Molecular Detection and Assessment of Associated Risk Factors for Newcastle Disease Virus in Non-vaccinated Village Chickens in Central Rift Valley of Oromia, Ethiopia

By:

Ashenafi Milkesa Fekensa

A Thesis Submitted to the Department of Applied Biology School of Applied Natural Science

Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Biology

(Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

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Candidate's Declaration

First, I hereby declare that this M.Sc. thesis entitled **"Molecular Detection and Assessment of Associated Risk Factors for Newcastle Disease Virus in Non-vaccinated Village Chickens in Central Rift Valley of Oromia, Ethiopia"** is my bona fide work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for M.Sc. degree in Biotechnology at Adama Science and Technology University, School of Applied Natural Science, Department of Applied Biology. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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School of Applied Natural Science, Department of Applied Biology; Adama, Ethiopia.

Date of Submission: _____

Advisor's Approval Sheet

To: Department of Applied Biology

Subject: Thesis Submission

This is to certify that the thesis entitled **"Molecular Detection and Assessment of Associated Risk Factors for Newcastle Disease Virus in Non-vaccinated village Chickens in Central Rift Valley of Oromia, Ethiopia"** submitted in partial fulfillment of the requirements for the degree of Masters in Applied Biology (Biotechnology), Graduate Program to the Department of Applied Biology and has been carried out by **Ashenafi Milkesa Fekensa**, **ID No: A/PR15399/10**, under my supervision. Therefore, I recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the department.

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Approval of Board of Examiners

We, the undersigned members of the Board of Examiners of the final open defense by **Ashenafi Milkesa Fekensa** have read and evaluated his thesis entitled **"Molecular Detection and Assessment of Associated Risk Factors for Newcastle Disease Virus in Non-vaccinated Village Chickens in Central Rift Valley of Oromia, Ethiopia"** and examined the candidate. Therefore, this is to certify that the thesis has been accepted in partial fulfillment of the requirement of the Degree of Master's in Applied Biology (Biotechnology).

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Advisor	Signature	Date
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Dedication

I dedicate this project to Jesus Christ Almighty my creator, my strong pillar, my source of inspiration, wisdom, knowledge and understanding and above all my everything. He has been the source of my strength throughout this program and on His wings only have I soared. I also dedicate this work to my father; Milkesa Fekensa (Milk) who has encouraged me all the way and whose encouragement has made sure that I give it all it takes to finish that which I have started and to my mother Alem Haile, my brothers Addisu Milkesa, Tsegahun Milkesa and Hambisa Milkesa and my little sister Tensay Milkesa, and my best wisher Dr. Alemu Disasa who have been affected in every way possible by this quest. Thank you. God bless you.

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Lists of Acronyms and Abbreviations

AGIDT	Agar Gel Immunodiffusion Test
APMV	Avian Paramyxovirus
BoFED	Bureau of Finance and Economic Development
cDNA	Complementary Deoxyribonucleic acid
CFSPH	Center for Food Security and Public Health
cRT-PCR	Conventional Reverse-transcriptase Polymerase Chain Reaction
CSA	Central Statistical Agency
Ct	Cycle Threshold
ddNTP	Dideoxynucleotide Triphosphate
ELISA	Enzyme Linked Immuno Sorbent Assay
F	Fusion Protein
HI	Hemagglutination Inhibition
HN	Hemagglutinin Neuraminidase
ICPI	Intracerebral Pathogenicity Index
ICTV	International Committee on Taxonomy of Viruses
IDT	Integrated DNA Technologies
L	Large RNA Polymerase
M	Matrix Protein
MoLF	Ministry of Livestock and Fisheries
mRNA	Messenger of Ribonucleic Acid
NAHDIC	National Animal Health Diagnosis and Investigation Center
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NP	Nucleoprotein

NVI	National Veterinary Institute
nvNDV	Neurotropic Velogenic Newcastle Disease Virus
NVSL	National Veterinary Services Laboratories
OIE	Office International des Epizooties
ORF	Open Reading Frames
ORS	Oromia Regional State
P	Phospho-protein
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
rRT-PCR	Real Time Reverse-transcriptase Polymerase Chain Reaction
RT-PCR	Reverse-transcriptase Polymerase Chain Reaction
SPF	Specific Pathogen Free
ssRNA	Single-stranded Ribonucleic Acid
VTM	Virus Transport Media
vvNDV	Viserotropic Velogenic Newcastle Disease Virus

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Abstract

Newcastle disease (ND) is a major infectious disease of poultry caused by a virulent strain of Avian Paramyxovirus – 1. It is a major threat to the poultry industry in many countries of the world including Ethiopia. The aim of this study was to detect Newcastle disease virus by molecular tools and identify its risk factors in non-vaccinated village chicken in Central Rift Valley of Oromia, Ethiopia. For this purpose, a total of 84 pooled in five swab samples from 420 cloacal and tracheal chickens were sampled and RNA was extracted from the 84 pooled samples to carry out qPCR reaction. A conventional PCR along with purification was also done for 10 positive samples that were having best Ct. value in order to send to Germany for sequencing. Out of the 84 pools of five swab samples tested for matrix gene (M-gene) using qPCR, 16.7% (14/84) samples were detected which included 13 positives and 1 negative for NDV. The prevalence of ND in males was found to be 16.10% and that in females was 14.67%. On the other hand, the prevalence of ND was higher in Adult (16.59%) compared to old chicken (11.11%) ($p < 0.05$). Although the overall ND prevalence was 15.48% (13/84), the highest score was recorded in Adama, 42.86% (6/14), and no positive case was detected in Bote and Bishoftu ($p <0.05$) while intermediate scores were obtained from Batu, Arsi-negelle and Shashemene. Based on the questionnaire interview, 71.1% (128/180) of the respondents indicated that ND was the leading disease and 98.3% (177/180) indicated that disease occurrence was higher during rainy season. Accordingly, the highest correlation value of $R= 0.62$ between the monthly rainfall data and disease outbreak was obtained. In general, the present study provides important information on epidemiology of NDV in Central Rift Valley of Oromia, Ethiopia, and highlights the importance of implementing surveillances and biosecurity practices in live poultry markets and village chickens.

Key words: Central rift valley; Newcastle disease; RNA; qPCR; Village chickens.

1. INTRODUCTION

1.1. Background of the Study

Newcastle Disease (ND) is an acute, mild to severe, highly infectious and pathogenic disease of domestic poultry, caged and aviary birds as well as wild birds caused by specified viruses of the Avian Paramyxovirus Type 1 (APMV-1) (Alexander *et al*., 2003; Amarasinghe *et al*., 2017). It is caused by Newcastle disease virus (NDV) which is a highly infectious agent capable of causing high mortality in non-vaccinated chickens. The sub clinical forms of ND in vaccinated and/or NDV exposure flocks which could have synergistic effect with other bacterial or viral infections (Capua and Alexander, 2009; Perozo *et al*., 2012). After 90 years since the first discovery of Newcastle disease (ND) in England and Indonesia (Kraneveld, 1926; Doyle, 1927) and the introduction of vaccine in 1950s to control the disease (Alexander, 2008), ND is still the most significant avian disease that continued to cause huge economical loss to the poultry industry. Because of its worldwide outbreaks and geographical spread, World Organization for Animal Health (OIE) has listed ND as a notifiable disease (OIE, 2013).

