the isolated viruses. The test was done using the selected allantoic fluid samples by inoculating in the rain of day old chickens. The experiment was done according to the OIE protocol (OIE, 2021). Fresh infective pooled sample with an HA titer ($> 2^4$) was diluted 1:10 in sterile isotonic saline

without antibiotics. Chickens were inoculated with 0.05 ml virus into the nares of ten day-old SPF chickens (age > 24 and < 40 hours) using sterile graduated insulin syringe (gauge 30G x 8mm). Chickens were fed and watered ad libitum. Chickens were examined once daily 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 8-days period. The most virulent viruses give ICPI values approaching the maximum score of 2.0, while lentogenic viruses were give a values approach to zero value OIE (2021).

3.4.7. Molecular identification

RNA virus extraction was conducted from infected DF1 cell culture and allantoic fluid confirmed positive for HA and HI tests. Accordingly, extraction was performed as described by the manufacturer (QIAGEN, Germany) RNeasy Mini Kit. Pooled infected Cell culture and allantoic fluids samples were thawed and vortexed. 350 µl of the homogenized samples were transferred to Eppendorf tubes and 350 µl of RLT buffer was added and homogenized by gentle pipetting and 350 µl of 70% ethanol was added to the homogenized lysate and mixed by pipetting 1050 µl of the sample was transferred to RNeasy spin column, centrifuge for 30 sec at 13000 rpm for 30 seconds, and the flow through liquid were discarded. Add 700 µl buffer RW1 to the RNeasy spin column and centrifuge for 30 sec at 13000 rpm to wash the spin column membrane and discard the flow-through liquid. Then, RPE buffer (500 µl) was added to RNeasy spin column centrifuged for 30 sec at 13,000 rpm and discarded the flow-through liquid. Second round RPE buffer (500 µl) was added to RNeasy spin column and centrifuged for 2min at 14,000 rpm and discarded the flow-through liquid and place RNeasy spin column in a new 2ml collection tube. Then, RNeasy spin column was placed in a new 2 ml collection tube and centrifuged for 1 min at 14,000 rpm and discarded the flow-through liquid. Finally, the spin column was placed in a new 1.5ml Eppendorf tubes and 40 µl elution buffer was added onto the membrane of the spin column, centrifuged for 1 min at 14,000 rpm and collected RNA at the bottom of the Eppendorf tubes (RNeasy Mini Handbook, 2019).

3.4.8. *Master Mix preparation*

The reaction mix was prepared for selected allantoic fluids and CPE positive infected DF1 cell culture at passage three and four, respectively. The following sequence primers were used for amplification of the Matrix gene. An amplicon size around 1100p primers forward (5 pm/μl) 5'- AGTGATGTGCTCGGACCTTC - 3' a volume of 2μl and Reverse primer(5 pm/μl) 5'- CCTGAGGAGAGGCATTTGCTA - 3' a volume of 2 μl matrix gene were used. (5μl), 5X RT-PCR buffer (5 μl), Q solution (5 μl), 10mM dNTPs mix (1μl), one step RT-PCR mix (1μl) and Template RNA extracts (5μl) were mixed for one reaction volume of 26 μl (RNeasy Mini Handbook, 2019).

3.4.9. *Polymerase chain reaction*

One step reverse transcriptase polymerase chain reaction (RT-PCR) was conducted by synthesis of cDNA. A reaction temperature of 50°C for 30 minutes for one cycle, initial denaturation at 95°C for 15 minutes for one cycle then primary denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, and elongation at 72°C for 1min . A total of 35 cycles, final elongation at 72°C for 10 minutes for was performed for the amplification of the M-gene of APMV-1 using thermal cycler (Applied ecosystems 2720 Thermal Cycler) and QIAGEN One-step RT-PCR Kit (25) Cat. No. / ID: 210210 were used.

3.4.10. *Gel electrophoresis*

A 1.5 % agarose gel was prepared, and 4 μl gel red with loading dye was added. 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-Acetate-EDTA (TAE) buffer in agarose gel electrophoresis System (IO - RAD - USA, Thermo EC - 2060) for one hour and 20 minutes at 120V electric current supply. A DNA ladder started with 100-p increments was used as a molecular weight marker. Afterwards, gels were visualized under 27 UV light using UV trans-illuminator (Uvi-tec UK, Cambridge C4 - 1Q - UK) and photographed y a digital camera (Uvitec, UK).

3.5. Animal management and vaccine efficacy test

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 80 chickens were assigned randomly into four experimental groups each having twenty chickens. Experiment group 1, 2, and 3 were vaccinated and group 4 was left as control. Chickens were vaccinated via intra-ocular route of administration. Vaccine immunization protocol was adopted from NVI vaccine schedule catalogue (Table 2).

Three types of live attenuated Newcastle disease vaccines product of NVI produced from the lentogenic strains (Lasota, H1, and Thermostale) were evaluated for their protective immunogenicity. A total of 80 day old chickens were used for vaccine efficacy test except for the control group all groups were vaccinated at day 0, first boost 14, and second boost day 35 with normal dose. Serum was collected at day 0 (before vaccination) and day 14, 35, and 42 (post vaccination) from each group. Immunogenicity progresses were evaluated using HI test and finally both vaccinated and unvaccinated groups were challenged by injection of active NDV (the circulating isolated virus). Onwards mortality and morbidity of unvaccinated but challenged chickens were thoroughly followed for clinical signs and finally postmortem examination and molecular detection of the agent was assessed.

Table 2: Type of vaccine and time of vaccination

Experimental Groups	No. of chickens	Priming vaccine (Day 0)	first booster (Day 14)	second booster (Day 35)	Administration route
Group 1	20	NCH1	NCTH	NCL	Intraocular
Group 2	20	NCH1	NCTH	NCTH	Intraocular
Group 3	20	NCTH	NCL	NCL	Intraocular
Group 4	20	-	-	-	-

3.6. NCL: Newcastle lasota vaccine, NCH1: Newcastle Hichener 1 vaccine, NCTH: Newcastle thermostale vaccine for each group given dose $10^{6.5}$ EID₅₀.

