Molecular Characterization for Strains of Newcastle Disease Virus Circulating in Chickens in Selected Migratory Birds Abundant Areas in the Mid Rift Valley and Central Part of Ethiopia

By: Esubalew Endale Alemu

A Thesis Submitted to the Department of Applied Biology in Partial fulfillment of the requirement of the Award of Degree of Master of Science in Applied Biology (Biotechnology)

School of Applied Natural Sciences

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

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Co-advisor: Melaku Sombo (Assistant Professor)

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APPROVAL SHEET

This is to certify that the thesis prepared by **Esubalew Endale Alemu** entitled as "**Molecular Characterization for Strains of Newcastle Disease Virus Circulating in Chickens in Selected Migratory Birds, Abundant Areas in Mid Rift Valley and Central part of Ethiopia**" submitted in partial fulfillment of the requirement for the master of degree of science in Applied Biology (Biotechnology) complies with the regulation of Adama Science and Technology University and meets the accepted standards with respect to originality.

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DECLARATION

I here declare that this thesis entitled as "Molecular Characterization for Strains of Newcastle Disease Virus Circulating in Chickens in Selected Migratory Birds Abundant Areas in Mid Rift Valley and Central part of Ethiopia" submitted to Adama Science and Technology University for the award of degree of M.Sc. in Biotechnology is the result of the research work carried out by me. I affirm that I have cited and referenced all sources used in this document. Brief quotations from this thesis may be used without special permission provided that an accurate and complete acknowledgement of the source is made. To the best of my knowledge, I sincerely declare that this thesis has not been submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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First, I declare that this thesis is my *bona fide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Adama Science and Technology University, School of Applied Natural Science and is deposited at the University/School 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. Brief quotations from this thesis are allowable without special permission provided that an accurate acknowledgement of the source is made. Requests for permission for an extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the School when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

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ACKNOWLEDGEMENTS

First of all, thanks to almighty God next, I would like to express my special words of thank to Professor Hunduma Dinka, for his constructive guidance, advice, and critical suggestions through my research work. Secondly, I would like to give deep thank to my Co' advisors Mr. Melaku Sombo and Dr. Tesfaye Rufahel for their allowance, facilitating and authorizing me to do my laboratory work at the molecular biology laboratory of the National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta, Oromia, Ethiopia. Thirdly, I would like to thank Mr. Dawit Hailu for allowing, facilitating, and authorizing the sequencing process at Armauer Hansen Research Institute (AHRI). Fourthly, I would like to give deep thank to Mr. Bayeta Senbeta and Mr. Chala Guyassa for their help during sample collection. Lastly, I would like to thank Applied Biology department staffs for their help with constructive guidance, and critical suggestions throughout my study.

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LIST OF ABBREVIATIONS

AI	Avian influenza
APMV	Avian Paramyxovirus
ATJK	Adami Tulu Jido Kombolcha
cDNA	Complementary Deoxyribonucleic acid
CSA	Central Statistical Agency
CS	Cloaca Swab
Ct	Threshold cycle
ddNTP	Dideoxynucleotide Triphosphate
ELISA	Enzyme Linked Immuno Sorbent Assay
F	Fusion protein
FDRE-MOA	Federal Democratic Republic of Ethiopia – Ministry of Agriculture
HA	Haemagglutination
HI	Haemagglutination Inhibition
HN	Hemagglutinin neuraminidase
L	Large RNA polymerase
M	Matrix protien
Mab	Monoclonal antibody
MoLF	Ministry of Livestock and Lisheries
mRNA	Messanger of Ribonuclec acid
NAHDIC	National Animal Health Diagnosis and Investigation Center
ND	Newcastle disease

NDV	Newcastle disease virus
NP	Nucleoprotein
NVI	National Veterinary Institute
OIE	Office International des Epizooties
ORF	Open reading frame
P	Phospho protein
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
PI	Percentage of inhibition
PPMV	Pigeon paramyxovirus
RBCs	Red blood cells
rRT-PCR	Real time reverse transcriptase polymerase chain reaction
RT- PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SPF	Specific Pathogen Free
ssRNA	Single-stranded Ribonucleic Acid
SNNP	Southern Nation Nationality and people
TS	Trachea Swab
VTM	Virus transport media
vvNDV	Viserotropic Velogenic Newcastle Disease Virus

ABSTRACT

Newcastle disease (ND) is a highly infectious chicken disease that causes major economic losses worldwide. The disease caused by Avian paramyxovirus type 1 (APMV-1). A cross-sectional study was conducted from February 2020 to October 2020 to investigate Newcastle virus strains circulating in chickens from selected migratory birds abundant areas in the mid-Rift Valley and the central part of Ethiopia. A total of 98 samples: 78 tracheal and cloacal swabs from chickens pools of five, and 20 tissue samples were collected. The main functional region of the F gene has been amplified (270 nucleotides) by qRT-PCR and sequenced by Sanger. Accordingly, 13.26% samples were positive for NDV base on the qRT-PCR test. Phylogenic analysis of the partial cleavage site of F-gene indicated that one field isolate of the Ethiopian NDV isolates had a velogenic motif 112R-R-Q-K-R-F117 at the fusion protein cleavage site and clustered within the lineage 5 (genotype VIIg) whereas three of the isolates had a lentogenic motif 112G-R-Q-G-R-L117and belonged to the sub-genotype of lineages 1, 2, and 3 (genotype I, II, and III). The genotype VIIg isolate was genetically similar to Egypt and Iran NDV isolates, indicating the possible epidemiological relation of NDV outbreaks in the study area were are due to seasonal migratory wild birds. The current vaccine strain (HB1 vaccine) used in the study area grouped into lineage 2 (genotype II) showing 73% to 89% similarity to some of the field isolated Newcastle disease viruses and shared similarities with many countries in Africa, Asia, Europe, North, Central, and South America. The current analysis showed that different NDV genotypes circulated in the middle Rift Valley and central part of Ethiopia. The virulent NDV continues has been an issue in the poultry sector in Ethiopia, and its continuous circulation in the village and commercial poultry needs better surveillance and effective vaccine.

Keywords: *F gene, Mid Rift Valley, Migratory birds, Newcastle disease virus, Phylogenetic tree, qRT-PCR*

1. INTRODUCTION

1.1 Background of the Study

Newcastle disease is an infectious disease in avian species, caused by Newcastle Disease Virus (NDV), which is one of the most important poultry diseases that highly affect the poultry sector in terms of production and productivity at domestic and international level (Alexander and Aldous, 2001; Samrawit and Mulat, 2018). It is a highly contagious viral disease affecting all avian species including wild and domestic avian species (Banur *et al*., 2013). The impact of Newcastle Disease (ND) has estimated great economic losses through many epidemics associated with very high mortality, high morbidity, and many other productions related losses (Mark *et al*., 2003). Newcastle disease has included among the list of diseases that require immediate notification upon recognition by the World Organization for Animal Health (OIE) (Muhammad *et al*., 2018).

Reported by Aldous *et al.* (2003) the virus that causes Newcastle disease is grouped under Phylum Negarnaviricota, Order Mononegavirales, family Paramyxoviridae, genus Avulavirus, and species Avian paramyxovirus type 1 (APMV-1) or Newcastle disease virus. It is an RNA virus and negative-stranded, whose genome size is about 15.2 kb, in which the genome is non-segmented and organized into six genes translating six structural proteins, namely: RNA-dependent RNA polymerase (L), nucleoprotein (NP), matrix protein (M), phosphor protein (P), hemagglutinin-neuraminidase (NH), and fusion protein (F) (Alexander and Aldous, 2001). Additionally, Proteins V and W are also encoded by RNA editing of P protein (Palva *et al*., 2012). From these proteins, the fusion protein is mostly considered as a molecular marker of virulence paramyxovirus type 1 (NDV), the diagnostic dilemma considering the F protein cleavage site is a trusted molecular indicator of NDV virulence that sufficiently predicts the virulence potential of NDV isolates and important for the complete genome analysis (Mark *et al*., 2003 and Murtadha and Mahdi, 2016). However, recent studies show that the HN and L proteins are directly associated with NDV virulence (Alsahamia *et al*., 2018).

Based on their pathogenicity, NDV strains are classified into three major groups such as velogenic (highly virulent), mesogenic (moderate virulence), and lentogenic (low virulence)

strains (Orsi *et al*., 2009 and Ahmed *et al*., 2017). The common clinical symptoms of NDV are a respiratory disorder, drop in appetite, decreased egg production, as well as disturbances of the nerve system, but the observed clinical symptoms in infected animals are very similar to the three different strains of NDV and another avian disease such as avian influenza and mark's disease. Because of this, it is difficult to distinguish between pathotypes only based on the clinical symptoms. To solve this kind of problem, researchers and scientists are developing different kinds of molecular techniques that are used for the diagnosis of these diseases (Murtadha and Mahdi, 2016).

Molecular diagnosis based on polymerase chain reaction (PCR) modifications involves the direct detection of nucleic acids of viral genomic RNA by converting to complementary deoxyribonucleic acid (cDNA) which is followed by RT-PCR amplification (Alsahami *et al*., 2018). RT-PCR method amplifies the target DNA fragment that can be subsequently via digestion using a restriction enzyme to digest DNA fragments of RT-PCR product, and then followed by DNA sequencing. Although this method is a more accurate, reliable, and time-saving method, its cost is higher compared to nonmolecular techniques (Aris *et al*., 2016). The development of a molecular method to study the pathogenesis of NDV has been done by the characterization of fusion (F) protein, which is a major determinant of NDV pathogenicity (Orsi *et al*., 2009). Early detection of the circulating strain of the virus is essential in the controlling of the Newcastle disease in terms of vaccine design. There are live (thermostable) and inactivated vaccines, derived from the lentogenic strain of NDV, that are used in Ethiopia to prevent the outbreak of ND (NVI, 2019).

Newcastle disease virus infects a wide extend of domestic and wild bird species around the world. More commonly affected species include chicken, turkeys, ducks, pigeons, guinea fowl, Japanese quail, and many wild birds of all ages (Alexander, 2009). The interaction of domestic poultry with wild birds has been recognized as a possible source of avian disease in domestic flocks around the Rift Valley region (MoA, 2018). Furthermore, the role that migratory birds play a pivotal role in the transmission of avian disease, according to the phylogenetic comparison of the viruses obtained from different hosts suggests that migratory birds carry the virus and reach it to domestic birds. Isolates of NDV have been obtained

frequently from migratory wild birds, especially feral waterfowl and other aquatic birds. Genotypes for class II NDV isolates comprise the majority of virulent NDV strains and some avirulent NDV strains. Viruses of genotypes VII, VI, and II of class II were identified and responsible for the prevalence of Newcastle disease in Ethiopia (Tsegaw *et al*., 2014 and Aldous *et al*., 2003). Occasionally, virulent viruses have been detected in wild birds, but usually, these were in birds found dead near infected poultry (Aldous *et al*., 2003 and Gelana, 2017). Therefore the purpose of this study is to investigate the strain of Newcastle disease virus that circulates in chickens in selected migratory birds' abundant areas in the mid- Rift Valley of Ethiopia and test whether this strain is matching with the vaccine currently in use.

1.2. Statement of the problem

Different pieces of evidence have shown that the prevalence of ND is high in Ethiopia. The serological, virological, and molecular data evidences are indicating the prevalence level of the disease in different parts of the country. For example, 11% of NDV prevalence was reported in southern Ethiopia (Belayheh *et al*., 2014) and 11.6% of prevalence was reported in Rift Valley area (Dehinenet *et al*., 2015). There are different factors that play a role in the prevalence of Newcastle disease such as lack of routine sanitary and hygienic practices, backward waste disposal, weak housing management, lack of appropriate transportation for poultry and poultry related products, inadequate feed and water in terms of quality and quantity, presence of connection with wild birds, lack of insurance and finance support, inadequate veterinary service, and inadequate vaccination practices of chickens. Despite live and inactive lentogenic vaccines are used in Ethiopia; however, there are complaints with these vaccines as not producing active immunity to block NDV, the method of diagnosis, and lack of sufficient and organized data about the disease. Because of those aforementioned reasons, Newcastle disease represents the most severe poultry disease responsible for the most economic losses across the country (Dehinenet *et al*., 2015).

There is a scarcity of information on the molecular status of the viral strain and the vaccine in use in the village and commercial chickens in the study area. To solve all problems, there is a need for sufficient and accurate data that are produced by molecular diagnosis methods to design effective controlling and eradication strategies for ND. Hence, this study was

investigated NDV strains that circulate in chickens in selected migratory bird abundant areas and test whether this strain is matching with the vaccine currently in use in Mid-Rift Valley and central part of Ethiopia.

1.3 Objectives of the study

1.3.1 General objective

- To characterizing Newcastle disease virus strains that circulates in chickens in selected migratory birds' abundant areas and test whether this strain is matching with the vaccine currently in use in the mid Rift Valley and central part of Ethiopia by using molecular tools.

1.3.2 Specific objectives

- To identify the NDV virus strain that circulates in non-vaccinated village and commercial chickens in the study area.
- To determine the prevalence of NDV at the interface of non-vaccinated village chickens and vaccinated commercial chickens in the study area.
- To assess the similarity between the circulating strains of NDV and the strain of the vaccine in use in the study area.
- To determine the prevalence of NDV based on sample types, sex, and age chickens in the study area.

1.4 Significance of the study

Newcastle disease is economically important, due to the fact that it causes high morbidity and mortality rates, reduces egg production, deteriorates egg quality, and impairs life performance (Orsi *et al*., 2009). The importance and impact of a Newcastle disease virus are directly related to its virulence. Major efforts to reduce the outbreak and prevalence of Newcastle disease have been undertaken in the last decades (Ashenafi, 2000). The main challenge in Newcastle disease is the lack of updated and accurate data that are important for diagnosis, vaccine

production, and developing a control strategy. This study addresses the more reliable and updated information about the circulating strain of the virus and fills the gap of information about why vaccination failure happened for the currently in-use vaccines.

1.5 Scope of the study

The principal concern of this study was to investigate the circulating strain of Newcastle disease virus in chicken in selected migratory birds' abundant areas in Rift Valley. Due to time and financial constraints, the current study could not cover the country wide samples to compare the whole picture of situation in Ethiopia.

1.6 Hypotheses

1. Ho = There are the same strains of Newcastle disease virus present in chickens and the current vaccine in use against ND in Ethiopia.
2. Ha = There are different strains of Newcastle disease virus present in chickens and the current vaccine in use against ND in Ethiopia.

2. LITERATURE REVIEW

2.1 General information on NDV

Poultry plays an important economic, nutritional, and socio-cultural role in the livelihoods of poor rural households in developing countries, including Ethiopia (Central Statistical Agency, 2018). Poultry rearing is particularly important to women, local communities, and urban communities for raising incomes and reducing poverty (Delesa *et al*., 2014). Poultry production is challenging due to infectious diseases such as Newcastle disease, Infectious bronchitis, Marek's disease, Infectious Bursal Disease, Salmonellosis, Mycoplasmosis, and Pasteurellosis (Zerihun and Bihonegn, 2018). Newcastle disease (ND) is one of the influential avian diseases that seriously affect poultry production all over the world. This disease is one of the major problems in village and commercial chickens in most parts of Ethiopia (Delesa *et al*., 2014 and Nesradin and Nejash, 2017). The disease has become endemic in the poultry population and causes heavy losses every year (Samrawit and Mulat, 2018). From March to May, the highest rate of ND outbreaks was present in chickens and this may be associated with the movement of chickens from the rural area to town for searching the chicken market, Easter Market, favoring the spread of diseases all over the country (Zerihun and Bihonegn, 2018). In Ethiopia, the first evidence of an ND epidemic was discovered in 1971 (NVI, 1974).The virus strains involved in those early outbreaks were the velogenic ones and caused up to 90% mortality. The disease spread throughout the country in the following years, with reports coming from various agro-climatic zones. Those early outbreaks had occurred in Addis Ababa, Alemaya, and Debre Zeit. After applying routine vaccinations with Lasota and HB1 strains, at least nine outbreaks of ND have been reported in three big poultry farms such as Dembi, Shola, and Lemlem between 1983-1995 (MoA, 2018). Newcastle disease has accounted for extremely large economic losses through numerous epidemics associated with very high mortality, high morbidity, and much other production related losses (Gelana, 2017).