Based on pathogenic and virulence properties, NDV is categorized into three major pathotypic strains i.e. lentogenic, mesogenic and velogenic strains (Vienna and Sarah, 2017). The amino acid sequence at the fusion cleavage site is related to virulence as specific sequences can be cleaved systemically as compared to others which can only be cleaved in specific host tissues leading to a local infection (de Leeuw *et al*., 2003). The OIE has enunciated out as it is vitally important for avian species and products in a specific geographical location where it often leads to trade restrictions and embargos due to sanitary and phytosanitary measures in Global trade (Leighton and Heckert, 2007).

Newcastle disease is regarded as one of the most important diseases of poultry throughout the world, not only due to seriousness of the disease and high flock mortality that may result from some ND virus infections, but also through the economic impact that may ensue due to trading restrictions and embargoes placed on areas and countries where outbreaks have occurred (Aldous and Alexander, 2008). The problem has also been documented in Africa (Naveen *et al*., 2013). From the limited reports to the OIE, it also appears the most important poultry disease in Ethiopia,

recurring every year (http://web.oie.int/wahis/public.php). In Ethiopia, the poultry industry is dominated by the traditional sector where free-range poultry keeping is the most common in the country (Ashenafi, 2000). The total poultry population in Ethiopia is estimated around 43 million of which 97% is village chickens, while only 3% chickens are kept under intensive management system in commercial farms (CSA, 2012). Diseases and especially the devastating Newcastle disease (ND) are perceived to be the main constraint (Tadesse *et al*., 2005) which frustrates any poultry investment in the country. In Ethiopia, village poultry production has a long traditional practice which is mainly used as an immediate cash income for the rural communities, where women are more involved in keeping village chickens (Eshetu *et al*., 2001).

Laboratory diagnosis for NDV includes virus isolation, serological assay and molecular test such as Reverse Transcription Polymerase Chain Reaction (RT-PCR). RT-PCR assay is more sensitive, specific and less labor intensive as compared to other conventional methods (Tang *et al*., 2012; Saif *et al*., 2005; Shahzad *et al*., 2011). It is also a useful tool to achieve without propagation in embryonated eggs allowing subsequent sequencing of the amplified nucleic acid and pathotyping even if the virus is found in minute quantities or has lost infectivity (Gohm *et al*., 2000; Singh *et al*., 2005). Knowledge on the molecular detection using RT-PCR is essential in order to introduce curative measures for post infection and to prevent the rapid spread of the disease.

Despite the wide spread problem of ND in Ethiopia in general and Oromia in particular, limited researches (Chaka *et al*., 2013; Terefe *et al*., 2015; Miressa, 2016) have been done so far in Central Rift Valley of Oromia, Ethiopia, to detect ND virus and assess the risk factors associated with Newcastle Disease. Therefore, the aim of this study was to detect the existence of Newcastle Disease virus by molecular tool, determine its prevalence and risk factors in non-vaccinated village poultry in central rift valley of Oromia, Ethiopia.

1.2. Statement of the Problem

Newcastle disease is one of the biggest challenge of poultry that contributes a lot for economic losses of the globe (Alexander, 2008). Despite the international trade regulations, national control plans, biosecurity procedures, diagnostic and monitoring tools and techniques, as well as the widespread use of various vaccines, and vaccination programs, ND is still listed among the most devastating poultry diseases considering both its clinical and economic consequences (OIE, 2013).

Such a problem is especially endemic in several developing countries, and has the greatest impact on villages where people's livelihood depends upon poultry farming (Mohamed *et al*., 2011; Rezaeianzadeh *et al*., 2011). Annual losses caused by this disease worldwide accounts millions of dollars (Susta *et al*., 2010; Waheed *et al*., 2013). Therefore, it is devastating disease and a major threat to the poultry industry (Narayanan *et al*., 2010). The most striking problem in village chicken production systems is the high mortality rate which could reach as high as 80–90% within the first few weeks after hatching due to diseases and predation (Dessie and Ogle, 2001). ND is highly infectious and causes more losses than any other diseases in the tropics. ND spreads rapidly through the flock and the mortality could reach up to 100% along with decrease in egg production (Nigussie *et al*., 2003; Serkalem *et al*., 2005; Nwanta *et al*., 2008; Choi *et al*., 2010; Haque *et al*., 2010).

The total poultry population in Ethiopia is estimated around 43 million of which 97% is village chickens (CSA, 2012). Since, ND primarily targets poultry livestock, it causes an impact on financial, nutritional and socio-cultural livelihoods of poor rural households in developing countries, including Ethiopia. Therefore, from the economic viewpoint, ND endangers poultry rearing which is predominantly important to rural women who support their families economically from chicken production. ND is capable of infecting humans (Zoonotic potential) and infection typically results in conjunctivitis and/or influenza-like symptoms in humans, when a person has been exposed to large quantities of the virus (Swayne *et al*., 2003; Capua and Alexander, 2004).

According to a report from Ethiopian Ministry of livestock and fishery (MoLF), several regions were recorded for the NDV outbreaks during various periods (2012-2015) that indicates the highest outbreaks occurred in the Oromia region in general (286) and central rift valley in particular (MoLF, 2018). Hence, central rift valley of Oromia region was selected for the current study area for substantial laboratory investigations of NDV.

1.3. Objectives of the Study

1.3.1. General Objective

- To detect Newcastle disease virus by molecular tools and identify its risk factors in non-vaccinated village chickens in Central Rift Valley of Oromia, Ethiopia.

1.3.2. Specific Objectives

The specific objectives of this study were:

- To detect Newcastle disease virus at the molecular level in non-vaccinated village chickens in Central Rift Valley of Oromia.
- To estimate the prevalence of Newcastle disease in non-vaccinated village chickens in Central Rift Valley of Oromia.
- To assess the associated risk factors for the occurrence of Newcastle disease virus infection in non-vaccinated village chickens in Central Rift Valley of Oromia.

1.4. Significance of the Study

Because of high prevalence of ND in Oromia region (MoLF, 2018), there is a need of technologies for accurate and rapid identification of the virus. Therefore, in this study, RT-PCR techniques was used to detect ND in non-vaccinated village chicken in Central Rift Valley of Oromia, Ethiopia. Above and beyond, the information generated provides some insights for researchers working in poultry disease control programs. From economic aspect, poultry production makes significant contributions to the livelihood of smallholders and the rural poor to increase farm income, while it also diversifies farm income, thereby reducing risk and vulnerability, especially in regions where crop production is limited. Therefore, this study will contribute towards designing appropriate control strategies of ND through early detection by molecular techniques and determining the associated risk factors.