3.6. Blood Collection

Blood was collected from all experimental chicken using sterile disposable syringe gauge 22Gx1¼ (3ml). Each chickens were properly handled, the site of blood collection disinfected with 70% ethyl alcohol and blood was collected from the wing vein. A volume of 1-2 ml blood collected from chicken, the syringes were laid 45° in horizontal position 3-4 hour at room temperature. Serum was harvested in sterile cryovial and stored at -20°C for further analysis (Kelly, L. and Alworth, L, 2013).

3.7. Experimental animals challenge

Two weeks after the last booster dose vaccination, both vaccinated and control group chickens were challenged with 0.2 ml of $10^{10.6}$ ELD₅₀ y inoculating through the oculonasal route administration of the locally circulating isolated NDV. based on the definition adopted y OIE, (2021) for incubation period of ND the chickens were followed for 21 days post challenge (dpc) y supplying feed and clean water adlibitum. Clinical signs, was recorded and samples for postmortem examination were collected from chickens showing clinical signs from non-vaccinated or control groups. Laboratory confirmation was 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 was determined y absence of clinical signs or absence of death within 21 dpc.

3.8. Data management and analysis

Obtained data from all experimental activities were recorded in excel spread sheet. Statistical data analysis was conducted by using STATA software/IC version15.0 (2017). The mean HI titer and standard deviation of the groups was compared y Generalized Linear Model with 95% confidence interval and 5% alpha value. ANOVA were used to compare means between and within experimental groups ‘differences of HI titer and lentogenic vaccines used. Statistical significant was considered at P -value < 0.05 difference between test groups.

4. RESULTS

In this study a total of 57 samples were analyzed. The findings of SPF embryo inoculation and DF1 cell line infection of allantoic fluid revealed in figure 2 that 29 (50.88%) samples were positive for HA and HI assay and 28 (49.12%) samples were found negative for NDV. Correspondingly, DF1 cell line showed CPE in 18 (31.58%) and confirmed positive with HA and HI test and 39 (68.42%) were found negative.

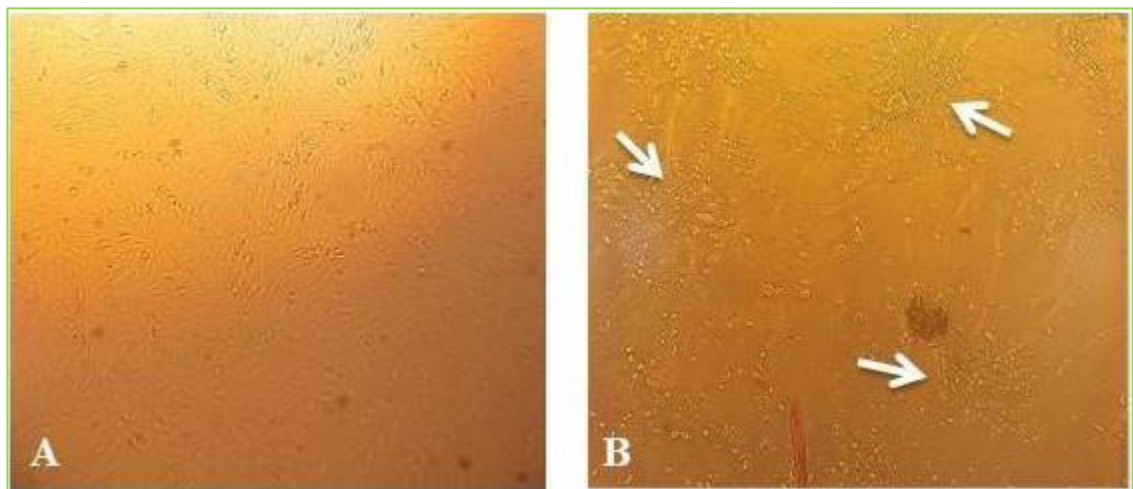


Figure : NCD virus isolation DF1 cell line where, (A) Normal cell culture (B) anormal cell (CPE) on inoculated cell culture, arrow showing aggregation and disruption of the monolayer).

4.1. Embryo means death time determination

Allantoic fluid sample with HA titer $> 2^7$ and infected DF1 cell with CPE higher than 60% during passage four were used for embryo mean death time determination. The experiment was conducted using 60 embryonated SPF eggs. The result in **tale 3** indicates that 21 (84%) allantoic fluid inoculated embryos were died and 5 (20%) DF1 cell inoculated embryos were died within 48 hours. The mean death time findings showed that the isolated virus was very virulent. Besides, these result revealed that Newcastle disease virus replication was by far better in embryonated

chicken eggs than DF1 cell.

Tale 3: Embryo mean death time from allantoic fluid and infected DF1cell culture samples.

Dilution	After 24 hrs		After 48 hrs		After 72 hrs		After 96 hrs	
	Allantoic fluid	DF1 Cell	Allantoic fluid	DF1 cell	Allantoic fluid	DF1 Cell	Allantoic Fluid	DF1 Cell
	10 ⁻⁵	0	0	5	2	0	2	0
10 ⁻⁶	0	0	5	2	0	1	0	2
10 ⁻⁷	0	0	4	1	3	2	0	2
10 ⁻⁸	0	0	5	0	0	2	0	3
10 ⁻⁹	0	0	2	0	0	0	1	5

For allantoic fluid samples 25 SPF embryos were used, for DF1 cell pooled sample 25 SPF embryo were used and for oth control 10 SPF embryos were used

4.2. Pathogenicity index

ICPI test was conducted in a total of 60 SPF chickens (age 33 hours). Chickens were assigned randomly into six groups of 10 chickens. Pooled allantoic fluid samples (passage four) with HA titer ($> 2^4$) were used for pathogenicity assay. Samples were revealed an ICPI score ranging from 1.6125 to 1.875. These findings showed that NDV isolates for challenge were found to be velogenic.