2.2 Classification of NDV

Classification of Newcastle disease virus based on restriction fragment length polymorphism analyses grouped into six distinct genotypes (I, II, III, IV, V, and VI). This classification was validated and further improved by phylogenetic analysis of the partial and complete sequence of the F protein gene (Dimitrov *et al*., 2019). All avian paramyxoviruses (APMV) are part of the genus Avulavirus. There are eleven serotypes of avian paramyxoviruses, still, all isolates of Newcastle disease virus belong to serotype 1 (APMV-1) (Aldous *et al*., 2003).

ND viruses are clustered into one genotype in class I, NDV which has a genomic size of 15,198 nucleotides and eighteen (I-XVIII) (Liu *et al*., 2007), is sometimes isolated from domestic poultry and wild aquatic birds, mostly it is not virulent to chickens. Genotypes for class II NDV isolates comprise the majority of virulent NDV strains and some avirulent NDV strains and a genomic size of 15,186 nucleotides. This class is further subdivided into 11 genotypes (I-XI) and group VI and VII can further be subdivided into sub-genotypes VI-a to VI-h and VII-a to VII-I (Aldous *et al*., 2003). A panzootic and significant economic loss in poultry sector caused by viruses of class II (Bello *et al*., 2016). According to Alexander (2009), class II subgenotypes II, III, and IV viruses are responsible for the first panzootic during the 1920s to 1960s. Figure 1 shows Viruses of genotypes VII, VI, and II of class II were identified and responsible for the prevalence of Newcastle disease in Ethiopia (Tsegaw *et al*., 2014 and Gelana, 2017).

Figure 1: Phylogenetic relationship between the Ethiopia NDV isolation (Tsegaw *et al.*, 2014).

2.3 Molecular biology of Newcastle Disease Virus

The genetic material of NDV is a negative-stranded non-segmented RNA virus that has a total size of 15198, 15186 or 15192 bp based on the genotype of the NDV (class I, II) (Aldous *et al*., 2003). The genome is divided into six genes, namely, nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large protein (L) (Figure 2). The P gene is unique in that translational editing of its mRNA results in two nonstructural proteins, V and W. The V protein plays a direct role in virus replication and host range restriction as well as serving as a virulence factor (Mia *et al*., 2008).

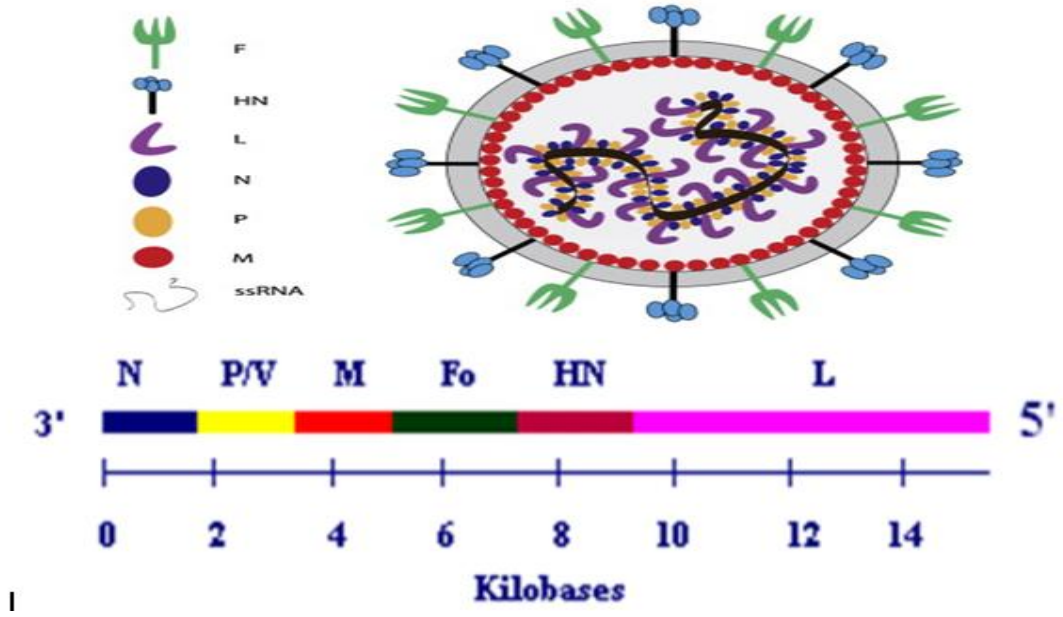

Figure 2: Structural features of Newcastle disease virus (Bello *et al*., 2018).

Principal pathogenic marker of NDV exists in F protein; a disulfide linkage is present between F1 and F2. These proteins empower the virus to attach to the host cell membrane. At the cleavage site, the F0 protein has two pairs of basic amino acids that can be cleaved by the host proteases. The highly virulent Newcastle disease virus has three (3) or more basic amino acids, which are lysine (K) or arginine (R) present at 113-116 residues and phenylalanine (F) at position 117. The cleavage of the F0 protein is due to the presence of these basic amino acids in virulent NDV. Cleavage of the precursor F0 into the F1 and F2 products is necessary

for viral spread to other cells and it has been found that low virulent virus has 112G/E-K/RQ-G/E-RL117 and virulent viruses have 112R/K-R-Q-K/RR-F117 amino acid sequence at the cleavage site (Figure 3).

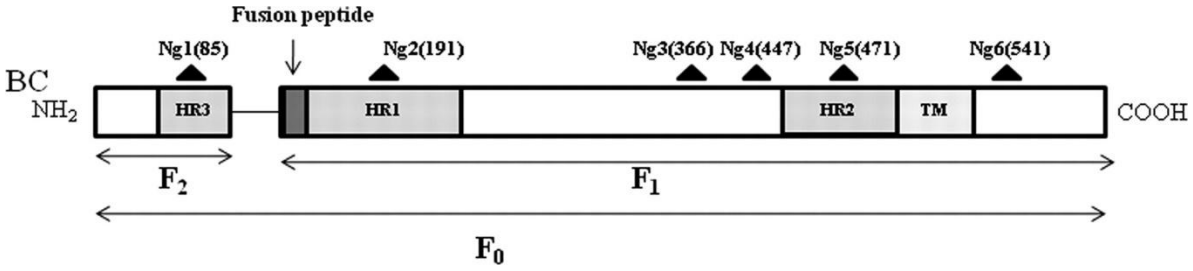

Figure 3: Structure of the fusion protein and its cleavage site (Orsi *et al*., 2009).

The F and HN surface glycoproteins are the foremost antigens that elicit a protective immune response. All other viral genes are mono cistrons encoding a single structural protein. Termination of the viral genome constitutes the leader and trailer regions, which accommodate the regulatory signals for virus transcription and replication. Morphologically, NDV appears to be a pleomorphic enveloped particle with projections of F and HN spike glycoproteins that participate in the initiation of the virus infectious cycle (Tanjina *et al*., 2009). Immediately beneath the viral envelope is the M protein, which is known to maintain the shape of the virus and assist in the packaging and release of the newly assembled viruses. The other three proteins are personally related to the viral genome and are known to perform replication-related functions. In particular, the NP precisely covers the entire viral RNA to form the ribonucleoprotein (RNP), which is the minimum template required for virus replication and mRNA biosynthesis (Bello *et al*., 2016). The P protein is the cofactor of the polymerase, while the L protein is the RNA-dependent RNA polymerase that serves as the viral replicate and transcriptase during the infectious cycle reported by Mia *et al*. (2008).

2.4 Epidemiology and Pathotypes of Newcastle Disease Virus

The first outbreak of Newcastle disease (ND) occurred in 1926 in Java, Indonesia. In the last ninety-three years, several majors economically devastating NDV panzootic have occurred worldwide (Muhammad *et al*., 2018). Consequently, several economic losses in a vast number of countries across South East Asia, the middle East, Europe, Africa, and America (Figure 4).

This particular panzootic was caused by the genotype class II, VII group of NDV, which currently constitutes the most rapidly evolving strain of the virus (Dimitrov *et al*., 2019). Newcastle disease can infect over 240 species of birds. When infected birds are introduced into susceptible flocks, all birds will be infected within two to six days. The disease is transmitted through direct contact with infected or carriers' birds. Transmission can also occur through contact with secretion and excretion of infected birds and contact with contaminated materials. Another important route of transmission is through air, fleas, rodents, insect, and dogs can also transmit NCD virus mechanically from infected fecal (Aldous *et al*., 2003 and Nesradin and Nejash, 2017).

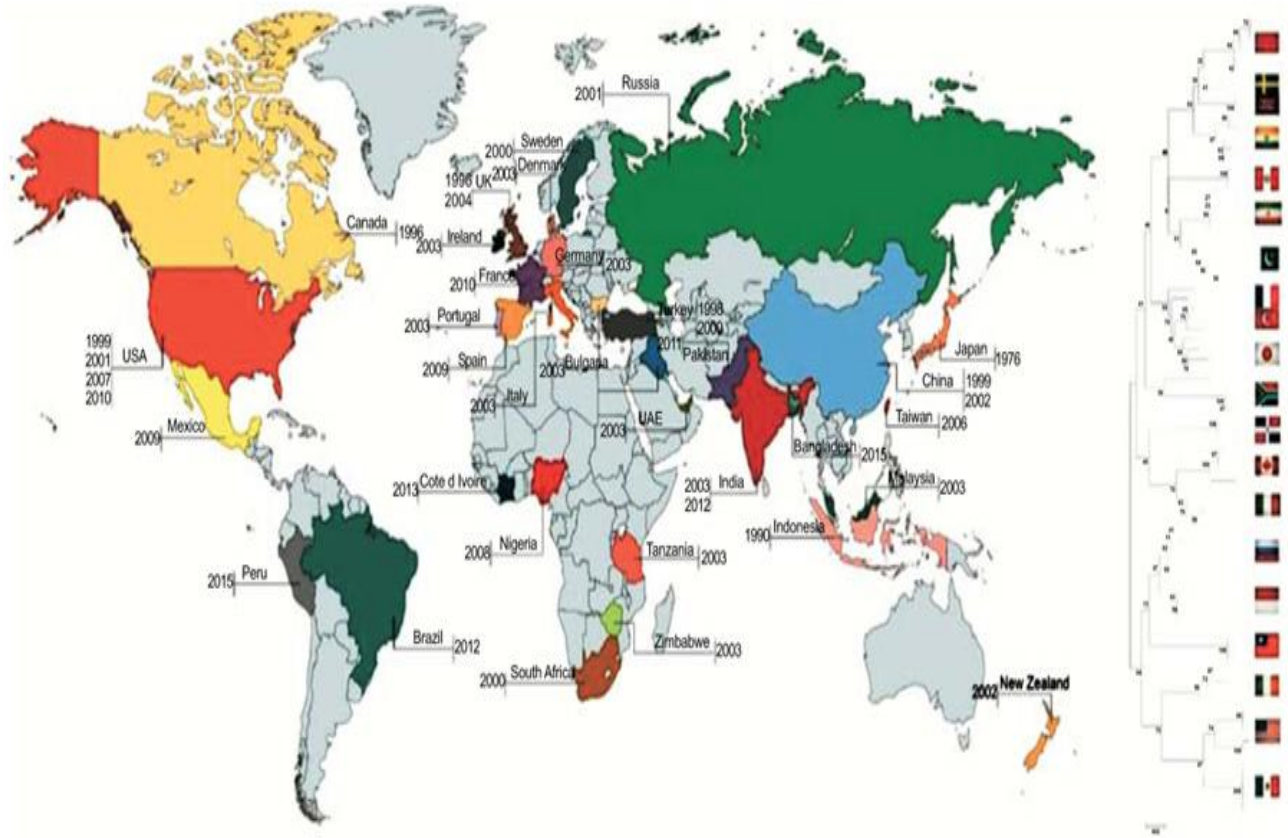

Figure 4: Newcastle disease virus (NDV) outbreaks in different parts of the world in the last decades (Rahman *et al*., 2018).

Based on their pathogenicity, NDV strains are classified into three major groups such as velogenic which is a highly virulent NDV infection results in acute onset, highly lethal disease. Mesogenic is a moderate virulence that causes acute, moderately lethal disease with nervous and respiratory signs. Lentogenic is low virulence strains (Orsi *et al*., 2009 and Ahmed *et al*., 2017). Velogenic and mesogenic forms are widespread in Asia, Africa, and Latin America.

2.4.1 Velogenic Newcastle Disease Virus

This form of virulence is characterized by high flock morbidity accompanied by respiratory and nervous signs and very high mortality. In egg-producing flocks, it causes shellless eggs and reduces egg production. The repeated sign of velogenic Newcastle disease is depression, nervous signs, increased respiration, inappetence, greenish-yellow diarrhea that leads to dehydration and collapse, swollen heads, and cyanotic combs. Infected birds usually die within one or two days and the mortality rate reaches near 90% (Abdisa and Tagesu, 2017). The mortality rate is usually higher in young birds compared to the adult.

2.4.2. Mesogenic Newcastle Disease Virus

Mesogenic virulence Newcastle disease is characterized by a moderate mortality rates, nervous and respiratory signs, an acute drop in egg production due to the presence of shell less eggs, loss of weight, depression, and signs of the nervous system that can develop late in the disease (Samrawit and Mulat, 2018).

2.4.3. Lentogenic Newcastle Disease Virus

Lentogenic ND may be capable of drops of egg production without showing clinical signs of an incompletely immunized commercial layer or breeder flock (Daniela *et al*., 2000). The morbidity rate is moderate to high. The lentogenic Newcastle disease doesn't show nervous signs but shows inapparent respiratory signs and a small drop in egg production (Samrawit and Mulat, 2018).

2.5 Status of poultry production and Newcastle Disease in Ethiopia

Ethiopia has approximately 60% of the total poultry population of East Africa; it was estimated at over 60 million in 2019 (Central Statistical Agency, 2020). In terms of the production system, 95% of the total poultry populations are contributed by village poultry production while only 5% is from commercial poultry farms. Concerning the breed of poultry were reported 81.71 % indigenous, 10.86 % of hybrid, and 7.43 % exotic (Zerihun and Temesgen, 2018).A 96 % of the total national chicken population held by four regional states, Oromia region has the biggest number of chickens around 24 million, followed by Amhara, SNNPR, and Tigray. The remaining 4 % are mainly distributed among rest regions.

The main challenge in the poultry industry is high chicken mortality due to diseases and predators prevalent in the different production systems (Alexander, 2009). For instance, about 32 million birds died due to disease in 2016-2017 (Figure 5). Like Newcastle disease (ND), Salmonellosis, Marek's disease, Infectious bursal disease (IBD), Mycoplasmosis, Colibasilosis, Coccidiosis, Heliminthosis, and Toxoplasmosis (FAO, 2019). According to Samrawit and Mulat (2018), ND is identified to be the most important disease in all production systems, being responsible for the largest proportion of morbidity and mortality in all parts of the country. The National Veterinary Institute (NVI) produces different kinds of vaccines, including against major poultry diseases such as ND and fowl pox. The institute produces a range of ND vaccines such as HitchnerB1, LaSota, and Thermostable I2 (NVI, 2019).