1.5. Scope of the Study

Molecular analysis along with further characterization of NDV field isolates would have been a useful tool in order to know the type of strains circulating in a particular geographical area. However, due to very low quality of sequencing result we have got from a company in Germany (LGC genomics) we couldn't able to analyze further. Therefore, the current study concentrated on/delimited to molecular detection using real-time and conventional RT-PCR and determination of the Prevalence of Newcastle Disease in some selected areas in Central Rift Valley of Oromia, Ethiopia. In addition, due to time and resource limitation, diverse agro-ecologies were not also covered in the present study.

2. LITERATURE REVIEW

2.1. General Overview on Newcastle Disease Virus

Newcastle disease is an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) while it was previously known as avian paramyxovirus type 1 (APMV-1); however, due to changes in taxonomy is now referred to as avian avulavirus (Amarasinghe *et al*., 2017). Avian paramyxovirus serotype 1 (APMV-1) viruses have been placed in the genus Rubula virus, sub-family Paramyxovirinae, family Paramyxoviridae (Mayo, 2002). Newcastle Disease Virus (NDV) is an enveloped, non-segmented, single-stranded negative-sense RNA virus with a helical morphology. Its genome has six open reading frames (ORF), which encode for the following proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and RNA-dependent RNA polymerase (L). During P-gene transcription, two additional nonstructural proteins, the V and W proteins are also generated through RNA editing (Steward *et al*., 1993; Han *et al.,* 2008; Chang and Dutch, 2012).

Based on genomic size and the nucleotide sequences of F and L genes, NDVs can be categorized as class I or class II viruses (Figure 1). Class I NDVs are occasionally isolated from wild aquatic birds and domestic poultry and are mostly avirulent to chickens (Kim *et al*., 2007). Class II contains viruses that have been isolated from multiple wild birds and poultry species. Most viruses within this group are virulent and cause significant economic losses to poultry industry worldwide (Miller *et al*., 2009). These viruses are also highly diverse and continuously evolving (Czeglédi *et al*., 2006; Diel *et al*., 2011). The key contributor to NDV pathogenicity is the formation of an active fusion protein upon cleavage of F protein precursor (F0) which is facilitated by the presence of a number of basic amino acid residues in fusion protein cleavage site (Toyoda *et al*., 1987; Glickman e*t al*., 1988). Wild birds seem to be the reservoir of avirulent strains, whereas poultry are the most important host of the virulent viruses but both hosts exchange viruses (Cardenas *et al*., 2013).

According to the strain variation of NDV, the rate of morbidity and mortality of poultry in a flock due to ND varies from 90-100%, thereby poultry industry all over the world facing serious economic losses every year (Gohm *et al*., 2000). In Ethiopia, ND is one of the most important diseases inflicting heavy losses of chickens (Chaka *et al*., 2012; Mazengia, 2012). Newcastle

disease (ND), called "*Fengil*" in Ethiopia, is the most important cause of economic loss in poultry production. Newcastle disease was first reported in 1971 from a small poultry farm in Asmara, then part of Ethiopia (Bawek *et al*., 1991). In 1972, outbreaks occurred in Addis Ababa and in 1974 in the poultry farm of the Agricultural University at Harer and in the Shola and Debrezeit poultry farms (NVI, 1974).

Although virus isolation followed by identification using most of the serological tests such as enzyme linked immunosorbent assay, fluorescent antibody technique, serum neutralization test, plaque reduction neutralization test and AGIDT are considered as the standard methods for the confirmation of the disease (OIE, 1996) but these are always considered as time consuming compared to that of genome detection of the virus as a single method. Therefore, genome detection of NDV both from the clinically sick and dead birds is very important for early and rapid confirmatory diagnosis of ND in poultry during outbreak of the disease compared to any other single or combined conventional methods of diagnosis (Jestin and Jestin, 1991; Pedersen *et al*., 2000). The reverse transcription polymerase chain reaction (RT-PCR) has established its position top over other confirmatory test methods in the field of diagnosis of ND (Singh *et al*., 2005; Krzysztof *et al*., 2006).

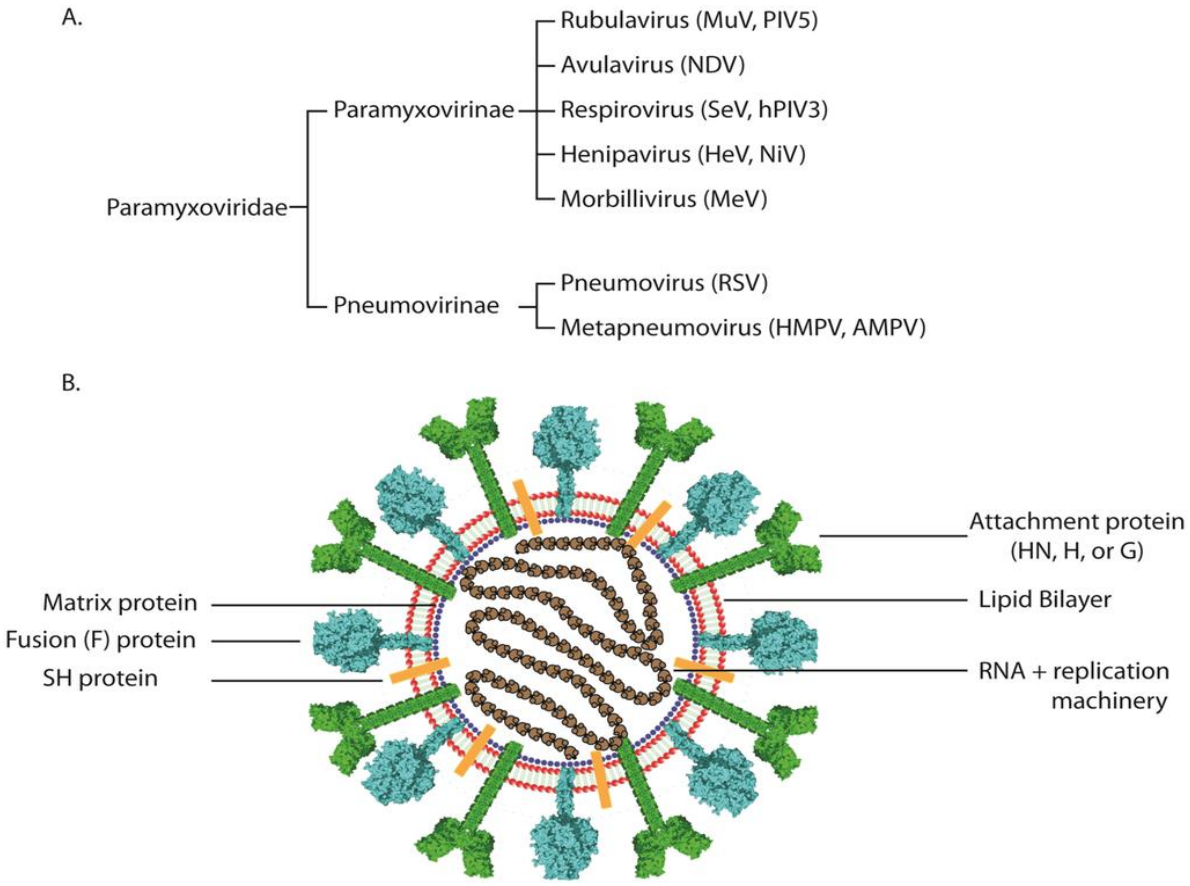

Figure 1: (A) Classification of representative members of the paramyxovirus family, the genus of ND Avulavirus. (B) Schematic of a paramyxovirus. Genomic RNA is wrapped by nucleocapsid core proteins (brown), which are connected to the viral envelope (red) by the matrix protein (blue). The attachment (green), small hydrophobic (present only in certain paramyxoviruses, orange), and fusion proteins (cyan) are depicted at the virus surface (Han *et al*., 2008).