Table 4: Experimental allantoic fluid samples (NDV isolates) and its corresponding ICPI score

Sample code	Original Sample source	ICPI score
AC-8	Rain	1.65
SS-4	Spleen	1.6125
AC-10	Rain	1.875
SS -16	Cloacal swap	1.7625
SS-22	Rain	1.662

SS=Stored sample, AC=Active case, ICPI= Intracerral pathogenecity index

4.3. Vaccine efficacy and immunogenicity evaluation

The maternal antibody (HI titer) in serum of unvaccinated (control groups) were gradually decreased from day 0 to day 42. While, vaccinated chickens antibody level showed a rise during the experiment period. In the first group (NCH1-NCTH- NCL) vaccination-boost schedule, the group HI geometric mean titer (GMT) \pm standard deviation (SD) and *p* value were 197.88, 305.86, and 0.000, respectively. While, in the second group the HI GMT \pm SD and *p*-value for NCTH-NCL-NCL schedule of priming-boost vaccination revealed 207.52, 320.87, and 0.000, respectively. Whereas, in the third group (NCH1-NCTH-NCTH) priming-boost- vaccination schedule revealed that the group HI GMT \pm SD and *p*-value were 138.24, 236.20, and 0.000, respectively (Tale 5 and Figure 3). The immune response for groups 1, 2 and 3 at day 14th, 35th and 42nd were gradually increased and these findings showed that the mean HI titer \pm SD was higher than \log_2^3 which was found to be protective.

Table 5: HI titer group mean, standard deviation and p-value

HI titer			
NCH1- NCTH – NCL	Mean	SD	<i>P-value</i>
At day 0	6.2	2.04	
At day 14	23.2	8.17	
At day 35	268.8	174.47	
At day 42	691.2	288.92	
Group HI titer	197.88	305.86	0.000
NCTH-NCL-NCL			
At day 0	5.6	2.01	
At day 14	46.4	25.90	
At day 35	249.6	167.28	
At day 42	736.0	310.43	
Group HI titer	207.52	320.87	0.000
NCH1-NCTH-NCTH			
At day 0	5.6	2.01	
At day 14	36.0	17.89	
At day 35	131.2	70.34	
At day 42	518.4	289.14	
Group HI titer	138.24	236.20	0.000
Control group			
At day 0	5.8	2.04	
At day 14	2.9	1.02	
At day 35	0	0	
At day 42	0	0	
Group HI titer	2.18	2.67	0.000

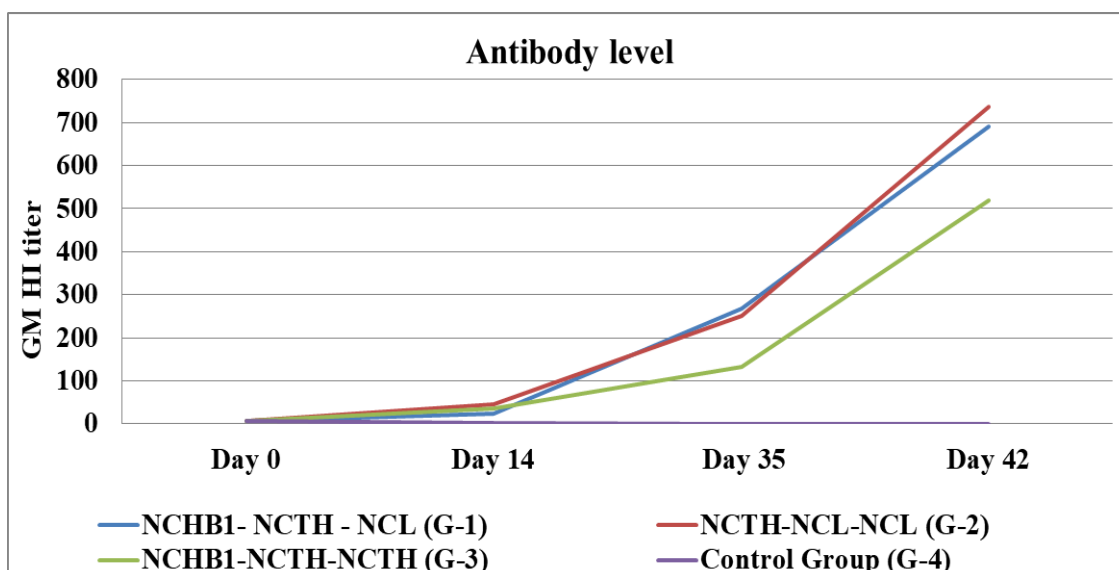


Figure : Geometric mean HI titer of vaccinated and control group chickens (G-1: group one; G-2: group two; G-3: group three; and G-4: group four).

4.4. Experimental animal challenge

Based on the ICPI score the challenge strain of NDV was identified. ICPI score of 1.875 (titer $10^{10.66}$ ELD₅₀/ml) was used as a challenge strain. It was also confirmed that clinical sign or deviation was not observed in each experimental group before challenge. The findings revealed that, clinical signs were not observed in all vaccinated and challenged groups till 21 days post challenge, while depression, loss of appetite, nervous signs, torticollis, and dizziness was observed in control group at the 3rd day post challenge (dpc) and death was observed in 9 (45%), 7 (35%) and 4 (20%) chicken at the 4th, 5th and 6th dpc, respectively (Figure 4). These findings showed that control group chickens were died (100%) after challenge while vaccinated groups were remained protected during the study period.