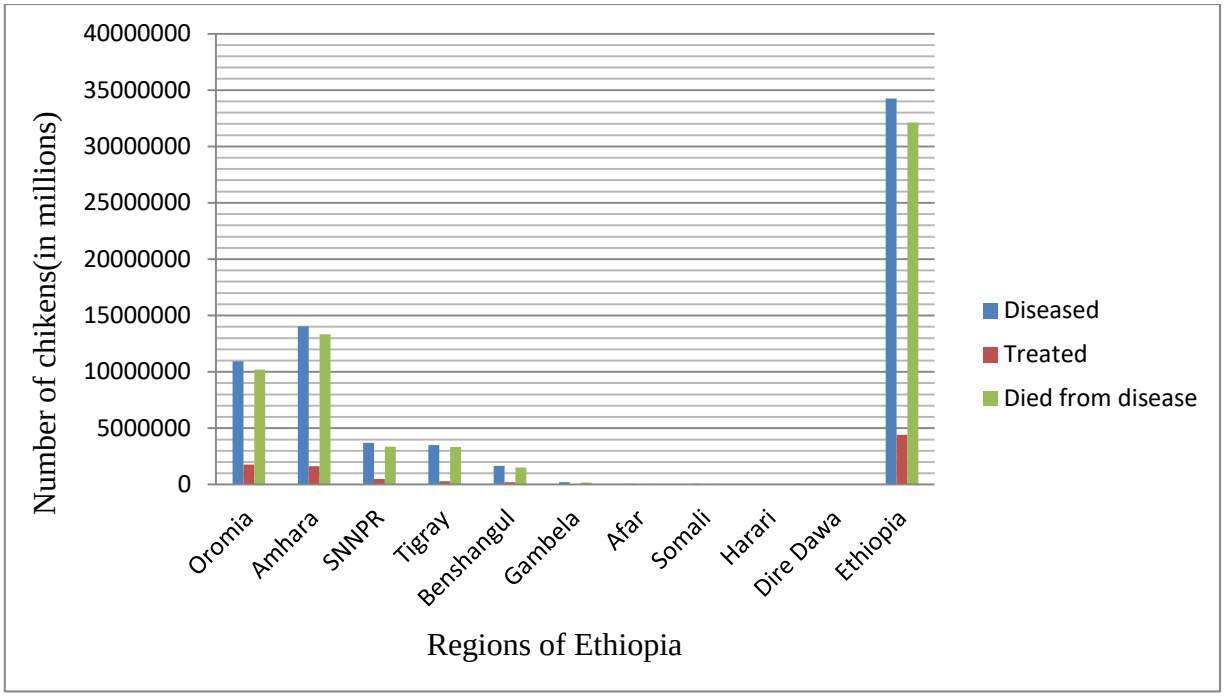

Figure 5: Number of chickens afflicted, treated, and died chicken due to disease in different parts of Ethiopia, 2016 (CSA, 2018 and FAO, 2019).

In Ethiopia, in 1971, the first outbreak of Newcastle disease was reported from a small poultry farm in Asmera, Eritrea (National Veterinary Institute (NVI, 1974), and the strain of this ND was a velogenic type, which was classified according to the virulence of the strain and caused about 80%-100% mortality (Ashenafi, 2000). In the following years, the disease was widely distributed in all areas of Ethiopia in 1972, outbreaks had been reported in Addis Ababa and in 1974 at the Haramaya College of Agriculture poultry farm reported by Ashenafi, (2020). And in 1995, ND outbreaks in the surrounding areas of Bishoftu, Adama, and Addis Ababa killed almost 50% of the village chickens (Ashenafi, 2000). The highest rate of outbreaks from March to May is suggested to be associated with the high rate of chicken marketing for Easter (Samrawit and Mulat, 2018). End of May and beginning of June are the start of the main rainy season. (Zerihun and Temesgen, 2018). Ashenafi (2000) has also reported the occurrence of the disease all year round.

Variance prevalence's had been reported for NDV antibodies in previous research, ranging from 5.6% (Belayheh *et al*., 2014) to 47.6% (Zeleke *et al*., 2005). According to the MOA (2018), ND outbreak was reported 205 times from 2011 up to 2015, among this, many reports from Oromia region 74.6%, followed by Amhara 11.7%, Addis Ababa 10.5%, SNNP 6.3%, Benishangul Gumuz 3.9%, and Tigray 2.9% but the rest regions have not report of ND outbreak (Table 1).

Table 1: Molecular and sero-prevalence of NDV in the Rift Valley and surrounding areas of Ethiopia.

R. No	Study area	Prevalence (%)	References
1	Rift Valley	22.5	Ashenafi (2000)
	Central high land	47.3	
2	Rift Valley and southern (Hawassa)	12.9	Zeleke *et al*. (2005)
	Over all	19.78	
3	Adama	38	Serkalem *et al*. (2005)
4	Rift Valley	11	Regasa *et al*. (2007)
5	ATJK	5.9	Chaka *et al*. (2012)
	Ada'a	6	
6	ATJK	16.73	Chaka *et al.* (2013)
	Ada'a	32.2	
7	Kersa Kondaltity	5.6	Belayheh *et al*.(2014)
8	Addis Ababa	30	Delesa *et al*.(2014)
9	Rift Valley	11.6	Dehinenet *et al.* (2015)
10	Ada'a	28.6	Desalegn (2015)
11	Bishoftu	20	
	Hawassa	15	Gelan (2017)
	Batu	15	
12	Central Rift Valley	15.48	Ashenafi (2020)

2.6 Host factor and NDV in wild birds

Newcastle disease infects a wide range of domestic and wild bird species worldwide, more commonly affected species include chicken, turkeys, ducks, pigeons, guinea fowl, Japanese quail, and many wild birds of both sexes and all ages (Alexander, 2009). Most of the avian species are vulnerable to NDV including chickens and some mammals like humans, cats, and dogs. Class I NDVs are mostly avirulent for chickens, and it is occasionally isolated from wild aquatic birds and domestic poultry. Class II contains most viruses that are virulent and cause significant economic losses to the poultry industry worldwide (Dehinenet *et al*., 2015), which have been isolated from multiple wild birds and poultry species. Isolates of NDV have been obtained frequently from wild birds, mainly migratory feral waterfowl and other aquatic bird viruses have been detected, as it is low virulence for chickens, but sometimes virulent viruses have been detected in wild birds and were those dead birds found near infected poultry. The most significant outbreaks of NDV reported in double-crested cormorants (Phalacrocoraxauritus) in North America since the 1990s in wild birds (Alexander, 2009).

Newcastle disease virus was responsible for most outbreaks in the UK in 1997 had introduced by migratory wild birds (Alexander *et al*., 2000). The same virus responsible for the outbreak in Denmark in 1996, it kills many free-living pheasants, this virus are closely related and were isolated from goosander in Finland in 1996 and a cormorant from Denmark in 2001 (Alexander *et al*., 2000 and Aldous *et al*., 2003), perhaps significantly for the poultry industry. In 2005 in Great Britain and France, NDV has been reemergence in pheasants that as a genetically very closely related virus in French (Loire Atlantique), some hypothesize led to that the virus may be established in some species of wild birds in Europe and come to the farm to a lake (Alexander, 2009).

According to Giovanni *et al*. (2011), the advancement and improved techniques for nucleotide sequencing and analysis, the availability of sequence data of NDV placed in computer databases, and the demonstration that even relatively short fragments or sequence lengths could give meaningful results in phylogenetic analyses have led to a considerable

increase in such studies in recent years. Mostly genetic diversity has been detected and classified with the virus, but viruses sharing antigenic, temporal, geographical, or epidemiological guideline tend to fall into specific ancestry or clades and this has shown valuable in assessing both the global epidemiology and local spread of ND (David *et al*., 2003).

2.7 Diagnosis

Newcastle disease can be diagnosed based on history, clinical signs, and laboratory tests. Newcastle disease clinically resembles highly pathogenic avian influenza, so during an outbreak rapid and accurate diagnosis is important to control and prevent dissemination of the disease (Ashraf and Shah, 2014). Different laboratory techniques such as virus isolation, serological (enzyme-linked immune sorbent assays (ELISA), immune diffusion test, agar gel precipitation, and molecular test (Reverse transcription-polymerase chain reaction (RT-PCR) are important for the diagnosis of Newcastle disease virus. Isolation of the ND virus is a definitive diagnosis of NDV (Mark *et al*., 2003).

2.7.1 Diagnosis based on clinical signs and post-mortem

The clinical sign of NDV depends on age, immune status of the host tissue tropism, and virulence of the virus strain. Sudden high mortality in a flock in the absence of premonitory clinical signs occurs when susceptible species are exposed to highly virulent strains. In susceptible flocks, the mortality rate in full can reach 100% (Samrawit and Mulat, 2018). The incubation period of ND is usually about five days. In the chicken's nerve, respiratory and digestive signs may occur. There are different important clinical signs observed in Newcastle disease such as greenish-white diarrhea, with ruffled feathers; depression in birds, and a state of prostration, a condition known as torticollis or stiff neck (head turned to one side), and other neurological signs like paralysis of leg and wing. ND is an acute disease that can cause death within 2 to 3 days (Alexander, 2009). Necropsy lesions caused by velogenic APMV-1 virus have mainly been characterized in poultry, especially chickens (Delesa *et al*., 2014). Both the Viscerotropic velogenic and neurotropic velogenic strains of NDV cause

hemorrhagic lesions, particularly in the mucosa of the proventriculus, small intestine, and ceca, but in the respiratory tract, gross lesions are not observed. Hemorrhages of the thymus and bursa of fabrics are also possible in older birds, but they are less common (Stivalis *et al*., 2015).

2.7.2 Laboratory diagnosis

The clinicopathologic picture of ND gives important clues in making clinical diagnosis. However, different types of viral and bacterial diseases may be shown similar clinical features that could be confused with ND. The routine differentials of ND include highly pathogenic avian influenza, a diphtheritic form of fowl pox, infectious laryngotracheitis, and infectious bronchitis. Others include fowl cholera, mycoplasmosis, and psittacosis in psittacine avian species (Roy *et al*., 2000). Distinguishing ND from all these diseases is a crucial task in arriving at the tentative diagnosis.

2.7.2.1 Sample for laboratory diagnosis

The appropriate sample for diagnosis of Newcastle disease includes tissue samples (trachea, lung, spleen, soft palate, colon, bursa, and brain) (Bello *et al*., 2018), which are important for histopathology and cloacal swabs, oro-nasal swabs, and Serum sample (OIE, 2013). A blood sample is usually collected from a wing vein. When the fresh sample is collected, the lung and spleen should be wrapped in plastic and placed into a cold box on ice. To do serological tests, haemolysed or contaminated samples should not be used because it will give unreal results (Ahmed *et al*., 2017).

2.7.3 Virus isolation

The virus is an obligate intracellular parasite that requires living cells to replicate. For virus isolation may use cultured cells, eggs, and laboratory animals. For diagnosis APM-1 infection virus isolation in embryonated eggs or cell cultures serves as important for viral isolation (Bernd *et al*., 2009). Cloacal and Tracheal swabs are a good sample for viral isolation of NDV.

Samples from fresh feces may also use as an alternative. The collected sample should be transported at pH 7.0-7.4 in isotonic phosphate-buffered saline (PBS), containing antimicrobial drugs and media proteins. Higher concentrations of antibiotics should be used when the collected sample is from the feces of suspected chickens. Virus isolation in culture followed centrifugation of the sample from feces or tissue for 10 minutes at temperature 25°C to obtain the supernatants samples and measure 0.2 ml of sample and inoculated into the allantoic cavity of embryonated SPF fowl eggs of 9 -11 days' incubation then incubate for 4-7 days at 35-37°C. If the tests give a positive result, embryonated eggs will die. Finally, all eggs that remained from incubation should be chilled to 4°C and done for haemagglutination test (HA) (Mark *et al*., 2003 and Alexander, 2000).

2.7.4 Molecular based assays

According to Jingjing *et al*., (2017), Molecular diagnostics is one of the most energetic and transformative areas of diagnostics, principal to advances in research and treatment that are revolutionizing health care across an extensive set of diseases and health conditions. This diagnostic test assesses the features of the disease at a molecular level, detecting and measuring specific genetic sequences in deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or the proteins they express. In the last decade, molecular diagnostics has undergone a period of rapid development and growth. The introduction of modern high-complexity tests and the incorporation of new technologies into the clinical molecular diagnostics laboratory are essential for achieving the goal of precision medicine. Nowadays, this technology is applied for the identification and investigation of Newcastle disease. Molecular techniques such as polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR) have been used for rapid and sensitive detection of Newcastle disease (Ahmed *et al*., 2017).

2.7.4.1 Reverse transcription polymerase chain reaction (RT-PCR)

Molecular techniques like reverse transcription-polymerase chain reaction (RT-PCR) have been frequently used around the world to detect viruses from field samples (Alexander and

Senne, 2008). Reverse transcription polymerase chain reaction (RT-PCR) is used to detect RNA virus, which is a negative and single-stranded RNA virus. There are two different configurations of the RT-PCR assay. In the two-step RT-PCR configuration, the cDNA is synthesized in a different tube before performing a PCR assay. In contrast, a one-step RT-PCR firstly synthesizes the cDNA. The reverse transcriptase is inactivated and the polymerase is activated simultaneously and the PCR reaction is carried out in a single tube (Bello *et al*., 2016). This is rapidly becoming the assay of choice. Several important steps need to be considered in developing an RT-PCR protocol. The first aspect is the RNA extraction. This needs to be an efficient process that can extract RNA from the samples even when it's in low concentrations and eliminate contaminants that will degrade the RNA. Another important aspect is the gene being targeted and the choice of primers. This can have a profound effect on the efficacy of the assay. Bad designed primers can result in mispriming and amplification of nonspecific products or the formation of primer dimer (Giovanni *et al*., 2011).

2.7.4.2 Nucleotide sequencing

Nucleotide Sequencing (NS) is one of the most recent tools that have revolutionized the diagnosis of infectious diseases. In addition to that it is important for tracking disease epidemics, it also facilities the rapid, sensitive, and specific detection and differentiation of mixed infections within a single host. It can also be used to detect low-frequency variants that would otherwise escape detection using common diagnostic tools. In addition, it is currently the most throughput tool used in the discovery of novel viruses associated with unknown diseases (Palya *et al*., 2012). Hence, its application in the development of advanced diagnostics cannot be overemphasized. Although different platforms of NS are constantly emerging, they all technically involve three major steps: sample preparation, sequencing, and data analysis (Bari *et al*., 2009). Variations exist largely in the sequencing techniques. Currently, most sequencing platforms aimed at viral diagnosis are focusing on improved sequence reads and the speed of the assay. Recently, NS-based characterization of genotype VI NDV in the United States has been reported to reveal a previously unknown genetic diversity of the virus with evidence of its continuous evolution (Bello *et al*., 2018). In addition, NS has recently been applied to simultaneously characterize the genomic sequences

of multiple avian paramyxoviruses. More so, differentiation of virulent from avirulent strains of class II NDV was successfully and rapidly achieved using NS based on a pyrosequencing procedure (Murtadha, 2016). These attributes completely make NS the most promising emerging technique for NDV discovery and diagnosis. Therefore far, the only disadvantage of the assay is its perceived high cost of the sample run. However, with increasingly cheaper sequencing services, NS tools are likely to become routinely available for viral diagnosis given their speed, accuracy, and multiplexing ability (Aris *et al*., 2016).

2.8 Control and prevention

The comprehensive approach to the control of Newcastle disease are hygiene and vaccination, this is critical in the control of ND in all farming systems where birds are confined within a fenced yard or house (Daniela *et al*., 2000). According to Abdisa and Tagesu (2017), Hygiene measures include such as cleaning, disinfection, limiting access to wild birds, and personal hygiene of the farm staff, vaccination in combination with appropriate hygiene measures, remains the very powerful way of controlling ND (Orsi *et al*., 2009). According to Muhammad *et al*., (2018), Vaccination against ND would result in immunity against infection and replication of the virus, certainly, ND vaccination usually protects the bird from the more serious effect of the disease, but virus replication and shedding may still occur in some cases. The control of ND in village chicken farms by Applying strict quarantine or biosecurity measurements, but the result is not too much because of the difficulty in monitoring the movement of people, chickens and wild birds, inadequate management of the farm, market places, transportation, vaccination, and some environmental factors (rainfall, humidity, nutrition, disease by itself and others) (Zerihun and Temesgen, 2018).

2.8.1 Vaccination

Vaccination is the most important method of control and prevention Newcastle disease. Currently, both inactivated and live vaccines for NCD are available in Ethiopia produced by NVI (NVI, 2019). There is also a thermostable vaccine which was specifically developed to be used in village chicken. We can use variety of routes for the administration of live vaccines and schedules from hatching until grow-out. Killed virus oil emulsion vaccines are

administered parentally advanced to the beginning of egg production. Lentogenic virus vaccines are for the most part suggested in drinking water, by eye drops, by aerosols, or intranasally. A vaccine using a heat-tolerant V4 strain has been advanced for feeding to village chickens in countries where these account for a significant proportion of poultry production. Although proper vaccination protects birds from clinical disease, it does not prevent virus replication and shedding, which results in a source of infection (Alexander, 2000).