2.2. Etiological Agent

2.2.1. The Virion

NDV virions are 100 nm or more in diameter, pleomorphic, but mostly spherical in shape and consist of single stranded, non-segmented, negative sense RNA genomes. The virion is enveloped with a lipid membrane from the host cell plasma membrane. The envelope contains two transmembrane glycoproteins: the HN and the fusion (F) protein. These proteins are present as homooligomers and form HN spike projections of 8 nm length on the outer surface of the envelope that participate in the initiation of virus infectious cycle (Miller *et al*., 2009; Samal, 2011).

According to ICTV (2011), ND virions measure approximately 1,000–10,000 nm in length. Surface projections on the envelope are spaced widely apart, and haemagglutinin neuraminidase (HN) and fusion (F) glycoproteins are equally distributed on the surface, embedded in a lipid bilayer. This lipid bilayer is comprised of the HN and F glycoproteins. The capsid/nucleocapsid is elongated with a helical symmetry and is not segmented (Miller *et al*., 2009).

The genome houses six genes, namely, nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and the large protein (L) (Figure 2) apart from the P gene which can be transcribed into three different mRNAs encoding one structural (P) and two non-structural (V and W) proteins (Steward *et al*., 1993; Han *et al*., 2008).

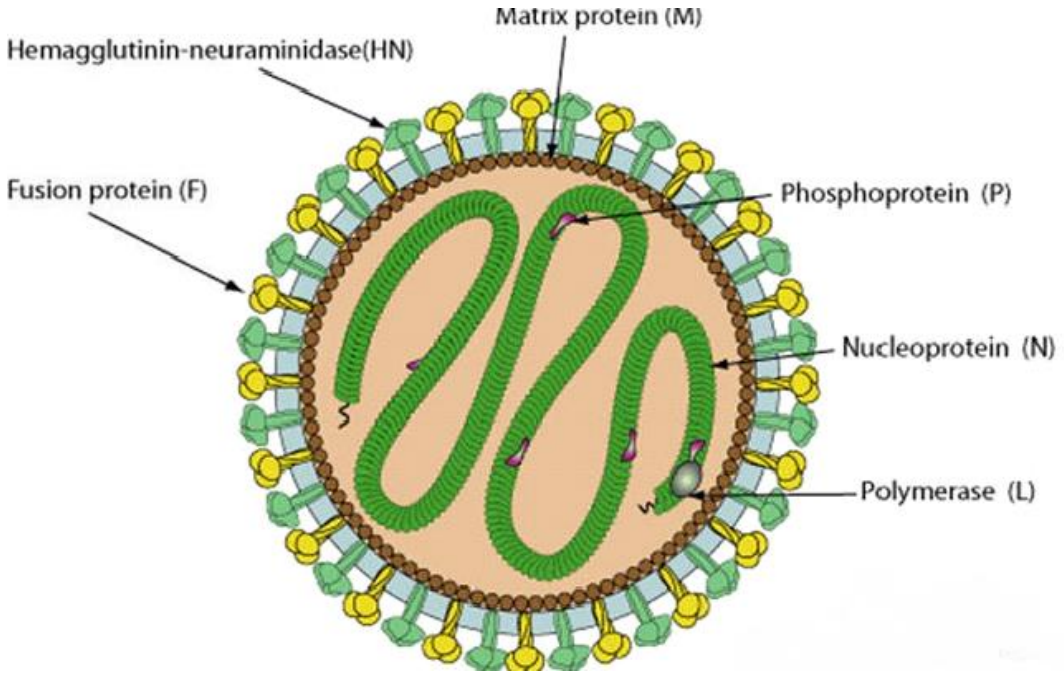

Figure 2: Schematic diagram of a paramyxovirus showing major components (Han *et al*., 2008).

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 (Battisti *et al*., 2012). The other three proteins are intimately associated with 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 (Conzelmann, 2004). The L protein is the RNA dependent RNA

polymerase that serves as the viral replicase and transcriptase during the infectious cycle, while the P protein is the cofactor of the polymerase (Dortmans *et al*., 2010).

2.2.2. The Genome Structures

The genome is about 15.2 kb in length that codes for six structural and two non-structural proteins (Choi *et al*., 2010). 'Rule of six' should be followed by genome because it should be of polyhexameric length to replicate rapidly. It encodes for six proteins in 3' to 5' direction; these are Nucleoprotein (NP), Large RNA polymerase (L), Fusion (F), Hemagglutinin Neuraminidase (HN), Matrix (M) and phosphoprotein (P) (Figure 3). The proteins W and V are additionally created within the P gene during transcription of mRNA at editing site by insertion of guanines (Linde *et al*., 2011).

Figure 3: Arrangement of the genes in the ND viral genome (Linde *et al*., 2011).

The 3' and 5' ends of the viral genome constitute the leader and trailer regions, which accommodate the regulatory signals for virus transcription and replication. There are at least three different genome lengths: 15,186; 15,192 or 15,198 bp, that strictly adheres to the rule of six (genome size divisible by six), with six genes that produce six structural proteins in a 3' to 5' order. Editing of P produces at least one other protein, the V protein, which has anti interferon properties (Czegledi *et al*., 2006).

2.2.3. Newcastle Disease Virus Entry and Replication

The envelope proteins are responsible for viral entry into a target cell. The HN protein mediates cell attachment, whereas the F protein is necessary for cell fusion (Figure 4). The HN protein requires sialic acid residues on the surface of the host cell in order to bind. It then also plays a role in viral release from the cell via its neuraminidase activity which removes sialic acid receptors (Jardetzky and Lamb, 2014). The fusion protein is a key component of the pathogenesis and virulence of the virus, as the ability of the virus to enter a cell is dependent upon the cleavage of

the inactive F0 protein by cellular proteases. After fusion of the virus envelope with the host cell membrane, the viral nucleocapsid is released into the cytoplasm (Maclachlan and Dubovi, 2010).