Figure : Control groups showing clinical sign of torticollis

4.5. Post Mortem diagnosis on experimental groups

The post mortem examination of unvaccinated-challenged chickens revealed some pathological lesions. Thus, enlarged spleen, enlarged liver, petechial cutaneous hemorrhages of the head, hemorrhage of pharynx, proventriculus, trachea, intestine and cloaca, pinpoint hemorrhage at proventriculus and intestine including abnormal morphology and color change in visceral organs were observed (Figures 5-7). Samples from these tissues were processed and confirmed for presence of NDV using RT-PCR targeting M-gene (~1100 p) for APMV-1.

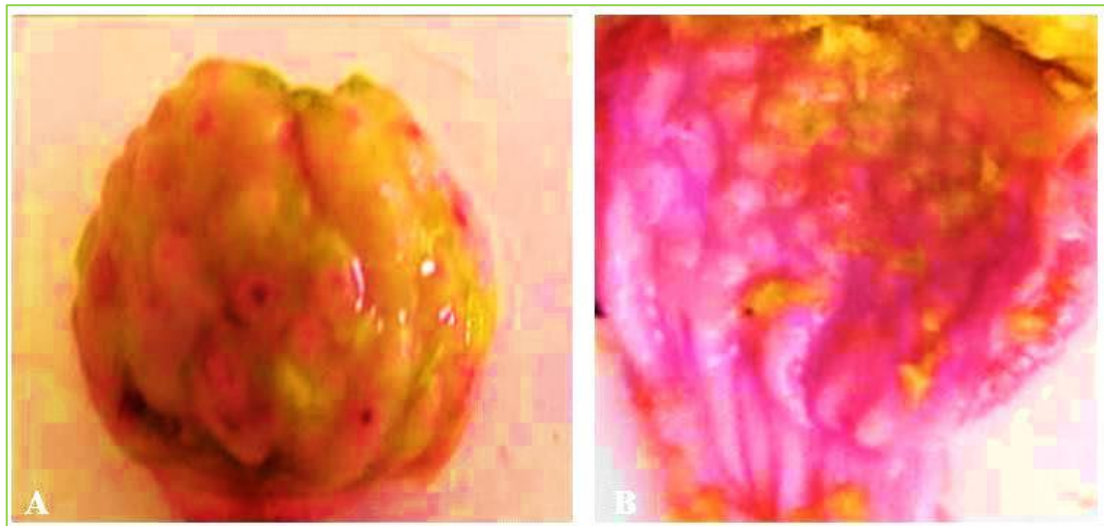


Figure : Hemorrhage of proventriculus (A) and Normal (B)



Figure : Enlarged Spleen (A) and Normal (B)



Figure : Enlarged liver (A) and Normal (B)

4.6. Polymerase Chain Reaction

Figure 8 illustrated that one step RT-PCR of 11 NDV suspected sample were positive around 1100 p for APMV-1 targeting primer designed to amplify the M-gene of NDV. Gel picture of NDV suspected samples from allantoic fluid and DF1 cell infected culture (passage four) and postmortem samples from unvaccinated-challenged chickens.

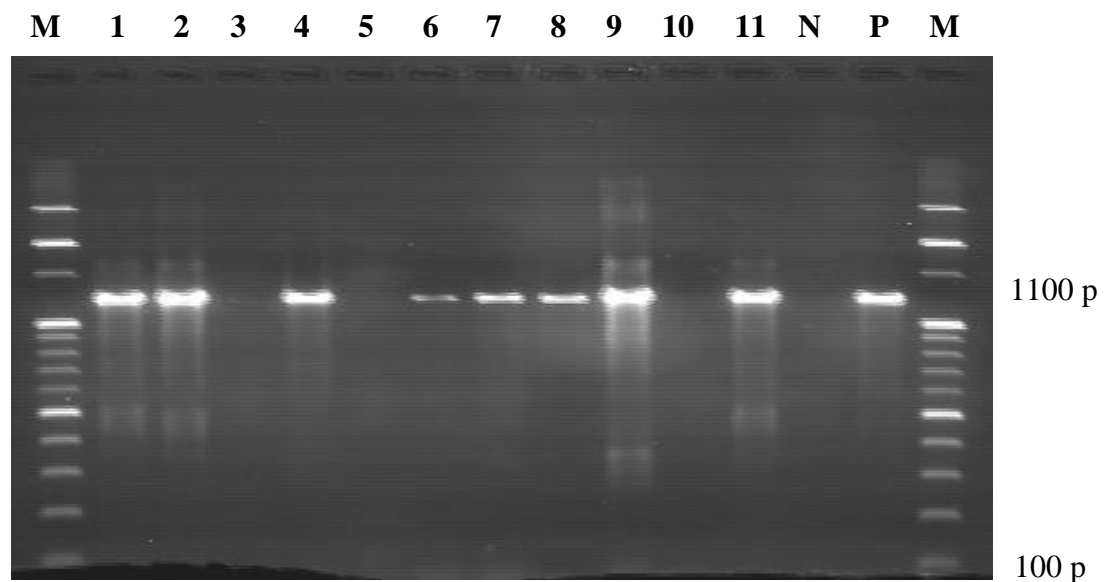


Figure : Agarose gel electrophoresis of PCR product (~1100 p) of NDV suspected samples.

Key: Lane M: Molecular marker (100plus); lane 1, 2, 4, 6, 7, 8, 9, and 11: positive samples; lane 3, 5, and 10: negative samples; lane N: negative control; lane P: positive control (Tale 6).