There are various reasons for vaccination failure, which may include: (a) high levels of maternal antibodies in young chickens which may interfere with the multiplication of live vaccines, thus reducing the amount of immunity produced (Sanda and Joshua, 2013). (b) Stress which reduces the chickens' ability to mount an immune response. According to Abdisa and Tagesu (2017), stress could include environmental extremes (temperature, relative humidity), inadequate nutrition, parasitism, and other diseases. (c) Inactivation of live vaccines due to improper handling such as not maintaining a cold chain (Nesradin and Nejash, 2017), (d) use of vaccines that do not contain the proper strains or serotypes of organisms required to stimulate protective immunity (Hailu, 2012). (e) immunosuppression due to infection with infectious bursal disease virus, Marek's disease virus, or chick anemia virus, and consumption of feed with high levels of mycotoxins. (f) use of vaccines that are of poor quality (low vaccine titer or contaminated). Vaccine efficiency is the vaccine itself - its titer, stability, serotype, quality, inactivation, and adjuvants (Palva *et al*., 2012). However, good manufacturing practices should ensure that vaccines are highly unlikely to be carriers of virulent NDV. However, in the past, birds have become infected when vaccines against other diseases have been contaminated with NDV, or as a result of failure to inactivate killed vaccines prepared from virulent NDV (Muhammad *et al*., 2018).

2.8.1.1 Live vaccine

Live vaccines are relatively cheap, sold as freeze-dried, easy to administer, and can be used for mass vaccination. These have been divided into mesogenic and lentogenic groups with their preferred mode of administration being eye drop, beak intranasal installation, or dipping for lentogenic vaccines, while the mesogenic vaccine requires an intramuscular injection.

Drinking water and aerosol administration can also be used. In most countries, Hitchner B1 and La Sota vaccines are used and are derived from the mesogenic strain of NDV (Bello *et al*., 2018). Some mesogenic vaccines may cause disease, especially in young birds. Because of heat liable, live vaccines are also having disadvantages under the village management system where transport and cold storage facilities are often inadequate (Alexander and Senne, 2008).

2.8.1.2 Inactivated vaccine

Inactivated vaccines can be used in situations unsuited for live vaccines and induce a high level of protective antibodies over a long period of time. These vaccines are produced from infective allantoic fluid of virulent NDV treated with B-propiolactone or formalin to kill the virus and then mixed with an adjuvant. The vaccine can administer by subcutaneous or intramuscular injection (Nesradin and Nejash, 2017).

3. MATHERIALS AND METHODS

3.1 Description of the study area

The current study was performed in selected Mid-Rift Valley areas (Bishoftu, Batu, Shashemene, Hawassa, and Arba Minch towns) from February 2020 to October 2020 by collecting swab samples from village chickens. Post mortem samples were also collected from chickens from central part of Ethiopia (Addis Ababa, Adama, and Bishoftu towns) (Figure 6). The Mid-Rift Valley areas are bounded by high land plateaus characterized by a semi-arid type of climate, with the mean annual rainfall ranging from 660mm to 1113mm. Around 583 species of birds were reported so far from the Rift Valley region of Ethiopia. It has been estimated that around 15 to 16 million chickens are found in this region (Zerihun and Temesgen, 2018) that are regularly exposed to migratory birds due to the existence of abundant lakes. Generally, these study areas were encompassing about 15-40km in width and more than five hundred kilometers in length.

Adama, Bishoftu, and Batu towns were located in the East Shewa zone, Oromia Regional State. The Zone extends between 7^0 33' N – 9^0 08' N and from 38^0 24' E – 40^0 05' E, with a total area coverage of approximately 8,370.90 square kilometers. Since a large part of the Zone is situated along with the Rift Valley system, rainfall ranges from 600 mm to 1000 mm with an average annual rainfall of 816 mm, with the temperature range from 10ºC in the uplands to over 30ºC in the depressions of the Rift Valley with a mean temperature of 20ºC. And the total population of poultry in the zone is around 1,701,129 (CSA, 2020).

Shashemene town is found in the West Arsi zone of the Oromia Regional State, located at 250 km south of the Addis Ababa, situated between 7^0 11'09" to 7^0 13'19"N latitude and 38^0 35'02" to 38^0 37'05"E longitude. Its annual average temperature ranges between 18ºC and 25ºC and has moderate annual rainfall ranging between 800 and 1300mm. The poultry population of the West Arsi Zone is estimated to be 731,044 (CSA, 2020).

Both Hawassa and Arba Minch towns are located in Southern Nation, Nationality and People

(SNNP) region. Hawassa town has a latitude and longitude of 7°3′N 38°28′E with an elevation of 1,708 meters, and the city is located at 273 km south of Addis Ababa. Arba Minch town is located at 500 kilometers south of Addis Ababa, at an elevation of 1285 meters above sea level. The total population of poultry in SNNP is around 8,147,205 (CSA, 2020). Addis Ababa is the capital and the largest city of Ethiopia and is situated between 9°01′01″ N and 38°45′08″ E (Chaka *et al*., 2013).

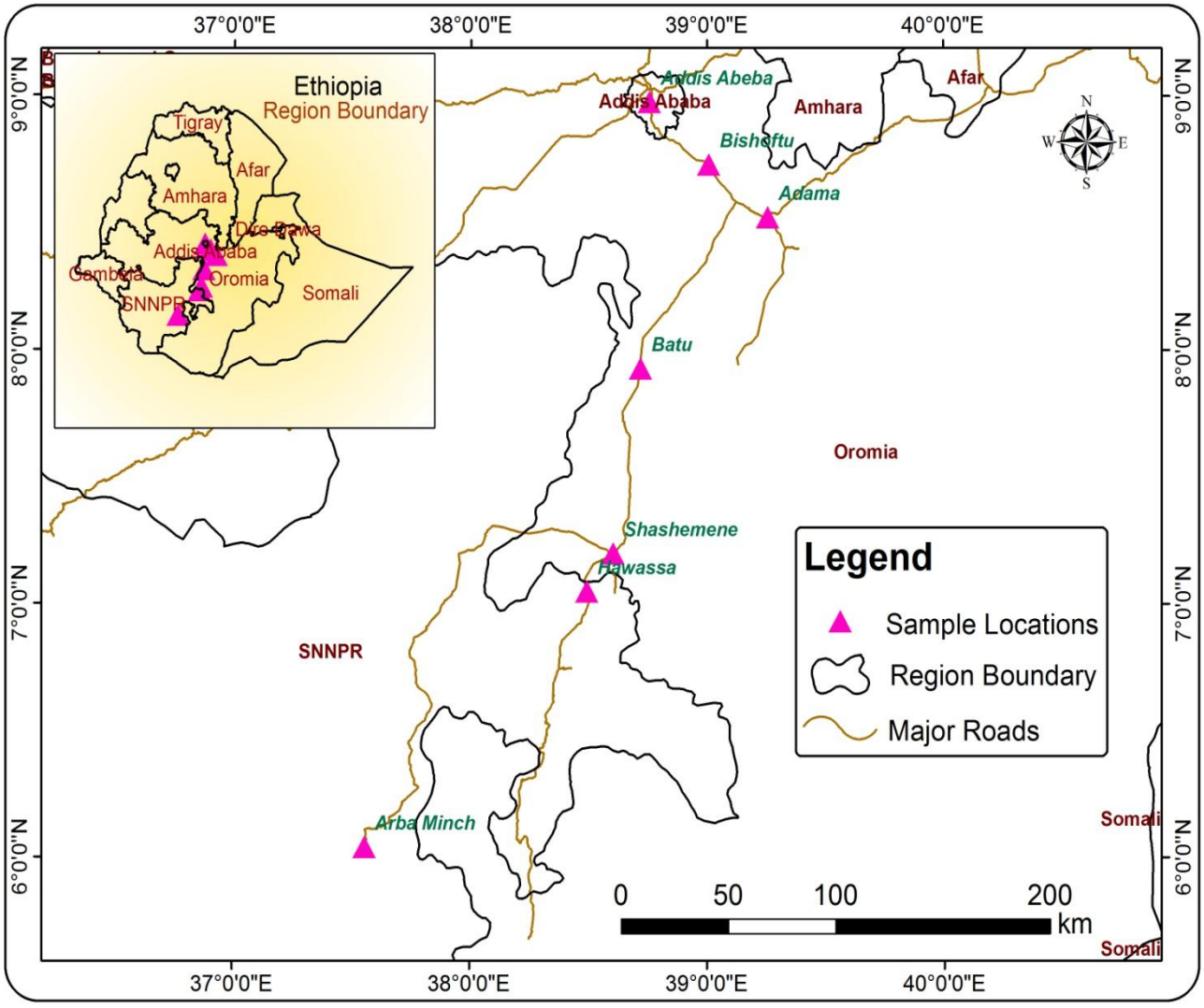

Figure 6: Map showing study areas in the mid Rift Valley area and central part of Ethiopia (by ArcGIS 10.4.1, Own Presentation).

3.2 Study population

The study populations were chickens in migratory birds' abundant areas in mid Rift Valley and central part of Ethiopia.

3.3 Study animals

The study animals were chickens selected from commercial and non-vaccinated village chickens from migratory birds' abundant areas in Mid-Rift Valley and central part of Ethiopia. Village and commercial chickens were sampled based on clinical signs and symptoms of Newcastle disease (Alexander and Aldous, 2001), districts (location), sex, age, breed, management system of the farm. Chicken's age category was determined based on their history from the owners (Belayheh *et al*., 2014 and Gelana, 2017): young 3 - 6 months and adults greater than six months. The management system of the farm under village or commercial was determined as per Kondombo *et al*. (2003) on the presence of wild birds on the farm.

3.4 Study design

A cross-sectional study design was performed for NDV detection and strain investigation from village (non-vaccinated) and commercial chickens located in the study area by collecting swab samples from February 2020 to October 2020, as well as post-mortem sample collection. The samples were collected randomly twice in a row from the local markets located in the study area.

3.5 Sample size determination

The sample size was calculated based on the prevalence of 16.7 % previously reported by Gelana (2017), using a 95% confidence level and 5% marginal error. Hence, the sample size was determined using the statistical formula described by Thrusfield (2005).

$$n = \frac{Z^2 P_{exp}(1-P_{exp})}{d^2}$$

Where;

n= Sample size

z= Statistic for a level of confidence

d^2= Required absolute precision

P_{exp}= Expected prevalence

Therefore, at the 95% confidence level, the sample size is:

$$n = \frac{1.96^2 Pexp\ (1\text{-}Pexp)}{d^2}$$

$$n = \frac{1.96^2 \times 0.167\ (1\text{-}0.167)}{(0.05)^2}$$

$$n = \frac{3.8416 \times 0.167\ (0.833)}{0.0025}$$

n= **214**

Hence, a total of 214 chickens were required to be sampled (tracheal and cloacal swabs) for determination of the molecular prevalence and strain identification of NDV. However, to improve the accuracy of the study, 390 chickens were randomly selected based on clinical signs and symptoms of NDV in from village, commercial, and poultry markets in the study area. To boost the study's accuracy, additional postmortem tissue samples were obtained from 20 chickens in Adama, Bishoftu, and Addis Ababa (Kality).

3.6 Sample collection

A total of 390 live chickens swab samples were collected using a random sampling technique from the village, commercial, and poultry markets of Bishoftu, Batu, Shashemene, Hawassa, and Arba Minch towns and equally distributed on consecutive market days in the study areas. Tracheal and cloacal swab samples were obtained from live chickens by inserting a sterile cotton-tipped swab into the trachea, gently swabbing the wall, and the cloacal swabs deep into the vent, swabbing softly through the vent wall. Both the tracheal and cloacal swab (pools of five) samples were put in separate sterile cryovials (2 ml volume) containing 500 µl of newly prepared viral transport media (VTM). All samples were labeled during collection (Annex III Figure 13 and 14), placed in an ice pack (4°C), and transported to the National Animal Health Diagnostic and Investigation Center (NAHDIC) molecular biology laboratory; and stored at -80°C before processing (Annex III Figure 15). Samples of sick and freshly dead birds from Adama, Bishoftu and Addis Ababa were collected for postmortem examination at NAHDIC. Brain, lung, tracheal, and tissue pools (of five) (from liver, kidney, heart, and spleen), and intestine were collected from twenty individual chickens and put in viral transport media (VTM). The collection and transport of samples were carried out according to the standard techniques recommended by the OIE (OIE, 2013).

3.7 Data collection by questionnaire survey

A total of 39 volunteer village chicken owners/individuals and sellers were interviewed by using a structured questionnaires (Annex II) to determine the risk factors for ND, such as wild bird cross-contact with village chickens, and individual respondents' knowledge of the causes, transmission, prevention, and control methods of ND; and to assess the socio-economic situations of village chicken producers.

3.8 RNA extraction

Viral RNA extractions from tracheal, cloacal swabs and postmortem tissue samples were performed using QIAamp Viral RNA Mini Kit as directed by the manufacturer (Qiagen,

2020). Briefly, samples collected with viral transport medium were centrifuged for 1 minute at 8000 rpm to obtain cell-free supernatant of the samples. Then, 140 µl of the samples were added in 560 µl of buffer AVL containing carrier RNA for lysis and then mixed. After 10 minutes of incubation at room temperature, 560 µl of 96 percent ethanol was added to the mixture and thoroughly mixed again. Then, the total samples were transferred to the mini-spin column for binding and centrifuged for 1 minute at 8000 rpm, replacing the old collection tube with the new collection tube. About 500µl (AW1) washing buffer was added to each sample and centrifuged for 1 minute at 8000 rpm by using a new collection tube again, to eliminate any unnecessary protein and others, a 500 µl washing buffer (AW2) was applied and centrifuged at a maximum speed of 14,000 rpm for 3 minutes. Again, without adding anything, the new collection tube was used and centrifuged for 1 minute at a maximum speed of 14,000 rpm. Following the addition of 60 µl of elution buffer (AVE) to the mini-spin column, a new and sterile 1.5 µl micro-centrifuge tube was used for final collection and incubated at room temperature for 3 minutes. Finally, for preservation, the eluted RNA was kept at -80°C for later analysis.

3.9 Real-time quantitative polymerase chain reaction (qRT-PCR)

Using an Applied Biosystems 7500 fast real-time PCR thermocycler, the following master mix reagents per 25 µl reaction: 5 µl of PCR buffer (5x), 0.5 µl of each primer (10 pmol), 1 µl of the probe (6 pmol), 0.8 µl of deoxynucleoside triphosphate (dNTPs), 1.25 µl of $MgCl_2$ (25 mM) and 0.5µl of RNase inhibitor (13.3 u/µl) and 1 µl of enzyme and 6.45 µl of the Rnase free water mix were used (QIAGEN). The following sequences of forward primer M+4100-5'-AGT GAT GTG CTC GGA CCT TC-3 ', reverse primer M4220-5'CCT GAG GAG AGG CAT TTG CTA-3' and a Primer Probe of M+4100- 5'FAM- TTC TCT AGC AGT GGG ACA GCC-TAMRA-3’ were used for M-gene assay. All reagents were mixed in a single reaction tube (master mix) to ensure the reagents were distributed homogeneously. Depending on the number of samples to be tested, aliquots of 17 µl of the prepared reagents were distributed into the applied biosystem plate. Finally, 8 µl of RNA template was added to each plate to obtain a total reaction volume of 25 µl. Known positive NDV antigen and Rnase free water were used as positive and negative controls, respectively. To avoid evaporation, the plates were sealed

with a sealer and placed in a thermal cycler machine that was connected to the computer with its own software (Applied Biosystems Sequence Detection Software Version 2.3), and the program was adjusted accordingly for each specific reaction. The reverse transcription (RNA to cDNA) stage was performed at 50°C for 30 min, followed by incubation at 95°C for 15 min. The cycling conditions for the M-gene assay were programmed for 40 cycles of 10-second denaturation at 94°C, 30-second annealing at 52 °C, and extension at 72°C for 25-second (Wise *et al.*, 2004). At the extension phase of each cycle, the reporter dye (FAM) and quencher dye (TAMRA) signals were measured, and the threshold cycle (Ct.) for each sample was determined. Samples with a Ct. value of greater than 35 were considered negative for M genes based on qRT-PCR (OIE, 2013).