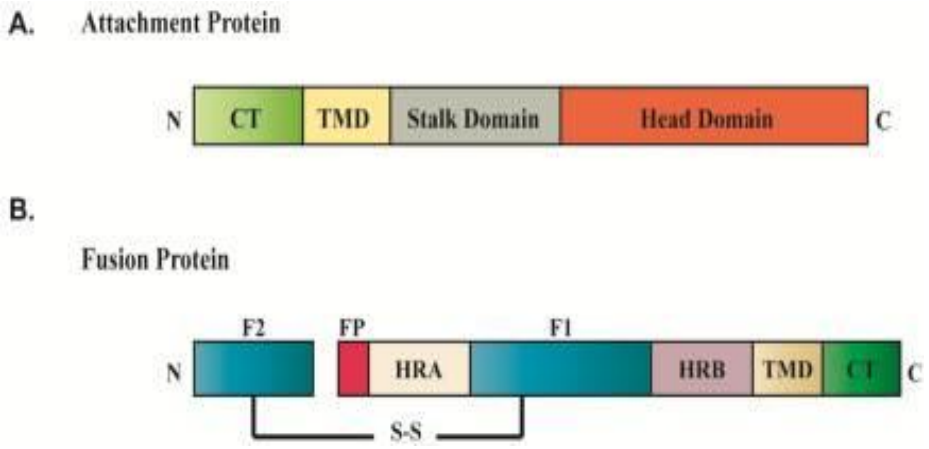

Figure 4: Structure of fusion and attachment protein (El Najjar *et al*., 2014).

Viral attachment is mediated by the hemagglutinin neuraminidase (HN) protein. This binds to gangliosides and N-glycoproteins containing a distinct structure of sialic acid and sugars. Virus to cell binding is followed by the activation of the viral fusion protein F. Fusion of the viral and the host cell membrane, then allows the transfer of the viral genome into the host cell‘s cytoplasm. There, the 15 kb non-segmented negative single-stranded RNA (ssRNA) is transcribed into mRNAs and is translated into viral proteins (Fournier and Schirrmacher, 2013).

Intracellular virus replication takes place within cytoplasm. Because the virus RNA has negative sense, the viral RNA-directed RNA- polymerase (transcriptase) must produce complementary transcripts of positive sense that may act as messenger RNA and use the cell’s mechanisms, enabling the translation into proteins and virus genomes. The F protein is synthesized as a nonfunctional precursor, F0 that requires cleavage to F1 and F2 by this cleavage has significance in the pathogenicity of NDV strains. The HN of some strains of NDV also requires posttranslational cleavage (Alexander, 2003).

2.2.4. Assembly and Release

Nucleocapsids assemble in the cytoplasm of the host cell with initial attachment of the N protein to the RNA to form a helix followed by integration of the P and L proteins (Figure 5). The

nucleocapsids are then transported to the plasma membrane and are connected to the F and HN surface glycoproteins via the M protein. The viral envelope is formed during the process of budding from the host cell (Lamb and Parks, 2007; El Najjar *et al*., 2014).

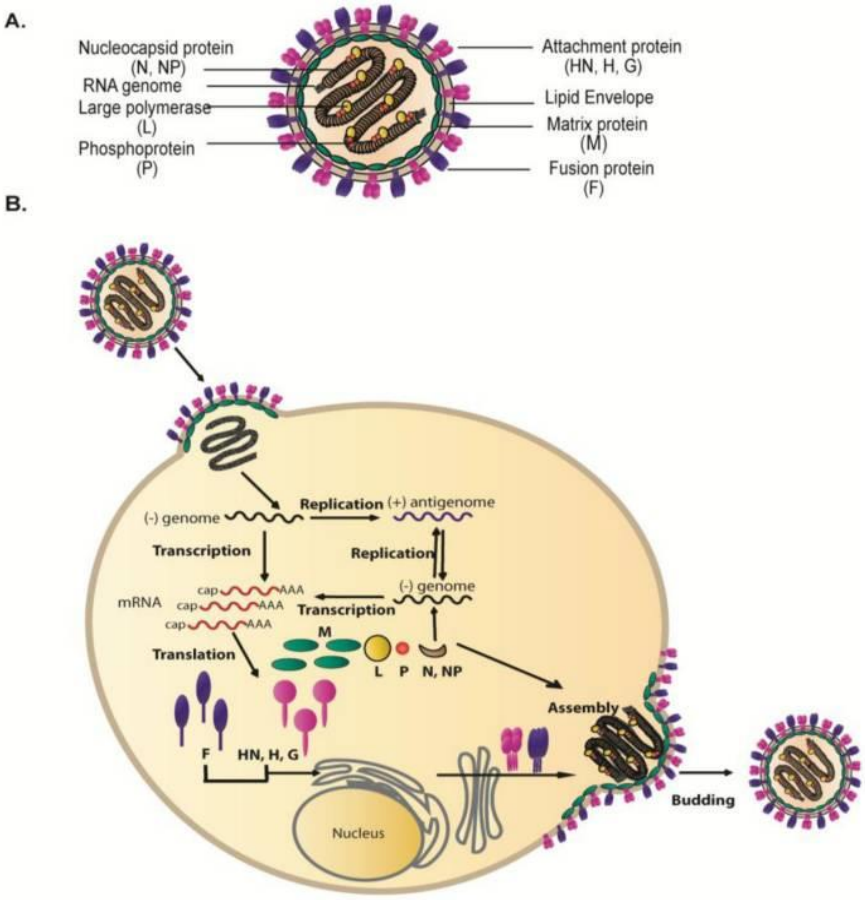

Figure 5: Summary on the life cycle of NDV (El Najjar *et al*., 2014).

2.3. Epidemiology of Newcastle Disease

Newcastle disease is distributed throughout the world (Figure 6). ND is epizootics in Central and South America, Asia, and Africa while sporadic epizootics occur in Europe (Naveen *et al*., 2013). Here in Ethiopia also a number of studies have been conducted to determine the prevalence of ND in different agro-ecology and season of Ethiopia (Nassir, 1998; Ashenafi, 2000; Serkalem *et al*., 2005; Zeleke *et al*., 2005; Mazengia *et al*., 2010; Chaka *et al*., 2012 and Belayheh *et al*., 2014).