Tale 6: Description of sample type and gel electrophoresis result

Lane	Sample type	Result
M	Molecular marker 100+	
1	Allantoic fluid sample	Positive
2	Allantoic fluid sample	Positive
3	Intestine sample	Negative
4	Liver sample	Positive
5	Df1 infected cell sample	Negative
6	Df1 infected cell sample	Weak positive
7	Cloacal swab	Positive
9	Trachea sample	Positive
10	Intestine sample	Negative
11	Df1 infected cell culture sample	Positive
N	Negative control	
P	Positive control	-

5. DISCUSSION

Newcastle disease is the most economically important endemic viral disease of chickens and a potential threat to village chickens and commercial poultry farms in Ethiopia (Chaka *et al.*, 2012; Mesfin and bihonegn, 2018). Although there are different strategic measures implemented to reduce the impact of this disease, it is still endemic and a priority disease in the country. Besides, ND remains the first and most common infectious disease with prevalence rates of 80% and 43% in commercial poultry farms and rural village chicken, respectively (Mesfin and bihonegn, 2018). This has been proved by the current study that NDV was characterized from suspected ND cases and detected molecularly.

Laboratory investigation revealed, NDV was isolated from 29 (50.88%) allantoic fluid harvest and 18 (31.58%) from DF1 cell culture. This finding was in agreement with previous studies of Mesfin and bihonegn (2018), who reported prevalence of 80% in commercial farms. Embryo MDT evaluation of the designated allantoic fluid with HA titer 2^7 and infected DF1 cell with CPE higher than 60% at passage four revealed 21 (84%) and 5 (20%) death within 48 hours, respectively. Besides, the MDT findings showed that the circulating virus was confirmed to be very virulent based on the standard by Hanson and Brandly (1955), embryo MDT within 60 hours considered as highly virulent pathotypes, while MDT 60 – 90 hours are categorized under mesogenic and MDT higher than 90 hours grouped as lentogenic viruses. The mean death time result showed that the isolated virus was very virulent and consequently the finding implied that NDV replication was considered to be better in embryonated chicken eggs than DF1 cell inoculation. This finding was aligned with the findings of Yinfeng *et al* (2016) who reported chickens and ducks are major hosts of NDV with distinct responses to infection.

Furthermore, the ICPI score revealed a value ranging from 1.6125 to 1.875 implied that the NDV isolate was categorized under velogenic pathotypes. This finding is in accordance with previous reports (Chaka *et al.*, 2013; Fentie *et al.*, 2013) and it was proved that the circulating virus was virulent NDV in the study area.

The maternal antibody HI titer in unvaccinated (control groups) progressively decreased during the experiment period. Whereas, vaccinated chickens antibody

level exhibited an increment. It was observed that higher geometric mean (GM) HI titer in the second experiment (NCTH-NCL-NCL) priming-boost vaccination schedule compared to the first experiment (NCH1-NCTH-NCL) vaccination- boost and the third experiment (NCH1-NCTH-NCTH) priming-boost vaccination schedule. Thus, in all groups the immune response at day 14th, 35th and 42nd were gradually increased and these findings showed that the GM HI titer \pm SD was higher than \log_2^3 which was found to be immunogenic to induce protective immune response. Furthermore, this study showed that the GM HI titer post second vaccination increased significantly ($P < 0.05$) in all vaccinated groups. The current findings implied that there was significance difference in HI titer due to booster vaccination. The findings are in accordance with earlier reports signifying booster vaccination enhance the immune response (Banu *et al.*, 2009).

In this study, it was detected that GM HI titer was peaked post-second-boost and revealed a significance difference with P -value (< 0.05). Hence, secondary boost produces higher antibody HI titer than the first boost. The result is in agreement with previous reports of Shuai *et al* (2003). In methodical comparisons of the GM HI titer \pm SD of all vaccinated groups were showed that chickens were acquired a protective level of HI titer against NDV. However, the maternal antibody (HI titer) of unvaccinated (control groups) were reduced from day 0 to day 35 and become insignificant to the threshold level at the end of the experiment period. These findings are in line with the reports of Banu *et al* (2009) and Geletu (2018) who described the detection of maternal antibody until 28th day.

In general, vaccinated and challenged chickens based on the NDV challenge strain ICPI score (1.875, titer $10^{10.66}$ ELD50/ml). It was evidenced that clinical signs or deviation due to challenge were not observed in all groups till 21 days post challenge. However, clinical signs of depression, loss of appetite, nervous signs, torticollis, and dizziness were observed in control group within three days of post challenge. Besides, death was observed in 9 (45%), 7 (35%) and 4 (20%) chicken at the 4th, 5th and 6th dpc, respectively. These findings showed that control group chickens were died (100%) after challenge while vaccinated groups were remained protected during the study period. Thus it can be concluded that the current ND vaccine is efficient to protect vaccinated chickens against the circulating NDV in the study area. Yet, it is important to consider vaccine effectiveness through considering

methods of vaccination, vaccine transportation and cold chain management, and route of administration. Further, future researches have to address the countries biosecurity and biosafety concern in this sector.

6. CONCLUSION AND RECOMMENDATIONS

In this study, it was identified that there is velogenic strains of NDV in the study area as it was confirmed by ICPI test. The current ND vaccine produced at NVI was efficient and determined to be protective against the circulating virus in the study area. The current recommended dose of $10^{6.5}$ EID₅₀ per chicken was effective to induce protective antibody level. Thus, priming-boost vaccination schedule allows chicken to raise their antibody level. The vaccination and challenge experiment after the secondboost evidenced that the vaccine protects chicken from infection.

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

- Sustainable surveillance of ND outbreak and molecular analysis relating on epidemiology of NDV, using gene sequence to understand the evolutionary relationship of the circulating NDV at national level.
- Vaccine effectiveness and evaluation of biosecurity measures should be addressed to overcome vaccine failure complaints from customers.
- Training and awareness creation about the disease and vaccine management (vaccine storage and transportation system, time and route of administration and lack of vaccination based on disease outbreak conformation) should be given to all stockholders' of the sector along the value chain.
- Employing modern technologies such as recombinant DNA vaccines, the current NVI vaccine should be improved in accordance with the circulating NDV strains in various region of the country.