3.10 Conventional polymerase chain reaction

Conventional PCR was performed using NDV positive samples that were identified by qRT PCR using NOH-5'-TAC ACC TCA TCC CAG ACA GG-3 'forward primer sequences and NOH-5'- AGT CGG AGG ATG TTG GCA GC -3' reverse primer for sequencing the PCR product to precisely detect pathogenic strains of NDV. The following quantities of reagents were used per 25 µl reaction: 5 µl of PCR buffer (5x), 0.25 µl of each primer (20 µM), 0.5 µl of deoxynucleoside triphosphate (dNTPs) (10Mm), 0.1 µl of RNase inhibitor (40 U/µl), 0.5 µl of RT-PCR enzyme mix and 13.4 µl of the Rnase-free water (QIAGEN). Both reagents were combined in a single reaction tube (master mix) and 5 µl of extracted RNA or template were applied to 20 µl of the mixed samples to achieve a total volume of 25 µl for each respective tube and tightly capped to prevent evaporation. NDV antigen and RNase free water were used as positive and negative controls, respectively. The thermal cycler or PCR system was programmed for reverse transcription (RNA to cDNA) steps after the tubing was positioned and incubated at 50 °C for 30 minutes, followed by 94 °C for 15 minutes. The cycling conditions consisted of 35 cycles and each cycle had 30 seconds for denaturation at 94 ° C, 1 minute for annealing at 55 ° C, and extension for 1 min at 68 ° C and 1 cycle at 68 ° C for 7 min final extension (OIE, 2013).

3.10.1 Gel electrophoresis

Two µl of loading dye and 8 µl of amplified PCR products were mixed and loaded on a 2% agarose gel supplied power and run for 45-60 minutes. Then the gel was mounted under the UV transilluminator or Gel doc system that was connected for imaging on the device with its respective software (Gel Doc^{TM} **XR+**, version 5.0 of Molecular Image Lab software). Finally, the band's appearance or absence was noticed and the picture was recorded.

3.11 Sanger sequencing

Sequencing reaction was performed with primers NOH-forward (5'-TAC ACC TCA TCC CAG ACA GG-3') and NOH- reverse (5'- AGT CGG AGG ATG TTG GCA GC -3') which encompass 270 bp region of the fusion (F) gene. The master mixes for sequencing reaction were as follows: PCR product 2 µl, primer (1uM) (3.2ul), and Big Dye Terminator mix (1ul), 5x Big Dye Terminator reaction buffer (4µl) were brought to 8.8ul-9.8 in distilled H_2O and amplified at the following condition: 1 min at 95^0C for 1 cycle, for 35 cycle (30 s at 96^0C, 15 s at 50^0C, and 4 min at 60^0C), and held at 4^0C. After the run was completed, the reaction products were purified using sodium acetate-ethanol purification method. The purified products of sequencing reactions were analyzed on an ABI3500 24 capillary genetic analyzer (Sanger Sequencing Machine).

3.12 Data management and analysis

Collected data were encoded on the Microsoft Excel 2016 spreadsheet and stored in a computer before it was transferred to SPSS Version 20 for statistical analysis. The outcome was presented using descriptive statistics such as percentage and P-value. For raw sequence data reading and editing, ChromasPor version 2.1.9, and BioEdit version 7.2.5 software's was utilized. BLAST was used for comparative studies to collect additional gene sequences for Newcastle Disease virus Fusion gene from GenBank. Multiple sequence alignments were performed for phylogenetic tree construction to align the sequences as nucleotides using the ClustalW algorithm in MEGA 10.2.4.4 (Bello *et al.,* 2016). The Neighbor-Joining tree

construction method was used with Jukes–Cantor and Kimura nucleotide substitution model with the pairwise deletion option. 1000 bootstrap replicates have been used for phylogenetic tree construction.

3.13 Ethics approval

Sampling from animals were carried out according to the experimental practice and standards approved by the University Ethical Review Board (UERD) from Research Affairs Dean Office, Adama Science and Technology University (ASTU), with the verification number RECSoANS/BIO/03/2020 (Annex V), that is in accordance with the international guidelines for animal welfare.

4. RESULTS

4.1. Real time quantitative PCR (qRT-PCR) based molecular detection of APMV-1 in chickens

Out of the 98 pooled samples (78 swab and 20 tissue samples) tested by qRT-PCR for M gene assay, 13.26% samples were identified as positive for APMV-1 in chickens in the study area (Table 2), where it was highest at Addis Ababa (60%), followed by Arba Minch (20%), and Adama (20%), and the least prevalence was recorded at Bishoftu (13.6%). The results of Ct. values when read from Applied Biosystems 7500 fast real-time PCR thermocycler were presented in Figure 7.

Table 2: Prevalence of Newcastle disease by qRT-PCR tests with Ct. Values for M- gene in the Mid-Rift Valley and central part of Ethiopia.

Sample ID	Sample type	Location of sample	live or died animal	Ct Values
12517	Trachea Swab	Arba Minch	Live	32.21
12518	Cloacal swab	Arba Minch	Live	34.09
12519	Trachea Swab	Arba Minch	Live	32.59
12521	Trachea Swab	Arba Minch	Live	33.96
12522	Trachea Swab	Arba Minch	Live	33.99
12523	Cloacal Swab	Arba Minch	Live	34.08
		Mean Ct. value for M-gene		33.48
41984	Brain	Bishoftu	Died	19.94
41985	Lung & Trachea	Bishoftu	Died	27.90
41986	Pool tissue	Bishoftu	Died	30.50
41992	Brain	Adama	Live	29.19
41997	Lung & Trachea	Addis Abeba	Died	28.24
41998	Pool tissue	Addis Abeba	Died	33.99
41999	Intestine	Addis Abeba	Died	31.56
NDV vaccine	Anti-gene	NVI	Live thermo stable	34.26
Positive control	NDV Antigen			21.64
Negative control	RNase free water			>35
		Mean Ct. value for M-gene		28.76

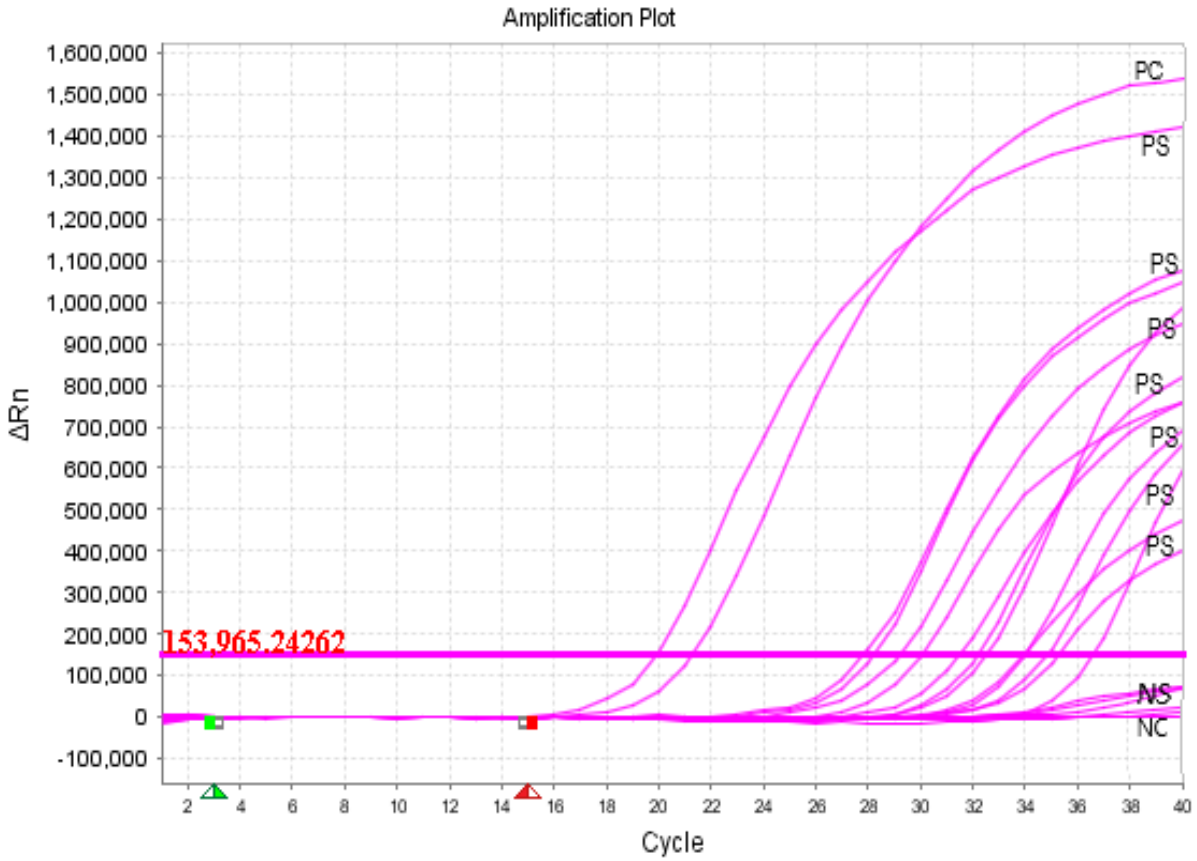

Figure 7: Amplification plot results of qRT-PCR with their Ct. Value positive sample (PS), positive control (PC), negative sample (NS), and negative control (NC).

Based on the swab samples, only the Arba Minch town chickens (20%) have NDV in the Mid-Rift Valley region with the mean Ct. value of 30.84 (Table 2) .Out of the 78 pooled swab samples tested based on the qRT-PCR of the M gene, 6 isolates were identified as positive for NDV. Among these, 4 (10.25%) tracheal swab pools (TS) were positive, although their corresponding cloacal swab pools (CS) of the same individuals were negative. Similarly, 2 (5.12%) CS was positive though their corresponding TS pools of the same individuals were negative (Table 3). There were no swabs that had shown a direct correlation where samples could be collected from both trachea and cloaca from the same individual animals.

Table 3: Detection rate of NDV by sample types (swab and tissue) in the study areas.

Sample type		No examined	No positive	Detection rate %	P-value
swab sample	Trachea swab	39	4	10.25	
	Cloacal swab	39	2	5.12	<0.05
Total		78	6	7.69	
Tissue sample	Brian	5	2	40	
	Lung & tracheal	5	2	40	
	Pool tissue (liver, kidney, heart, and spleen)	5	2	40	>0.05
	Intestinal	5	1	20	
Total		20	7	35	

Out of the 20 pooled tissue samples tested based on the qRT-PCR of the M gene, 7 isolates were identified as positive for NDV, where 2 (10%) were brain tissue pools, 2(10%) were lung & tracheal pools, 2(10%) were tissue pools (liver, kidney, heart, and spleen), and 1(5%) were intestinal pools (Table 3).

In the mid Rift Valley and central part of Ethiopia area, female chickens (11.47%) were infected than males (10.52%) for Newcastle disease; however, there was statistically no significant difference ($p > 0.05$) by sex (Table 4).

Table 4: Prevalence of ND in chickens by Sex and Age.

Categories	No. Examined	No. positive	Prevalence (%)	P-value
Sex				
Female	61	7	11.47	>0.05
Male	57	6	10.52	
Total	98	13	13.26	
Age				
Old	66	5	7.57	<0.05
Young	52	8	15.38	
Total	98	13	13.26	

The present finding revealed that the prevalence of ND was not similar in old and young chickens. 8.16% of young chickens were infected to the Newcastle disease virus out of the total population in the study area with a statistically significant difference by age ($p < 0.05$) (Table 4).

The current molecular findings indicated an overall prevalence of 16% (8/50) ND in village chickens, whereas 10.41% (5/48) overall prevalence in commercial chicken farms in the study area (Table 5). There was a statistically significant difference ($p< 0.05$) among the production systems. The highest prevalence of ND in village chickens was recorded in Arba Minch (41.66) followed by Adama (33.3%) whereas the highest prevalence of ND in commercial chicken farms was recorded in Addis Ababa (60%).

Table 5: Prevalence of ND in the production system (village and commercial) in the Mid-Rift Valley and central part of Ethiopia based on swabs and tissue samples.

	Districts	No. of chicken Examined		No. Positive		Prevalence (%)		p-value*
		Village	Commercial	Village	Commercial	Village	Commercial	
Swab Sample	Arba Minch	12	18	5	1	41.6	5.5	<0.05
	Shashemene	7	5	0	0	0	0	
	Hawassa	9	3	0	0	0	0	
	Batu	8	4	0	0	0	0	
	Bishoftu	6	6	0	0	0	0	
	Sub total	42	36	5	1	11.9	2.7	
Tissue Sample	Bishoftu	5	5	2	1	40	20	
	Addis Ababa	-	5	-	3	-	60	
	Adama	3	2	1	0	33.3	0	
	Sub total	8	12	3	4	37.5	33.3	
	Grand Total	50	48	8	5	16	10.41	

Based on the geographical location of the study area, the overall prevalence of ND in the Mid-Rift Valley area was found to be 10.75 % (Table 6) where the highest was at Arba Minch (20%) and Adama (20%) followed by Bishoftu (13.6). The overall prevalence of ND in the central part of Ethiopia was found to be 22.2%. There was a statistically significant difference ($p< 0.05$) among the study geographical locations.

Table 6: Prevalence of NDV based on the geographical location of the study area

Geographical location	No. Examined	No. Positive	Prevalence (%)	P- value
Mid Rift Valley of Ethiopia				
Arba Minch	30	6	20	
Shashemene	12	0	0	
Hawassa	12	0	0	
Batu	12	0	0	
Bishoftu	22	3	13.6	<0.05
Adama	5	1	20	
Total	93	10	10.75	
Central part of Ethiopia				
Bishoftu	22	3	13.6	
Addis Ababa	5	3	60	
Total	27	6	22.2	
Overall	120	16	13.3	

4.2 Conventional PCR- based molecular detection of NDV

Fourteen samples (thirteen isolates were positive for NDV and one Vaccine of NDV) were run by conventional PCR for sequencing purposes to precisely detect pathogenic strains of NDV (Figure 8).

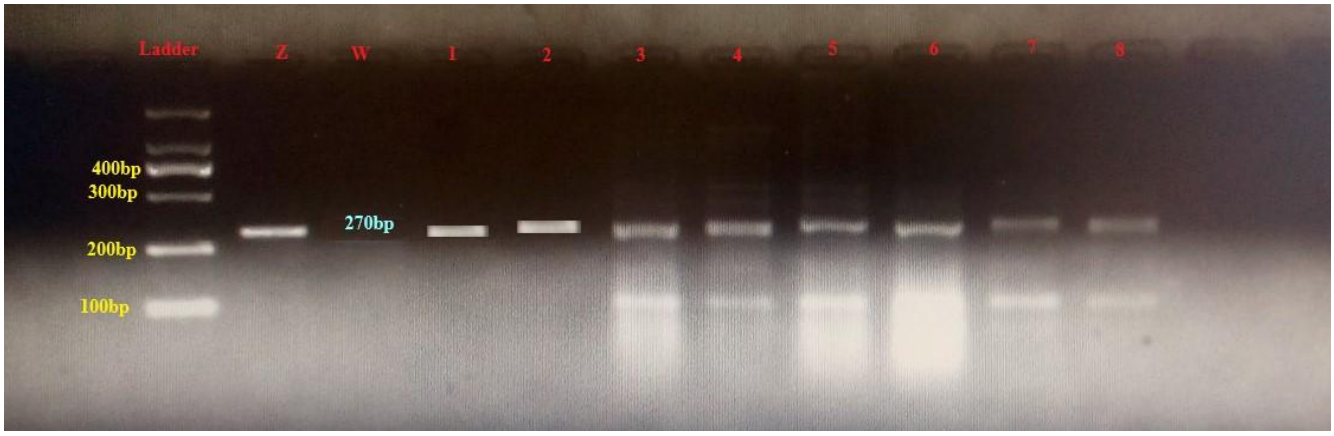

Figure 8: Gel electrophoresis in 2% Agarose gel for PCR amplification of F genes (270bp). The left lane is a marker or ladder of 1000bp, lane 1 up to lane 8 are samples, lane z is the positive control, whereas lane w is the negative control.