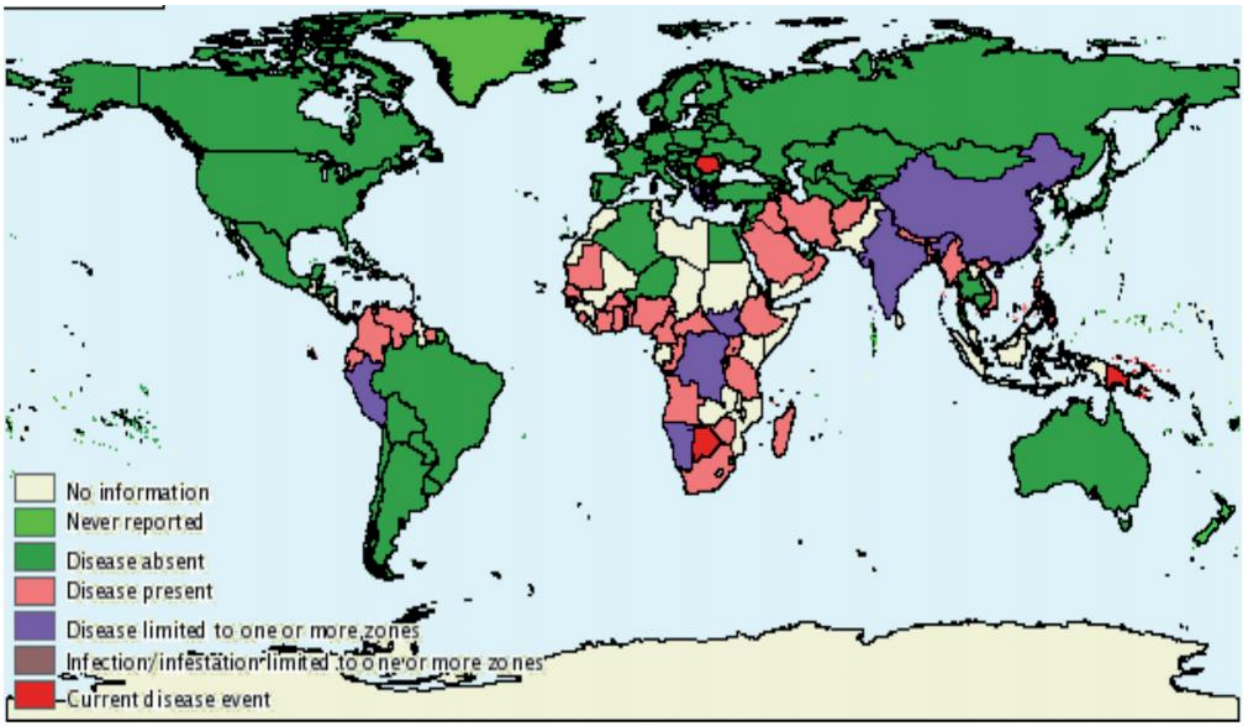

Figure 6: Prevalence of Newcastle disease in 2015.

Source: World Animal Health Information Database (WAHID Interface), Version 1 Copyright © World Organization for Animal Health (OIE); www.old.caribvet. net/en/diseases/newcastlediseases/geographical-distribution.

2.3.1. The Spatial Distribution of Newcastle Disease in Ethiopia

ND is widely distributed in all areas of Ethiopia, although the level of the disease prevalence may show momentous variations across the different farming systems and agro-ecological zones of the country. The national picture of ND status by summarizing the outbreak data reported to Ministry of Livestock and Fishery (MoLF) /formerly said Ministry of Agriculture and Rural Development (MoARD) from 2012 - 2015 was presented in Table 1 and monthly ND outbreak reports in Table 2. The highest rate of outbreaks from March to May suggested to be associated with high rate of chicken marketing for Easter (Alders and Spradbrow, 2001).

Table 1: Newcastle disease reports from seven national regional states of Ethiopia (2012 to 2015).

Region	Number of outbreaks	Number of cases	Number of deaths	Population at risk
Oromia	286	14604	5084	1885704
Amhara	54	3461	1540	178038
South Nation and Nationalities	32	2258	1263	465876
Tigray	17	7267	6179	218733
Addis Ababa	1	20	19	400
Benishangul-Gumuz	14	359	3324	54173
Gambella	1	18	8	506

Source: Ministry of Agriculture and Livestock Resources (2018).

Table 2: Newcastle disease monthly outbreak reports from seven national regional states of Ethiopia (2012 to 2015).

Months	Number of outbreaks	Number of cases	Number of deaths	Population at risk
January	28	2042	562	141169
February	33	7788	3126	282707
March	38	9307	5109	544954
April	46	1855	725	423153
May	39	3686	2776	229131
June	37	3558	1546	290902
July	29	2285	1398	71860
August	26	1385	521	56828
September	28	1185	578	202423
October	24	844	206	58827
November	27	1095	675	78763
December	27	536	183	125044

Source: Ministry of Agriculture and Livestock Resources (2018).

Nasser (1998) reported 2.3% and 58.5% from state poultry farms and Dembi, respectively; Ashenafi (2000) reported 22.5% and 47.3% from Rift valley and central highland of Ethiopia; Serkalem *et al*. (2005) recorded 28.6%, 29.7% and 38.% at Debreberhan, Sebeta and Adama, respectively; Tadesse *et al.* (2005) reported 32.2% from central Ethiopia; Regasa *et al.* (2007) reported 11% from mid rift valley of Oromia; Mazengia *et al*. (2010) recorded 21.7% at Bahir Dar; Chaka *et al*. (2012) reported 5.14% and 7.12% from ATGK and Adea, respectively; Nega *et al*. (2011) reported 55.8% from selected districts of western Amhara; Chaka *et al.* (2013) reported 16.73% and 32.2% from AJKT and Adea, respectively; Belayheh *et al*. (2014) recorded 5.6% from Kersana Kondaltity; Terefe *et al*. (2015) reported 6%, 16.1% and 2% from Bishoftu, Tikurwuha and Ziway; Miressa *et al*. (2015) reported 26.7% from Adama and Bishoftu; Getachew *et al*. (2016) reported 26.2% from Alamata District, Southern Tigray.