7. REFERENCES

- Alders, R., and Spradrow, P. (2001): Controlling Newcastle disease in Village Chickens: a field manual. Australian Centre for International Agricultural Research (ACIAR).
- Aldous, E. W. (2001): Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathology*, **30**:117-128.
- Alemu, (2010): Drug Administration and control authority of Ethiopia. Standard treatment guideline for veterinary practice, 1st edition, Addis Ababa, Ethiopia.
- Alexander, D. J. (2004): Highly pathogenic avian influenza/Newcastle disease. In OIE Manual of diagnostic tests and vaccines for terrestrial animals, 5th ed. Office International des Epizooties, Paris, France, Pp. 258-282.
- Alexander, D.J. (1997): Antigenic diversity and similarities detected in avian paramyxovirus type 1 isolates using monoclonal antibodies. *Avian Pathology*, **26**: 399-418.
- Alexander, D. J. (2000). Newcastle disease and other avian paramyxoviruses. *Revue Scientifique et Technique-Office International des Epizooties*, *19*(2), 443-455.
- Alexander, D.J. and Parsons, G. (1986): Protection of chickens against challenge with the variant virus responsible for Newcastle disease in 1984 by conventional vaccination. *Veterinary Record*, **118**: 176-177.
- Alexander, D.J., Russell, P.H. and Collins, M.S. (1984): Paramyxovirus type 1 infections of racing pigeons and first Characterization of isolated viruses. *Veterinary Record*, **114**: 444-446.
- Al-Gari, S. (2003): Newcastle disease virus: immune reactivity and pathogenesis, PhD thesis, Utrecht University, Faculty of Veterinary Medicine, The Netherlands.
- Al-Haee, M.A., and Sharawi, S. (2013): Detection and characterization of Newcastle

disease virus in clinical samples using real time RT-PCR and

Amarasinghe G.K., Ayllón M.A., ào Y., asler cC.F., avari S., lasdell K.R., etal., (2019). Taxonomy of the order Mononegavirales: update 2019. Arch. Virol., 164, 1967–1980. doi: 10.1007/s00705-019-04247-4.

Ashenafi, H. (2000): Survey on identifications of major disease of local chickens in three selected agro climatic zone in central Ethiopia. DVM thesis, Faculty of veterinary medicine, Addis Ababa University, Dere Zeit, Ethiopia.

Attia, Y. A., Rahman, Md. T., Hossain, Md. J., asiouni, S., Khafaga, A. F., Shehata, A. A., & Hafez, H. M. (2022). Poultry Production and Sustainability in Developing Countries under the COVID-19 Crisis:

arman, (2002): An Epidemiological and Experimental study of New castle disease in village chickens of Bangladesh, Pp. 2 -28.

Beach, J.R. (1942): Avian pneumoencephalitis. Proceedings of the Annual Meeting of the US Livestock Sanitary Association, **46**: 203-223.

Beach, J.R. (1944): The neutralization in vitro of avian pneumoencephalitis virus by Newcastle disease immune serum. Science, 100: 361-362.

Bell, J.G., Fotzo, T.M., Amara., A. and Agede., G. (1995). —A field trial of the heat resistant V4 vaccine against Newcastle disease by eye-drop inoculation in village poultry in Cameroon, Preventative Veterinary Medicine, 25, 19– 25.

Bello, M. ., Yusoff, K., Ideris, A., Hair-ejo, M., Peeters, . P. H., & Omar, A. R. (2018). Diagnostic and Vaccination Approaches for Newcastle Disease Virus in Poultry: The Current and Emerging Perspectives. ioMed Research International, 2018.

Bhaiyat, M.I., Ochiai, K., Itakura, C. and Kida, H. (1994): Brain lesions in young roiler chickens naturally infected with a mesogenic strain of Newcastle disease virus. *J. Avian Pathology*, 23:693-700.

Boostani, A.R., Pourakhsh, S.A., Momayez, R. and Charkhkar, S. (2013): Molecular characterization and phylogenetic study of Newcastle disease virus isolates

from the 2010 to 2011 outbreaks in Shiraz, Iran. *Afr. J. Microbiology*, 7:657-660.

Cattoli, G., Fusaro, A. and Monne, I. (2010): Emergence of a new genetic lineage of Newcastle disease virus in West and Central Africa—implications for diagnosis and control. *Vet Microbiology*, **142**:168–176.

Central Statistical Agency (CSA) [Ethiopia]. (2007). The 2007 Population and Housing Census of Ethiopia: Statistical Report at Country Level.

Cho, S., Kwon, H., Kim, T. and Kim, J.H. (2008): Characterization of a Recombinant Newcastle Disease Virus Vaccine Strain. *Vaccine Immunology*, **15**:1572-1579.

Chume, A., Izquierdo-Lara, R., Tataje, L., Gonzalez, R., Criillero, G., González, A. E., ... & Icochea, E. (2017). Pathotyping and phylogenetic characterization of Newcastle disease viruses isolated in Peru: defining two novel sugenotypes within genotype XII. *Avian diseases*, 61(1), 16-24.

Collins, M. S., ashiruddin, J. . and Alexander, D. J. 1993: Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease virus showing variation in antigenicity and pathogenicity. *Arch. Virology*, **128**:363-370.

CSA (Central Statistic Authority) (2021). Federal Democratic Republic of Ethiopia Central Statistical Agency Agricultural Sample Survey 2020/21. Report on Livestock and Livestock Characteristics (Private Peasant Holdings), Addis Ababa, Ethiopia

David, E. and Daniel, J.K. (2003): Zoonosis update:Avian influenza and Newcastle disease. *JAVMA*, **222**:1534-1540.