4.3 DNA sequence result

The sequences of the current isolates (NDV41984 ethi2021, NDV 41997 ethi2021, NDV 12519 ethi2021, NDV 41992 ethi2021, and NDV24HB1 ethi2021) were from different areas). The chromatogram file Sanger sequencing results are presented in (Supplementary Figure 1) and the deduced amino acid sequences from positions 112 to 117 F gene sequences showed that the field isolate of NDV in this study, NDV41984 ethi2021, is belonging to velogenic strain (motifs RRQKRF) by BLAST analysis. Similarly, our analysis revealed that NDV 41997 ethi2021, NDV 12519 ethi2021, and NDV24HB1 ethi2021 (NDV vaccine) belongs to lentogenic strains of NDV with the motif GKQGRL (Figure 9), and the rest (NDV 41992 ethi2021) field isolate of NDV is not detected. The Newcastle disease viruses isolated from Arba Minch and Addis Abeba were found to belong to the lentogenic strain, and velogenic one isolate from the freshly dead chicken brain in Bishoftu. NDV41984 ethi2021 had amino acid sequences that were similar (78%) to reference sequences (Accession number: KC851848.1) in Ethiopia (Tsegaw *et al*., 2014), and more similar (98.32% to 97.49%) to reference sequences of the Iranian NDV strains (Accession number: KP771863.1, JX131358.1, JX131357.1, KU201415.1, KU201413.1, MG519857, KU201409.1, and KU201410.1). NDV of Ethiopian strains that were previously characterized and stored in the GenBank database

(KC851848.1), however, are less similar (61%) to the vaccine strain (NDV24HB1 ethi2021) widely used to vaccinate chickens in Ethiopia.

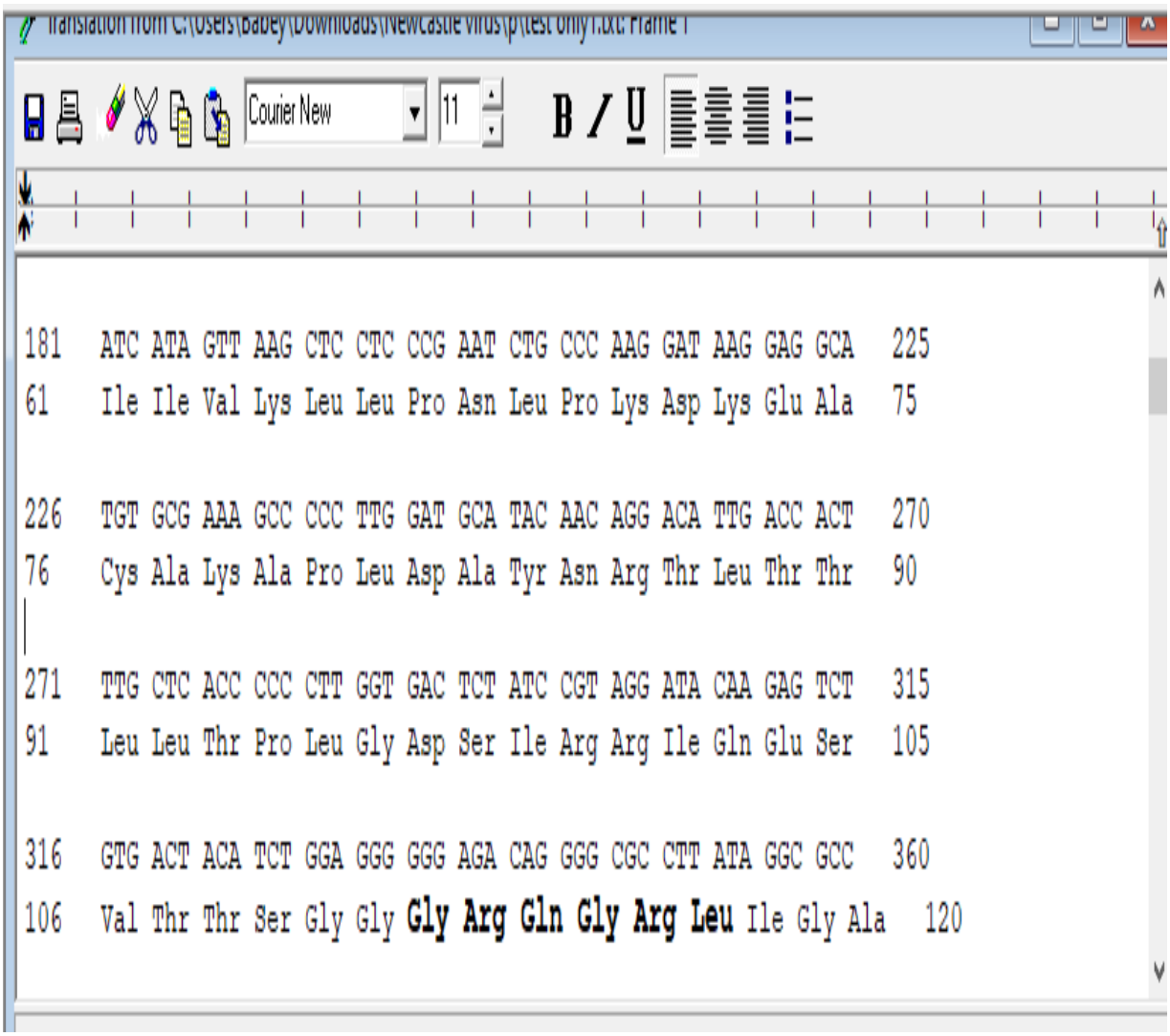

Figure 9: Amino acid sequences (112-117) of the partial F gene among field isolates of NDV strains viewed by BioEdit software.

Phylogenetic analysis of the five isolates was conducted on the basis of nucleotide sequence analysis of the variable region of the F gene. Their phylogenetic relationship has been compared to the NDV sequences available in GenBank (Figure 10).

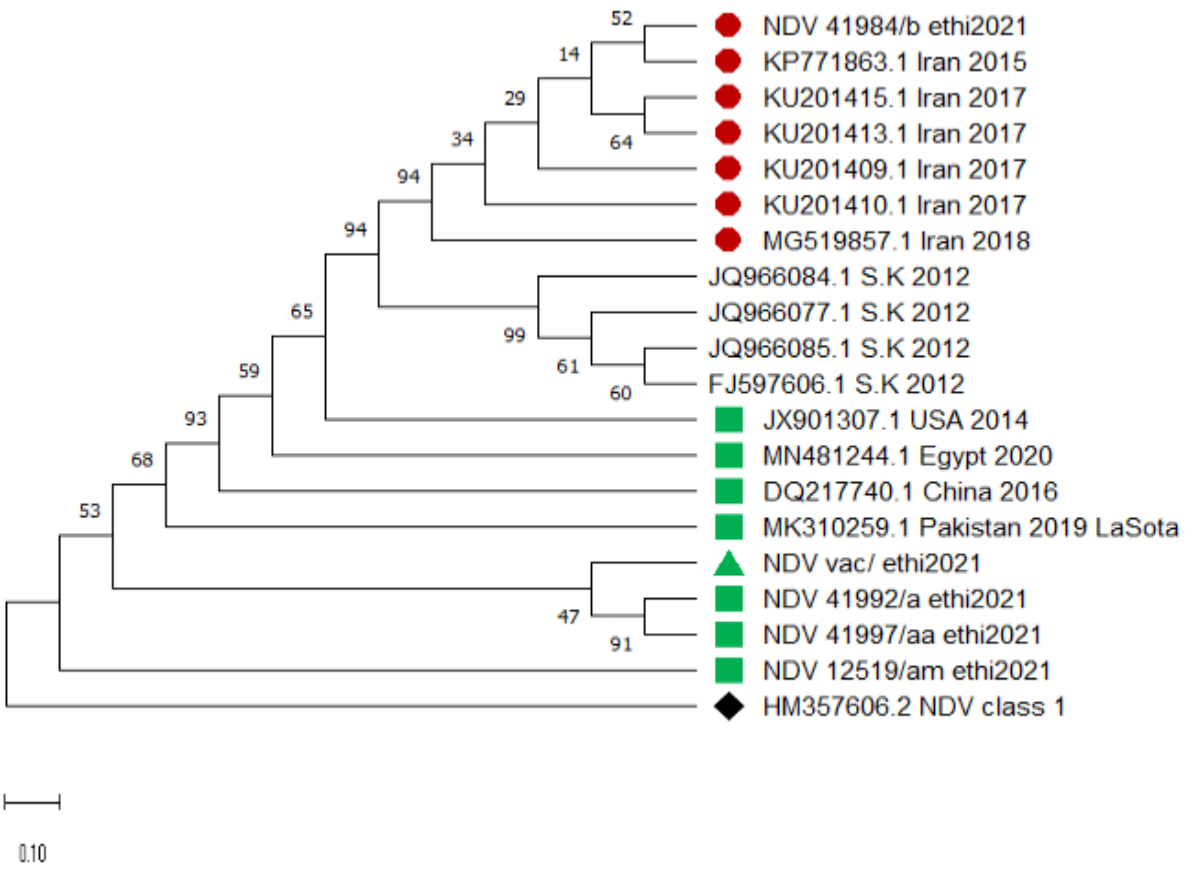

Figure 10: Phylogenetic relationships between the Ethiopian NDV field isolates (NDV 41984 ethi2021, NDV 12519 ethi2021, NDV 41997 ethi2021, NDV 41992 ethi2021, and NDV vaccine ethi2021) and five reference nucleotide sequences of velogenic strains (accession number: KU20145.1, KU20143.1, KU20149.1, KP771863.1, and KU201410.1) and four lentogenic strains of (accession number: JX901307.1, MK310259.1, MN481244.1, and DQ217740.1) for out group the accession number: HM357606.2 (Liu *et al*., 2007), NDV based on partial F gene sequence by MEGA 10.2.4.

The phylogenetic analysis demonstrated that NDV41984 ethi2021 is found in genotype VII and/or lineage 5 (Figure 11). The vaccine strain (NDV24HB1 ethi2021) was distantly associated with NDV reference sequences (GenBank accession numbers MK481244.1) and listed as genotype II and/or lineage 2.

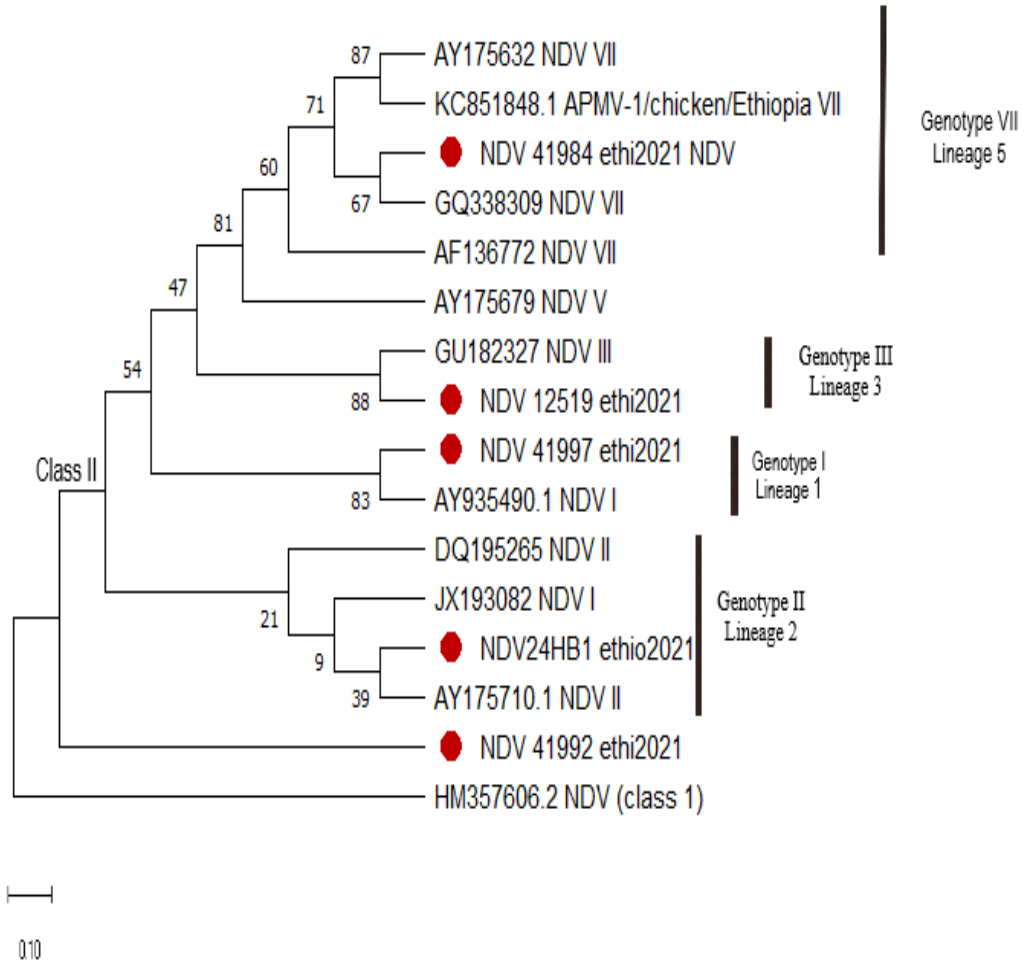

Figure 11: Phylogenetic relationships between the Ethiopian NDV isolates (NDV 41984 ethi2021, NDV 12519 ethi2021, NDV 41997 ethi2021, NDV 41992 ethi2021 and NDV24HB1 ethio2021) and reference with accession number: MK481244.1, GU182327.1, AY175710.1, AY935490.1, GQ338309.1, JX129807.1, and KC851848.1 (Tsegaw *et al*., 2014), the out group the accession number: HM357606.2 (Liu *et al*., 2007), based on partial F gene sequence, by MEGA 10.2.4.

4.4 Questionnaire survey result

Newcastle disease virus outbreaks were observed by 46.15% of respondents during the short rainy season, while 28.2 % of respondents observed during the long rainy season. Nevertheless, ND outbreaks were observed by 15.38 % in dry cool season and 5.1% of respondents throughout the year. For chicken health management, 61.53% of the respondents used traditional methods, 23% took sick chickens to a veterinary clinic, 10.25% did not use any medicine, and the remaining 5.1% used both veterinary clinic and traditional medication methods.

The current questionnaire survey revealed as chicken producers has a distinct awareness of ND cause, transmission, prevention, and control. Accordingly, 43.6%, 28.2%, 20.5% of the respondents had low, medium, and high information, respectively, while 7.7% did not respond. Out of 39 respondents, 51.28% and 48.7% said the occurrence of ND is more pronounced in layers and local breed birds, respectively. On the other hand, 71.8% of them answered as ND outbreak was observed in those chicks which had contact with wild birds (Supplementary Table 1). Others responded that the outbreak was observed within 10 km distance from the market (74.35%), and Lake (79.5%).

5. DISCUSSION

Chicken production is a significant economic activity in Ethiopia that serves chicken producers as a source of income, nutritional, and socio-cultural services (Solomon *et al*., 2013 and Getachew *et al*., 2016). The government of Ethiopia has declared chicken production a priority sector for food security with the aim to increase the number of small-scale and commercial farms for poultry farming (Samrawit and Mulat, 2018 and Zerihun and Temesgen, 2018). Control of poultry diseases such as NDV is of vital importance to achieving this objective. It is important to understand its epidemiology for effective control using vaccines from the strains circulating in the country. Based on the parameters used to classify virulent NDV (velogenic NDV), prevalence rates, nucleotide and amino acid sequences at key positions, and their phylogenetic status, the field strains isolated in this study are genotypically velogenic and lentogenic strains, which can break normally protective levels of the antibody. Very virulent strains of NDV have been reported in outbreaks involving up to 80% mortality of chicken flocks, resulting in severe losses (Chaka *et al*., 2012 and Gelana, 2017).