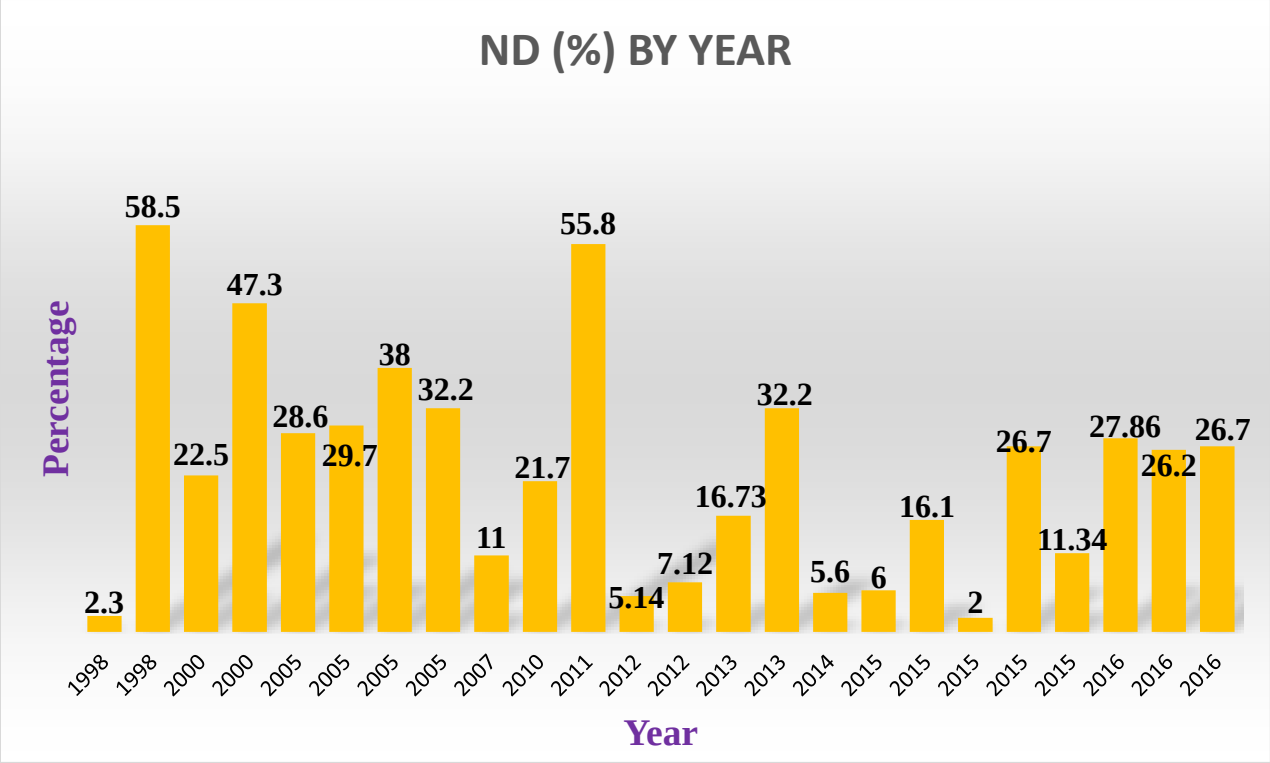

Figure 7: Yearly ND prevalence in Ethiopia.

2.4. Transmission

NDV can infect more than 240 species of birds and it spreads mainly through direct contact between healthy and infected birds. The disease transmits through droppings and secretions from the nose, mouth and eyes of infected birds. The disease spreads by contaminated water, feed and transport. Airborne transmission of the virus is also an important route of transmission for ND (Li *et al*., 2009). NDV as well transmitted via inhalation or ingestion of virus shed in feces and respiratory secretions by infected birds for variable lengths of time (Alexander, 1988; Leighton and Heckert, 2007). Some virus isolates have been found to be transmitted through the egg to the hatching chick (Roy and Venugopalan, 2005).

Mechanical transfer of infected feces occurs by rodents, insects, dogs, fleas, or scavenging animals. Infection takes place by virus inhalation, ingestion or by contact with conjunctiva. The disease may vary from subclinical with no mortality to severe infection, with 100% mortality (Ullah *et al*., 2004). The virus is also able to replicate in the intestinal tract which can then be excreted in the feces. It has been shown that large amounts of virus are commonly excreted in the feces of NDV infected birds (Alexander *et al*., 2004). Vaccination and insemination crews as well as veterinarians have been shown to transmit the disease from farm to farm due to improper cleaning and disinfecting of equipment (Li *et al*., 2009).

Live bird markets can also contribute to the persistence and spread of the virus. These markets may not follow appropriate cleaning and disinfecting techniques which allows for the possibility of environmental contamination. Live birds in the market are exposed to birds from multiple sources. These birds run the risk of disseminating the virus as they leave the market. Low virulent strains of NDV have been regularly isolated from wild birds by the NVSL. Migratory wild birds have been shown to transmit NDV to free range poultry through direct contact or by contamination of feed or water (Killian, 2009).

2.5. Signs and Symptoms of Newcastle Disease

Neurotropic velogenic ND virus (nvNDV) affects the nervous system and causes paralysis of legs and wings, the neck twists to one side the birds may depict the picture of star gazing like torticulus, birds move in a circle and exhibit opisthonic posture. Viserotropic velogenic ND

virus (VVNDV) affects the digestive tract of birds, results in decrease feed and water intake, and copious greenish white diarrhea. Pneumotropic velogenic ND virus (PVNDV) causes respiratory signs in chicks which result in difficult breathing (Oladele *et al*., 2005).

The incubation period of ND virus ranges from 2 to 15 days. Cough flu sneezing, tracheal rales, fever, depression, watery discharge from nose, decreased feed intake, decreased water intake, diarrhea, conjunctivitis, ruffled feathers, torticolis, and blindness is observed in birds. Diarrhea, nervous signs, shivering and paralysis of legs and wings has been observed in pigeons (Shaheen *et al*., 2005). In addition, the clinical signs and symptoms of ND also include depression, weakness, loss of appetite, dehydration, inability to stand, cyanosis of comb and wattle, greenish watery diarrhea, nasal and eye discharges, decreased egg production, loss of weight followed by death (Pansota *et al*., 2013).

There are many factors which effect severity of clinical signs of ND in birds mainly; age, route of infection, immune status and concomitant environmental stress. Young birds have more severe and acute disease in comparison to older birds. The intravenous inoculation /infection elicit neurologic signs and aerosolization of high viral doses impact the upper respiratory infection (Alexander, 2003). There is also species variation regarding expression of clinical signs in birds. The clinical signs in chickens are recognizable at second day of the onset of the disease (Susta *et al*., 2011), birds become off feed and dull on third day and chicks become severely depressed and inactive with hard ruffled feathers on fourth day, prostrated position and open mouth breathing starts on fifth day of ND infection. Nervous signs i.e. blindness and torticollis and incoordination on seventh day of ND virus infection (Susta *et al*., 2011).

As a general, the frequent clinical symptom of virulent ND; chicken fluffs its feathers and appears to have its coat dragging on the ground, lethargy and inappetence, respiratory signs such as mild rales and snick can be detected by careful observation, severe respiratory distress and gasping, swelling of the head and neck, pink eye and swollen eyelids with abnormal accumulation of liquid, foamy discharge from respiratory tract, greenish diarrhea. When the disease is advanced nervous signs of tremor, torticollis, convulsions and paralysis of wings and legs will be seen (Czegledi *et al*., 2006).

2.6. Pathogenicity

NDV can be categorized into highly pathogenic (velogenic), intermediate (mesogenic), and nonpathogenic (lentogenic) strains based on pathogenicity in chickens (Alexander, 2000). In addition, avirulent and virulent strains can be distinguished on the basis of the cleavage site sequence of their fusion (F) protein, one of the most important virulence factors (OIE, 2012). Cleavage of the F protein during viral replication in the host plays a major role in the virulence of the virus (de Leeuw *et al*., 2003). The virulence of NDV strains varies greatly with the host. Chickens are highly susceptible, but ducks may be infected and show few or no clinical signs, even with strains lethal for chickens (Dai *et al*., 2014).

The pathogenicity of the virus depends on multiple factors including host species, age, immune status, secondary infections, stress, environmental conditions, the amount of virus transmitted, and the route of transmission but most importantly the strain of the infecting virus (Alexander *et al*., 2004; Saif *et al*., 2008). In general, the younger the chicken, the more acute the disease is. With virulent viruses in the field, young chickens may experience sudden deaths without major clinical signs; however, in older birds the disease may be more protracted and with characteristic clinical signs (Zeinab *et al*., 2018). The virulence of NDV strains is determined by multiple factors: tissue or organ tropism, the host immune system and/or the efficacy of replication (Fan *et al*., 2016).