Dimitrov, K. M., Afonso, C. L., Yu, Q., & Miller, P. J. (2017). Newcastle disease vaccines—A solved prolem or a continuous challenge?. *Veterinary microiology*, 206, 126-136.

Doyle, T.M. (1927): A hitherto unrecorded disease of fowls due to a filter-passing virus. *Journal of Comparative Pathology and Therapeutics*, **40**:144-169.

- Doyle, T.M. (1935): Newcastle disease of fowls. *Journal of Comparative Pathology and Therapeutics*, **48**: 1-20.
- Fikre, (2003): Poultry Disease Lecture Notes, Proceedings of a Postgraduate Refresher Course Conducted at Mekelle, in May 2003. y the Tigray Agriculture and Natural Resources Development. Pp. 2-3.
- Ge, G., Zheng, D., Zhao, Y. and Li, H. (2012): Evaluating viral interference etween Influenza virus and Newcastle disease virus using real-time reverse transcription-polymerase chain reaction in chicken eggs. *Virology J.* **9**:01-08.
- Hadipour, M.M., Haii, G.H. and Shayanpour, N. (2011): The Role of Avian Influenza, Newcastle Disease and Infectious roncchitis Viruses during the Respiratory Disease Outbreak in Commercial roiler Farms of Iran. *International J. Anim. Vet. Advances*, **3**:69-72.
- Halasz, F. (1912). Contriutions to the knowledge of fowlpest. Veterinary Doctoral Dissertation, Communications of the Hungarian Royal Veterinary School, Patria, udapest. Pp. 1-36
- Herczeg, J., Pascucci, S., Massi, P and Lomniczi, . (2001) : A longitudinal study of velogenic Newcastle disease virus genotypes isolated in Italy between 1960 and 2000. *Avian Pathology*, **30**:163-168.
- Ibrahim, L.A., Khal, S.A., Omer S.A. and Adam S.E I. (1992): The toxicology of Azadarachta india leaves. *Journal of ethopharmacology*, **35**: 267-273.
- International Committee on Taxonomy of Viruses (ICTV) (2019). Orthomyxoviridae. Virus Taxonomy: 2019 Release. [https://talk.ictvonline.org/ictv-reports/ictv9threport /negative sense-rna-viruses2011/w/negrnaviruses/209/orthomyxoviridae](https://talk.ictvonline.org/ictv-reports/ictv9threport/negative-sense-rna-viruses2011/w/negrnaviruses/209/orthomyxoviridae)
- Kaletka, E.F. and aldauf, C. (1988): Newcastle disease in free-living and pet irds. In: Newcastle Disease, D.J. Alexander Ed., Kluwer Academic Pulishers, oston. Pp. 197-246.
- Khan, T.A., Rue, C.A., Rehmani, S.F., Ahmad, A. and Wasilenko, J.L. (2010):

- Phylogenetic and iological Characterization of Newcastle Disease Virus Isolates from Pakistan. *J.Clin. Microiol.* **48**:1892-1894.
- Kraneveld, F.C. (1926): A poultry disease in the Dutch East Indies. *Nederlands-Indische laden voor Diergeneeskunde*, **38**:448-450.
- Lam, R.A., P.L., Collins, D. and Kolakofsky, J.A. (2000): Family Paramyxoviridae. In M.H.V. van Regenmortel (ed) *Virus Taxonomy, Seventh Report of the International Committee on Taxonomy of Viruses*. Academic Press: New York. Pp. 549-561.
- Lancaster, J.E. (1966): Newcastle disease - a review 1926-1964. Monograph No 3, Canada Department of Agriculture, Ottawa.
- Macpherson, L.W. (1956): Some oservations on the epizootiology of Newcastle disease. *Canadian Journal of Comparative Medicine*, **20**: 155-168.
- Madadgar, O., Karimi, V., Nazaktaar, A., Kazemimanesh M.and Hojjati, P. (2013): A study of Newcastle disease virus obtained from exotic caged irds in Tehran between 2009 and 2010. *Avian Pathology*. **42**:27-31.
- Makkay, A. M., P. J. Krell, and E. Nagy. 1999. Antibody detection-based differential ELISA for NDV-infected or vaccinated chickens versus NDV HN-suunit vaccinated chickens. *Vet. Microiol.* **66**:209-22.
- Maqool, A. (2002): Marketing of commercial poultry, poultry meat and eggs in Faisalaad City. M.Sc. Thesis University of Agriculture Faisalaad, Pakistan.
- Maqool, A.A. and ukhsh, K. (2007): Issues and Economics of poultry production: a case study of Faisalaad, Pakistan. *Pak. Vet. J.* **27**:25-28.
- Mayers, J., Mansfield, K. L., & rown, I. H. (2017). The role of vaccination in risk mitigation and control of Newcastle disease in poultry. *Vaccine*, **35**(44), 5974-5980.
- Mayo, M.A. (2002): A summary of the changes recently approved by ICTV. *Archives of Virology*, **147**: 1655-1656.
- Miller, J. (2014): Newcastle Disease in Poultry: the Merck veterinary manual.

45:189-189.