The current study showed an overall molecular prevalence of 13.26 % using qRT-PCR, indicating NDV is an endemic disease. In Addis Ababa, Adama, Arba Minch, and Bishoftu we detected a prevalence of 60%, 20%, 20%, and 13.6%, respectively. The NDV distribution among study areas varies having a statistically significant ($P<0.05$) difference among the study districts. A 10.75% prevalence of ND in Mid-Rift Valley and 22.2% prevalence in the central part of Ethiopia are suggested by current molecular findings. The result of the study in Rift Valley was comparable with previous reports of 12.9%, 11%, 11.6%, and 12.7% by Zeleke *et al*. (2005), Regasa *et al*. (2007), Terefa *et al*. (2015), and Gelana (2017), respectively, from Mid-Rift Valley of Ethiopia. However, the current finding was lower than the previous report in Rift Valley Ashenafi (2020), 15.5%. The result of the study in the central part of Ethiopia did not agree with the findings of Yemsrach *et al*. (2016) 26.7% in (Adama and Bishoftu) and Gelana (2017) 40% in Bishoftu. This difference can result from the research methodology, study year, sample size, and distinct exposure in the research areas of NDV in domestic chickens.

In this analysis, it was found that there was a statistically no significant difference between the different sex (Male and Female) in the occurrence of NDV ($p>0.05$). A 10% prevalence of ND in female chickens and 5.25% prevalence in male chickens are suggested by current molecular findings. The current result did not agree with the previous report by Dehinenet *et al*. (2015), Gelana (2017), and Ashenafi (2020). Huchzermeyer (1993) noted that the high prevalence in female chickens may be due to layer chickens that may have survived previous ND outbreaks and produce chicks that are vulnerable to NDV after the maternal antibodies have waned. The exact cause of these apparent sex differences in NDV presence is unknown.

The result of the M gene assay showed that 7.69% of the trachea and cloaca swab samples obtained were positive for NDV from 78 pooled chickens. qRT-PCR was employed to check NDV using the swabs of chickens collected from Arba Minch, Shashemene, Batu, Hawassa, and Bishoftu, but the only positive result was detected from Arba Minch (20%) and the remaining swabs were negative for NDV. This result varies from other previous studies by Terefa *et al*. (2015), which recorded a total prevalence of 8.4% of the disease in swabs obtained from the Mid-Rift Valley area where 12% and 2% were detected from Bishoftu and Batu, respectively. In addition to this, Ashenafi (2020) has reported a prevalence of 15.47% in the central Rift valley and Gelana (2017) 16.7% in the Mid-Rift Valley region. In this analysis, it was found that there was a statistically significant difference between the different swab types (trachea swab and cloaca swab samples) in the occurrence of NDV ($p<0.05$). A 10.25% detection rate obtained from TS sample compared to 5.12% for CS samples which is in agreement with the report of Chaka *et al*. (2012). The increased of TS positivity may be due to the presence of velogenic strains (viscerotropic velogenic) of NDV in the study area (Gelana, 2017). However, no positive result has been obtained from both TS and CS of the same individual chickens, indicating that the sampling of both TS and Cs samples from a single chicken would increase the probability of detecting the virus.

In this analysis, it was found that there was no statistically significant difference between the different tissue samples type (brain, lung & tracheal, pool tissue, and intestinal) in the occurrence of NDV ($p>0.05$). NDV prevalence in brain (10%), lung & tracheal (10%), pool tissue (10%), and intestinal (5%). Hongbo *et al*. (2014), note that viral loads differed among

individual chicken in different tissue types. This variation may be due to the presence of different virulent strains of NDV in the study areas and factors that affect the replication of the virus.

Age variations play an important role in the distribution of the Newcastle disease virus. There was a statistically significant difference between the different age groups in the occurrence of NDV ($p<0.05$). The prevalence of ND was 10.81% in young chickens and 4.87% in old chickens. The current result did not agree with the previous report by Desalegn (2015) reported a 42.2% of young chickens and 27% of old chickens are positive for NDV. According to this analysis, the high prevalence of NDV in young chickens may be due to, as birds mature tolerance to the disease generally increases. They develop immunity against the disease as the age of the bird rises (Minda *et al*., 216). This may be the explanation why the incidence of illness declines with the age of birds increasing (Palya *et al*., 2012).

In this analysis, it was found that there was a statistically significant difference between the different management systems (village and commercial) in the occurrence of NDV ($p<0.05$). A 16% prevalence of ND in village chickens and 10.41% prevalence in commercial chicken farms are suggested by current molecular findings. The current result did not agree with the previous report by Dehinenet *et al*. (2015) reported an 8.4% ND in village chickens. Kondombo *et al*. (2003) and Serkalem *et al*. (2005), the high prevalence in the village production system may be due to poor sanitary conditions, persistent exposure of chickens to range conditions and wild birds, nutritional deficiencies, lack of vaccination, and contact of chickens in one village with those in other villages can facilitate the spread of NDV.

In this study, we characterized five NDV isolates obtained from the village and commercial chickens in the Mid-Rift Valley and central part of Ethiopia. These results have shown that two different strain of NDV (one velogenic and three lentogenic) and four separate NDV lineages/genotypes are present in Ethiopia and circulate in chickens, namely, 1/I, 2/II, 3III, and 5/VIIg, and are consistent with recent findings on the circulation of genotypes VI and VII in villages and live bird markets in Central Ethiopia (Chaka *et al*., 2013), genotypes 2II, VI and VIIf,g by Tsegaw *et al*. (2013) from rural chickens in the northwest of the country. NDV

41984 ethi2021 were velogenic isolate from the freshly dead chicken brain in Bishoftu belong to genotype VIIg, and similar to Ethiopia NDV strains (accession number KC851848.1) 78 %. NDV 12519 ethi2021were lentogenic isolated belong to genotype III and more similar to USA NDV strains (JX901307.1) 88.15%, China (DQ217740.1) 88.15%, and Egypt (MN481244.1) 87.68% Hualei *et al*., 2007. NDV 41997 ethi2021 were lentogenic isolated from the freshly dead chicken intestine in Addis Ababa belong to genotype I and more similar to the South Korea NDV strain (JQ966084.1) 96.43% (Byoung *et al*., 2012). NDV vaccine ethi2021 were lentogenic isolated belong to genotype II and more similar to the HB1 vaccine strains (MK796810.1) in India at 96.76% and LaSota vaccine strains (MK310259.1) in Pakistan at 96.76%. Genotypes 1/I reported by Byoung *et al*. (2012) isolates, wild birds, and live bird markets in South Korea.

The result of the Ethiopian isolates in this study and the existence of genotype VIIg circulating in many northeastern African countries nearest to the Middle East supports the suggestions of (Aldous *et al*. 2003), the virulent strain of NDV is normal and the outbreaks are frequently reported in different countries to the World Organization for Animal Health (Office of International Epizootics 2011). Different Velogenic Lines/Genotypes of NDV in Africa lineage 5 (genotype VIIb,d,f,g, and h) in Egypt, South Africa, Mozambique, Ugandan, and Burundi (Chantal *et al*., 2013 and Radwan *et al*., 2013), subgenotype VIIg emerged from the Middle East and Asia (Malaysia and China), causing outbreaks in eastern and southern Africa. According to Tsegaw *et al*. (2013), the close genetic similarity of the central and northern Ethiopian virulent isolates to the Sudanese and Egyptian isolates suggests possible epidemiologic connections between NDV outbreaks in these countries, most likely due to geographical proximity and cross-border movement of poultry.

NDV infects a wide variety of domestic and wild bird species across the globe (Alexander, 2000), in Europe, North, Central, and South America, and Asia, genotypes II and III were more widespread in domestic and wild birds. The association of domestic poultry with wild birds has been recognized as a potential source of avian disease in domestic flocks across the Rift Valley region (Gelana, 2017 and Ministry of Agriculture and Livestock Resources, 2018). Moreover, according to the phylogenetic comparison of viruses acquired from different hosts,

the role that migratory birds play in the transmission of Newcastle disease indicates that migratory birds carry the virus and enter domestic birds. In West Africa, several additional strains of avirulent and virulent NDV circulate in wild birds and pigeons, and the current high genetic diversity of NDV strains found in poultry Genotype VIh strains have likely been introduced in Africa and in Nigeria in particular on several different occasions (Chantal *et al*., 2013). Typical wild-type avirulence strains have also been found in wild birds. And genotype VIh was also found in wild pigeons in Ethiopia (Delese *et al*., 2016). However, we did not find strong evidence that wild birds were actively contributed to the spread of the enzootically circulating virulent genotype VIIg strains.

In this analysis, it was found that the field isolates are originated from a common ancestor and divergence from subgenotype II and VI class II in the study area, and those isolates are (73 % to 89 %) identical to the NDV HB1 vaccine strain, which is a commercially available NDV vaccine developed and used in Ethiopia. Their partial F gene sequences are similar to those of other lentogenic and velogenic NDV strains from Ethiopia, Africa, Asia, and Europe were reported to GenBank, but they vary from those of the NDV24 HB1 vaccine strains. In this study, the findings of the phylogenetic analysis are consistent with those of Aldous *et al*., 2003, Palya *et al*., 2012, Jingjing *et al*., 2017, and Alsahamia *et al*., 2018, showing the continuous evolution and mutation of NDV's F gene. This can influence the virus's antigenicity and pathogenicity. Mutations at amino acid positions 112 to 117 of the F gene have resulted in changes in virulence and have also affected the antigenicity of this protein and the effects of neutralizing antibodies (Ahmed *et al*., 2017). Vaccines made from lentogenic strains provide defense against virulent strains (velogenic NDV) (Mebrate *et al*., 2019). However, these vaccines are affected by maternally derived antibodies, meaning that velogenic NDV can possibly spill over into the vaccinated flock, eventually allowing vaccinated chickens to become infected and then remove viral viruses. This is considered one of the reasons why mutant vaccine strains tend to cause outbreaks in vaccinated flocks (Muhammad *et al*., 2018).

There have been records of adaptation of escape mutant vaccine strains in regions using vaccines prepared from lentogenic strains alongside indigenous field strains (Banur *et al*.,

2013). As has been observed in Europe, the use of live vaccines, especially imported ones without genotype matching, has resulted in genetic diversification through the assortment of circulating viruses (Jingjing *et al*., 2017). To assess the effectiveness of commercial vaccines to protect chickens from virulent field strains, further studies are required, but we suggest that effective protection against velogenic NDV strains could be achieved if vaccines are prepared from autogenous strains that trigger outbreaks. In the last 60 years, conventional ND vaccines have stood the test of time by proving a track record of protective efficacy (Palya *et al*., 2012). However, these vaccines cannot block the replication and shedding of most of the phylogenetically divergent virulent NDV isolates currently circulating. Even if clinical protection is achieved, the greatest drawback of these conventional genotype II-based NDV vaccines is their inability to avoid the shedding of the heterologous virulent NDV (Muhammad *et al*., 2018). While several factors may be involved in this post-vaccination shedding of virulent NDV, the genotype of mismatch between vaccine strains and challenge strains are believed to be a key player.

The current study showed that the need of biosecurity practices in poultry farms and market sites in the Mid-Rift Valley and the central part of Ethiopia that contribute to the spread of the diseases along with farms and the market chain. We observed several practices that could promote Newcastle disease and other infectious disease transmissions among chickens. These included limited cleaning and disinfection of the farms (house), site of the farm, market places, holding cages, mixing of birds from different origins (interaction of wild birds with the village chickens), inappropriate disposal of sick and dead birds, and poor waste disposal mechanisms. Most recent reports indicated that marketing systems play a considerable role in the dissemination of diseases over wide geographical areas in a relatively short period of time in Ethiopia (Belete and Tsegaw, 2020). A similar observation was reported from Addis Ababa to live poultry market sites (Delesa *et al*., 2014). The current finding is also in agreement with a report from eastern Shewa zone backyard chickens markets where poor biosecurity practices were suggested to play major roles in the transmission of Newcastle disease viruses (Chaka *et al*., 2012).

The majority (90%) of respondents mentioned that there is no habit of cleaning chicken house

once a day, where chickens from different origins are placed in the same cages (mixed) and sold to the customer this may prefer the chances of disease transmission among birds, as they may be the main source of infection. Studies conducted by Jingjing *et al*. (2017) have shown that clinically healthy chickens harbor a virulent virus that was responsible for the severity of NDV among bird flocks, while clinically healthy chickens may be a source of infection for poultry flocks. Ten percent of the respondents clean the chicken house and holding cages every day with clean water. Most of the respondents responded that they dispose of manure and dead birds either within the markets or on the roadside. Only twenty-three percent of them seek veterinary services when chickens are sick. This result was consistent with the findings of Belayheh *et al*. (2014) and Gelana (2017) who confirmed that the majority of the household did not use drugs for their sick birds in the mid-river valley. The possible explanation for this issue may be the lack of knowledge and the neglected follow-up of the chickens. The presence of several lakes in the study area tends to be closely associated with the possibility of an ND outbreak. This may be due to the seasonal migration of wild birds in search of feed, especially during times of scarcity; which predisposes chickens to NDV originating from wild birds (Gelana, 2017). A similar finding was reported by (Alexander *et al*., 2001), the virulent NDV had been isolated from both wild and domestic birds. In this respect, the bilateral transmission of the virus between wild birds and domestic chickens could play an important role in the likelihood of village chickens acquiring NDV infection from wild birds and vice versa.

6. CONCLUSION

The prevalence of the Newcastle disease virus is 13.26 % in both non-vaccinated village chickens and vaccinated commercial chickens in the study areas. RT-PCR and molecular characterization and diagnostic techniques have also verified that the disease is widespread in the mid-rift valley and central part of Ethiopia. This molecular characterization and phylogenetic study of the NDV strains of Ethiopia provides evidence of the presence of at least four distinct NDV genotypes circulating in the middle rift valley and in the central part of the country. It has been shown that virulent NDV continues to be a problem in Ethiopia as some of the identified isolates belong to the velogenic group, lineage 5 (genotype VII), which is the predominant virus variant responsible for ND outbreaks in poultry worldwide. We have reported variations in amino acids between field circulating strains and vaccine strains. The incidence of ND in the rift valley areas was attributed to the presence of numerous species of adoptive and migratory wild birds in search of their feed around water bodies; this may encourage contact with local scavenging village chickens. The free range of village birds poses a high risk of NDV transmission between wild birds and poultry in both directions, from wild to domestic and vice versa.

On the other hand, the molecular prevalence of NDV indicated widespread exposure of village chickens to mid-river valley areas from the market. In addition, exposure to wild birds, the introduction of new birds, to an existing flock, mixing with neighboring poultry, as well as aerosol contact between ND-infected chickens without any clinical signs, The lack of effective vaccination and biosecurity in the village of backyard chicken producers, and also the conventional way of raising poultry could worsen morbidity, mortality, and loss of output.

7. RECOMMENDATIONS

The following recommendations are forwarded based on the present findings.

- ✓ To prepare vaccines targeting the circulating field strains and genotypes of NDV.

- ✓ To avoid economic losses associated with outbreaks, strict NDV vaccination programs and biosecurity measures are mandatory for commercial and village poultry in Ethiopia.

- ✓ Further molecular characterization of NDV isolates from outbreak areas, the close phylogenetic relationship between Ethiopia's virulent NDV isolates, and those viruses from countries that are geographically close warrants continued surveillance of the early detection of virulent NDV in commercial and village poultry and understanding the role of wild birds.

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9. ANNEXES

ANNEX I

Supplementary Table 1: Questionnaire survey results on associated risk factors for ND in the study area.