- Mohamed, H.A., Kumar, S., Paldurai, A. and Samal, S.K. (2011): Sequence analysis of fusion protein gene of Newcastle disease virus isolated from outbreaks in Egypt during 2006. *Virology J.* **8**(237):01-04.
- Morgan, R.W., Gel, J. and Poppe, C.R. (1999): Efficiency in checking of herpes virus of turkey recombinant vaccine containing the fusion of ND virus.
- Napit, R., Poudel, A., Pradhan, S. M., Manandhar, P., Ghaju, S., Sharma, A. N., ... & Karmacharya, D. . (2023). Newcastle disease urden in Nepal and efficacy of Talet I2 vaccine in commercial and backyard poultry production. *Plos one*, 18(3), e0280688.
- Nolen, R.S. (2003): Emergency declaration: exotic Newcastle disease found in commercial poultry farms. *J. Am. Vet. Med. Assoc.* 222:411.
- NVI (2016): Product catalogue. User manual of ND vaccine, Debre Zeit, Ethiopia.
- OIE (2011): Geographical distrbition of Newcastle disease. Wesite: <https://www.google.com/snewcastle-disease-map/tm>.
- OIE (2012): Newcastle disease. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.3.14. <http://www.oie.int/international-standard-setting/terrestrial-manual/access-online>.
- OIE terrestrial manual (2021). Chapter 3.3.14, Newcastle disease (infection with Newcasle disease virus),
- Pham, H. M., Konnai, S., Usui, T., Chang, K.S. and Onuma M. (2005): *Archive Virology*. **150**:2429-2438
- Röme, A., Werner, J. Veits, T. and Mettenleiter, T. C. (2003): Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus Pathogenesity. *J. Gen. Virol.* **84**:3121-3129.
- Saifuddin, M.D., Chaudhury, T.I.M. and Arun, M.M. (1990): Protection confirmed y vaccination with lacksurg and Komarove strains of NDV against ND in angladesh. *Tropical Animal Health and production*, 22: 263-272.

- Seal, . S., King, D. J. and ennett, J. D. (1995): Characterization of Newcastle disease virus isolates y reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence dataase for pathotype prediction and molecular epidemiological analysis. *J. Clin. Microiol.* **33**:2624-2630.
- Serkalem, Hagos, Ashanafi and Zeleke (2001): International journal of applied reaserch in vet. Medicine: Vol.3 No.1, 2005: pp 25-29.
- Shim. J., So, H.H., Won, H.H. and Mo, I. (2011): Characterization of avian paramyxovirus type 1 from migratory wild irds in chickens. *J. Avian Pathol.* **40**(6):565-572.
- Spradrow, P.. (1987): In Newcastle Disease in Poultry. A new food pellet vaccine. J.W. Copland editor. Canerra, ACIAR. Pp. 12-18.
- Waheed, U., Siddique, M., Arshad, M. and Saeed, A. (2013): Preparation of newcastle disease vaccine from VG/GA strain and its evaluation in commercial roiler chicks. *Pak. J. Zool.* **45**(2):339-344.
- Wen, M., Chen, Z.T., Zhang, D.X., Yang, J.L. and Zhou, .J. (2007): Cloning and sequence analysis of F gene of Newcastle disease virus isolated from Guizhou province, China. *J. Food Agric. Environ.* **10**:484-486.
- Xiao, S., Paldurai, A., Nayak, ., Mirande, A. and Samal, S.K. (2013): Complete genome sequence of a highly virulent Newcastle disease virus currently circulating in Mexico. *J. Genome Announcements.* **1**(1):01-02.
- Yinfeng K., Minsha F., Xiaqiong Z., in X., Pei G., Yulian L., Yanling L., and Tao R. (2016). *Virology journal*, **13**: 49-58
- Zanetti, F., Rodriguez, M., Capua, I., Carrillo, E. and Seal, .S. (2003): Matrix protein gene sequence analysis of avian paramyxovirus 1 isolates obtained from pigeons. *Virus Genes.* **26**:199-206.
- Kelly, L. M., & Alworth, L. C. (2013). Techniques for collecting blood from the domestic chicken. *La animal*, 42(10), 359-361. <https://doi.org/10.1038/la-an.394>
- Yoriyo, K.P., Adang, K.L., Faiyi, J.P., & Adamu, S.U. (2008). Helminthes parasites

of local 519 chickens in auchi state, Nigeria. *Science World Journal*, 3(2), 35-37. 520. <https://doi.org/10.4314/swj.v3i2.51782>

Zelege, A., Sori, T., Gelaye, E., & Ayelet, G. (2005). Newcastle disease in village chickens in the southern and rift valley districts in Ethiopia. *International Journal of Poultry Science*, 4(7), 507-510.

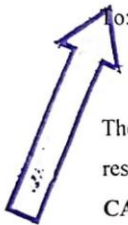
Ganar, K., Das, M., Sinha, S., & Kumar, S. (2014). Newcastle disease virus: current status and our understanding. *Virus research*, 184, 71-81.

ETHICAL CLEARANCE



ብሔራዊ የእንስሳት ጤና ጥበቃ ኢንስቲትዩት
NATIONAL VETERINARY INSTITUTE

ቁጥር
Ref. No ሀሀ/1349
ቀን
Date: 12/06/2023



To: Ayele Teshome

Subject: Letter of Ethical Clearance

The research ethical committee of the National Veterinary institute reviewed and discussed your research project entitled “ISOLATION AND MOLECULAR DETECTION OF NEW CASTLE DISEASE VIRUS AND IMMUNOGENECITY EVALUATION OF VACCINE IN AND AROUND BISHOFTU-ETHIOPLA” on Dec,2022. After discussion and review of your project proposal, it is found scientifically and ethically sound from relevance, originality and technical competence point of view.

Hence, the project proposal is allowed to be executed provided that:

4. All procedures and conditions stipulated in the proposal are respected and any deviations, variations or changes may be made only in consultation between and reported to the committee.
5. All comments given by the committee should be considered and fulfilled by the researchers.
6. The project activity is open for occupational supervision by the committee whenever this is deemed necessary.

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

Yours sincerely,


Derecha Bayisa (Dr)
Operational Deputy
Director General

Cc:

- > General Manager
- > Research & Development Directorate Director
NVI

☒ 19
ደብረ ዘይት ኢንዱስትሪ
Debre Zeit ETHIOPIA

☎ +251-11-433-84-11/16
☎ 251-911-51-08-94
FAX: +251-11-433-93-00

E-mail: nvi-rt@ethionet.et
info@nvi.com.et
Website: www.nvi.com.et