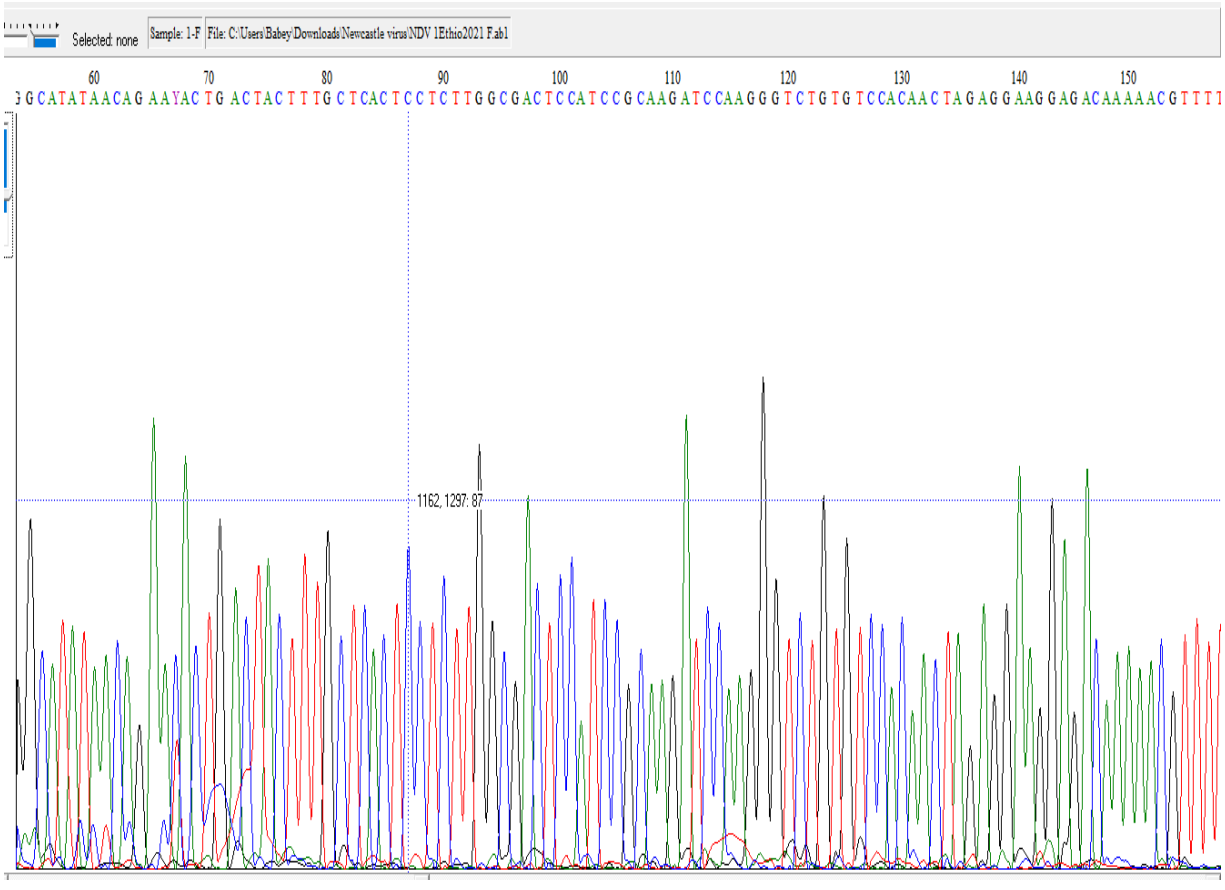
Category of variable	**N= 39**	**%**
Way of transportation		
Carry by hand	19	48.7
By cart	12	30.76
By car	8	20.51
Did you clean the chicken's room daily?		
Yes	4	10.25
No	35	89.74
Did you encounter ND outbreaks?		
Yes	34	87.17
No	5	12.82
Did you vaccinate your chicken for NDV?		
Yes	5	12.82
No	33	84.61
Season of ND outbreaks		
A short rainy season	18	46.15
Long rainy season	11	28.2
Dry cool season	6	15.38
Throughout year	2	5.1
How could treat your chickens?		
By tradition	24	61.53
Veterinary clinic	9	23
Mixed	2	5.1
Not use any medication	4	10.25

Knowledge about causes, transmission, prevention and control of ND		
Low (<10 question answered out of 30)	17	43.6
Medium(10 -20 question answered out of 30)	11	28.2
High (21-30 question answered out of 30)	8	20.5
Not respond	3	7.7
Age of poultry at ND more pronounced		
Starter	9	23
Layers	20	51.28
Broilers	1	2.56
Mixed	7	17.94
Not respond	2	5.12
Breed at ND more pronounced		
Local	19	48.7
Exotic	15	38.46
Cross	5	12.82
Did you observe an outbreak within 10 km distance from the lake		
Yes	31	79.5
No	8	20.51
Out-break of ND in chicken contact with wild birds		
Yes	28	71.8
No	3	7.69
Level of biosecurity system?		
Low	10	58.82
Moderate	7	29.4
High	2	11.76

Did you observe out-break (< 10 km distance from market)		
Yes	29	74.35

Annex II

Supplementary Figure 1: Chromatogram file sample for F gene sequence visualized by chromas software.



Annex III

Questionnaire format for survey

Part I: General Information

Date_____

Region _____, Zone _____, District_____ City/village_____

Name of the respondent_____

Geographical positioning system data collection in selected Rift Valley areas.

Location	North (latitude)	East (longitude)	Altitude

Part II: Information related to the research purpose

1. How long have you been working with chicken keeping? _____

2. How many which breeds and types of chicken do you have currently?

Chicken Type	**Number of chickens by breed**		
	Exotic	**Hybrid**	**Local**
Layer			
Pullets			
Chickens			
Cockerels			
Cocks			

3. Objectives of the farm? Egg production, meat production, breeding stock, mixed

4. Type of production system? Scavenging, semi-intensive, extensive
5. Level of biosecurity system? Low, moderate, high
6. Where do your chickens spend the day time (Housed/Scavenging)?
7. How far do your chickens move for scavenging and watering? _____
8. Is there any interaction of the chicken with other species of wild birds? Yes/No
9. Did you encounter a health problems in your chicken/farm? _____
10. Which disease causing high losses in your chicken/farm?
11. What is the major health problem affecting your chicken?
12. In which types of diseases and chicken mortality is more serious? _____
13. At what season do you frequently encounter the disease problems?
14. Where and when the chicken is bought for production purposes? Do you used DOC?

_____ _____

15. In which types of diseases and chicken mortality is more serious? ____
16. At what season do you frequently encounter the disease problems?
17. Do you vaccinate your chicken for these major diseases in your area? (Yes/No). If yes, which one? Source of vaccine?

Name of Major diseases (Local Name)	Affected Age	Affected Sex	Affected Breed	Affecting Season	No of Sick	Clinical sign	No of Dead

More about NDV

A. Way of transportation____ Carry by hand, By cart, By car
B. Did you clean chicken's room daily? YES/ NO
C. Did you encounter ND outbreaks? YES/ NO
D. Season of ND outbreaks? Form short rainy season, long rainy season, dry cool season, through out of the year.
E. The chickens get vaccination for NDV? If yes, which type of vaccine is used? ____
F. How could treat your chickens? By tradition, Veterinary clinics, Mixed, Not use any medication.
G. Knowledge about cause, transmission, prevention, and control of ND? Low, Medium, High, Not respond.
H. Age of poultry at ND is more pronounced? Starter, Layers, Broilers, Mixed, Not respond.
I. Breed at ND more pronounced? Local, Exotic, Cross
J. Did you observe outbreak (distance from a lake)? <10Km, 10-20Km, >20Km
K. Out-break of ND of chicken contact with wild birds? YES/ NO
L. Did you observe outbreak (< 10 km distance from the market)? Yes/No
M. What are the major losses of the disease (Production status/Morbidity/Mortality)? _____
N. What do you think about disposing of dead birds and other materials that facilitate the occurrence of major diseases?

Annex IV: Picture showing sample collection and processing the samples.

Figure 13: Swab sample collection in the mid Rift Valley (A, B,& C)

Figure 14: Tissue sample collection at NAHDIC, Sebeta (A, B,& C)

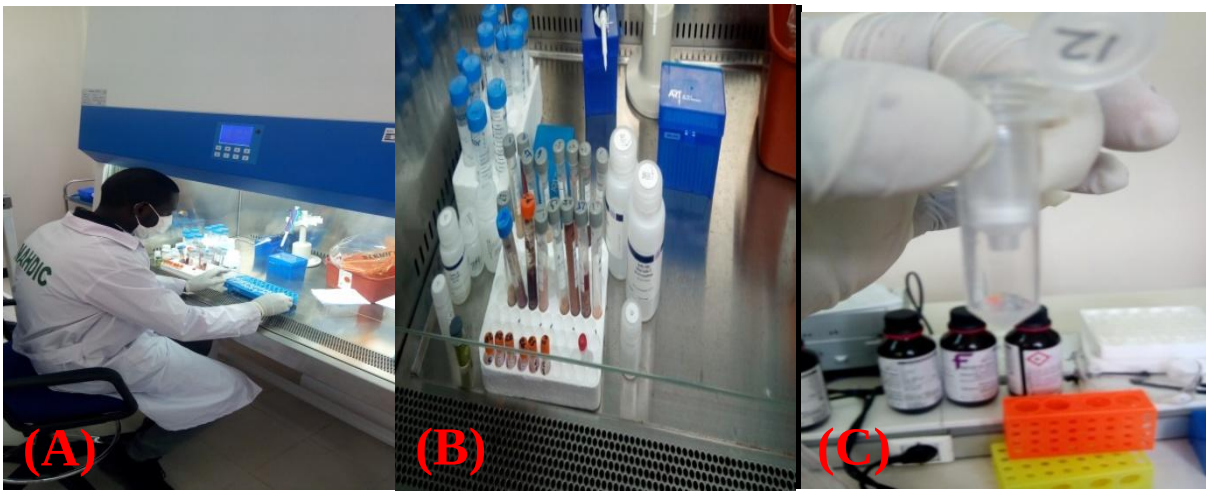


Figure 15: Sample processing for amplification and sequencing at NAHDIC, Sebeta (A, B,& C)

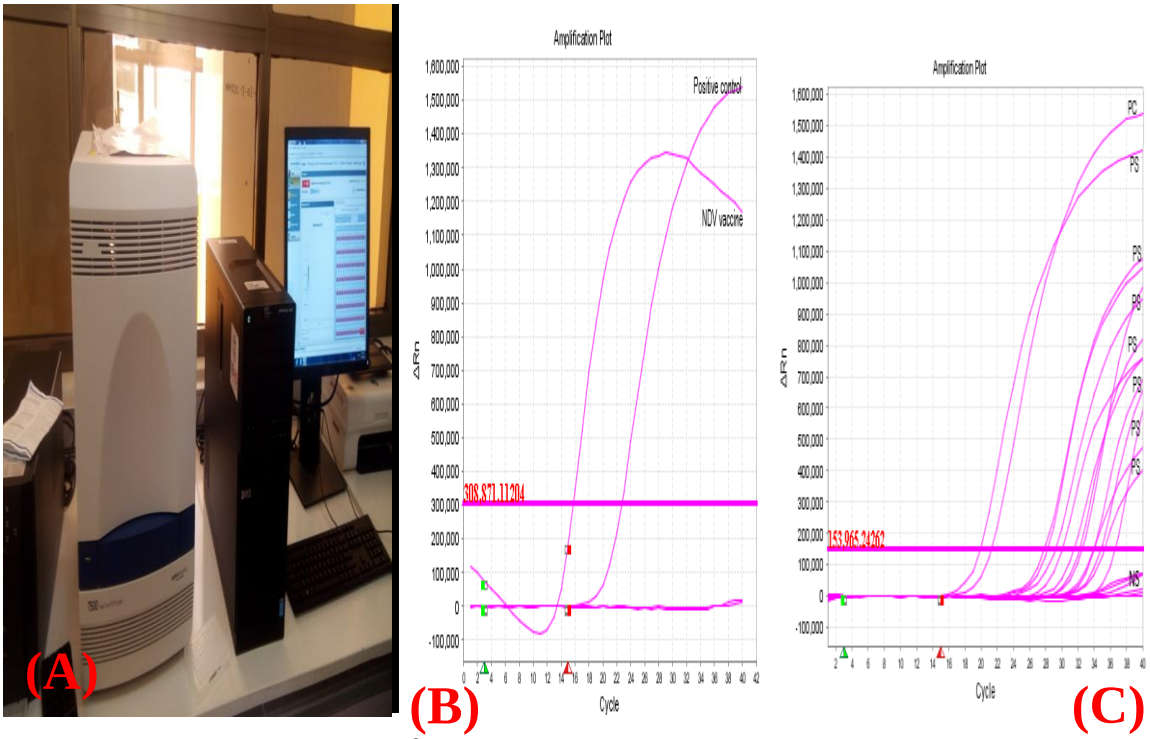


Figure 16: qRT-PCR and amplification result in NAHDIC, Sebeta (A, B,& C).

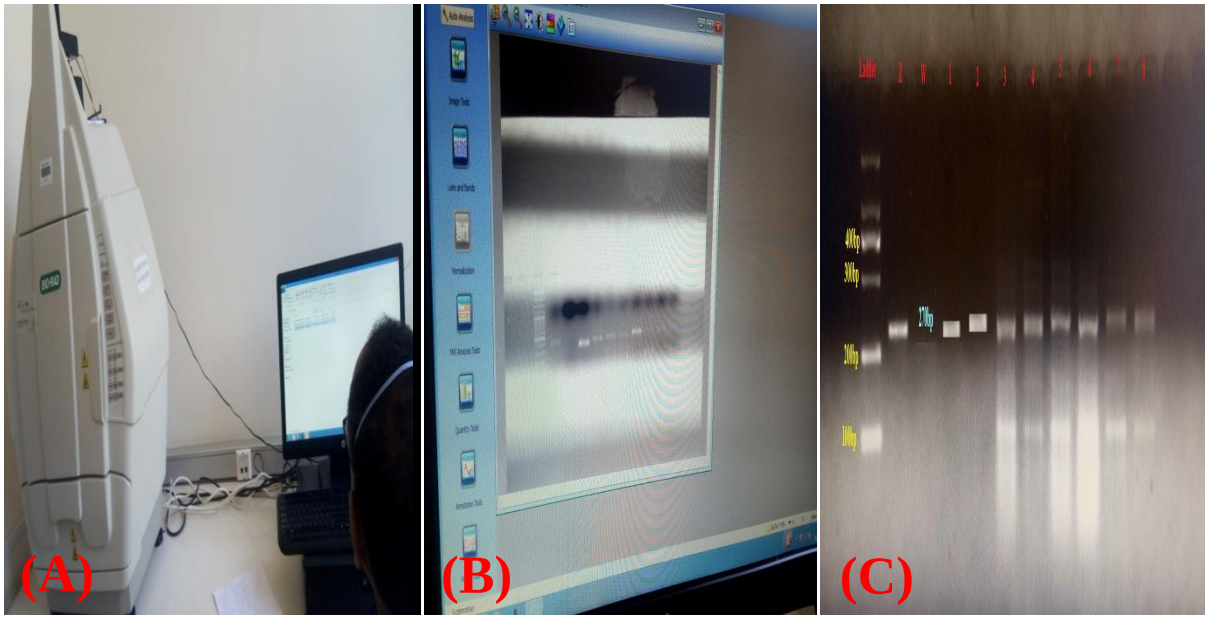


Figure 17: conventional PCR and amplification result in NAHDIC, Sebeta (A, B,& C).

Annex V: sing and symptoms of NDV in chickens during the sample collection

Figure 18: Picture shown paralysis of legs and wings, the neck twists on one side infected by ND. (A, B,& C).

Annex VI: Research Ethical Approval Letter

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

RESEARCH AFFAIRS DEAN OFFICE

UNIVERSITY ETHICAL REVIEW BOARD (UERB)

Phone: 1888

Fax: +2510221-10-00-46

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CERTIFICATE OF APPROVAL

To: Esubalew Endale Alemu

Department: Applied Biology

Program: M.Sc

Study Title: Molecular Investigation for Strain of Newcastle Disease Virus Circulating in Chickens (in Selected Migratory Birds Abundant Area in Mid Rift Valley of Ethiopia

Approval Date: January 10, 2020

Certificate Ref. No: RECSoANS/BIO/03/2020

Based on the request, the University Ethical Review Board (UERB) members reviewed the proposal taking into consideration the following key points:

1) Whether the intended research project have any risk to the research team, to the participants, data collectors, the data to be collected, the institutes involved in the research, project partners, funders involved, viz.
2) How, where, and by whom participants will be identified, approached, and recruited.
3) Type of people recruited.
4) The consequences of the research, risk of the data, how will it be protected and addressed
5) Consideration of anonymity and confidentiality

In light of the above key points, UERB members identified no any risk to any of the participants, institutions, data collectors and viz. Therefore, UERB recommend the implementation of the project as per the approved proposal.

Finally, I congratulate you for securing this Ethical Approval Certificate to precede your project.

Wishing you all the best!

Alemu Disassa Muleta (
Research Affairs D

Chairperson of University Ethical Review Board