2.7. Molecular Basis of Pathogenicity

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

presence of these basic amino acids in virulent NDV. It has been found that avirulent virus have 112G/E-K/RQ-G/E-RL117 and virulent viruses have 112R/K-R-Q-K/RR-F117 amino acid sequence at cleavage site (Boostani *et al*., 2013). Most of the pathogenic APMV-1 viruses for chicken have sequence 112R/K-R-Q/K/R-K/R-R116 Office of International Epizootics (OIE) accepts F cleavage sequence as determinant of primary virulence. However, if this cleavage sequence is not found, then an Intra Cerebral Pathogenicity Index (ICPI) is required for determination of the virulence (Wise *et al*., 2004).

2.8. Zoonotic Potential

Avian avulaviruses are capable of infecting humans and infection typically results in conjunctivitis and/or influenza-like symptoms in humans, when a person has been exposed to large quantities of the virus. Mostly, Laboratory workers and vaccinators are affected. The use of personnel protective equipment and biological safety cabinet has reduced the exposure of laboratory workers. Infection is rarely seen in the workers of a farm; moreover, persons handling or consuming poultry products do not appear to be at risk (Nolen, 2003). Exposure to a large amount of virus is necessary, thus human infections are most common in individuals working on poultry farms or in slaughterhouses. There has been one documented instance in which a 42-year old man with a history of non-Hodgkin's lymphoma developed a lethal pneumonia following a peripheral blood stem cell transplant (Goebel *et al*., 2007).

APMV-1 infection is known to cause mild disease in human beings. Most commonly, human infection is associated with conjunctivitis, which resolves within 1 week. Less often, it is associated with transient fever or chills (Swayne *et al*., 2003; Capua and Alexander, 2004). So far, only two fatal cases of human infection have been reported, one in the USA (Goebel *et al*., 2007) and the other in the Netherlands (Svraka *et al*., 2010). The conjunctivitis usually resolves rapidly, but the virus will be shed in the ocular discharges from 4 to 7 days. In some cases, mild, self-limiting influenza like disease with fever and headache has also been reported in humans. There is no evidence found to support human to human transmission but the potential for human to bird transmission exists (David and Daniel, 2003). Despite the ability for avian avulaviruses to infect humans this pathogen should be considered a low priority for public health agencies because of the high infectious dose and the mild clinical symptoms

observed in immune-competent individuals. Fomite transmission of NDV is a documented route of spread due to robust viral stability; thus, special precautions should be taken for individuals that work closely with domestic poultry and are immune-compromised or for workers that co-habitate with an individual that is immune-compromised (Leighton and Heckert, 2007).

2.9. Diagnosis

Newcastle disease can be diagnosed based on history, clinical sign and laboratory test. It clinically resembles highly pathogenic avian influenza so during outbreak rapid and accurate diagnosis is important to control and prevent dissemination of disease (Khan *et al*., 2010). Laboratory diagnosis for NDV includes 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). RT-PCR assay is more sensitive, specific and less labor intensives as compare to other conventional methods used for lab diagnoses such as virus isolation, Immuno-Fluorescence Staining, Neuraminidase Inhibition and ELISA (Saif *et al*., 2005; Shahzad *et al*., 2011; Tang *et al*., 2012). Using modern technologies, new diagnostic techniques are being developed for identification and differentiation of NDV strains (Rezaeianzadeh *et al*., 2011). Other molecular diagnostic tests like nucleotide sequence analysis are also important in viral disease diagnosis (Shahzad *et al*., 2011; Shabbir *et al*., 2012).

2.9.1. Diagnosis Based on Clinical Sign

The clinical sign of ND depends on age, immune status of the host, tissue tropism and virulence of virus strain. Sudden high mortality in a flock in the absence of premonitory clinical signs occurs when susceptible species are exposed to highly virulent strain. In susceptible flocks the mortality rate in fully can reach 100% (Quinn *et al*., 2002). The incubation period of ND is usually about five days. In chicken's nerve, respiratory and digestive sign may occur. The major clinical sign observed in Newcastle disease are: greenish white diarrhea, with ruffled feathers; depression in the birds and a state of prostration, a condition known as torticollis (the head turned to one side and other neurological sign like paralysis of leg and wing). ND is acute disease can cause death within 2 to 3 days (Hasan *et al*., 2012).

2.9.2. Diagnosis Based on Pathological Lesion

Infection with panzootic ND is commonly associated with necrosis of the intestinal wall or lymphoid tissues resulting in hemorrhagic lesions in the mucosa of the proventriculus, ceca, duodenum, jejunum, and ileum. Birds displaying neurologic symptoms do not have pathologic lesions in the central nervous system. Gross lesions of the respiratory tract may include hemorrhage of the respiratory mucosa, air sacculitis and congestion of the trachea but are not always seen (Hines, 2012). Secondary bacterial infection is a significant concern and may lead to thickened air sacs with catarrhal or caseous exudates. Infection in other organs may be marked by hemorrhage in the lower conjunctiva, Para tracheal edema, and necrosis of the spleen. Laying poultry infected with vND may demonstrate flaccid and degenerative ovarian follicles, hemorrhage of reproductive organs including the ovarian follicles and egg yolk in the abdominal cavity (Hines, 2012).

Examination by histopathology also yields a variety of descriptive lesions influenced by the virulence of the strain and route of introduction. Microscopic lesions may include cellular infiltration, oedema, hyperaemia, and necrosis. Neurologic lesions are comprised of encephalomyelitis with degeneration of the neurons, lymphocyte infiltration, and hypertrophic endothelial cells. These lesions are usually found in the cerebellum, midbrain, spinal cord, medulla, and brain stem. Complete loss of cilia in the respiratory tract can occur within days of infection. In the early stages of infection, lymphocyte and macrophage infiltration is common in the mucosa of the upper respiratory tract along with congestion and edema (Alexander and Senne, 2008).

Virulent strains can cause hemorrhages of the blood vessels in multiple organs especially the intestinal tract. The serosal and mucosal surfaces show marked necrosis in intestinal lymphoid aggregates. Necrosis can be seen in the cecal tonsils, and hyperplasia of monocytes is evident in the liver and other organs. The germinal centers of the spleen and thymus show marked focal vacuolation and lymphocyte destruction. Hemorrhages can also occur in the heart, gallbladder, skin, and eyelids leading to conjunctivitis. Petechiae of the wattle and combs and facial edema are commonly seen during infection (Hines and Miller, 2012). Diagnosis should not be based on pathogenomonic lesions or clinical signs because these types of symptoms

and lesions are not specific to any strain of NDV. Some lesions may be seen with infection of low virulent strains, and symptoms may be similar to those seen with more virulent strains. Pathology is a useful tool to guide disease diagnosis, but it cannot be used solely to diagnose ND considering these types of lesions were not unique to NDV infection (Hines and Miller, 2012).

2.9.3. Diagnosis Based on Laboratory Techniques

2.9.3.1. Sample for laboratory diagnosis

The appropriate sample for diagnosis in Newcastle disease includes: tissue sample (trachea, lung, spleen, soft palate, colon, bursa and brain) which are important for histopathology and cloacae swabs, oro-nasal swabs and Serum sample. Blood sample is usually collected from wing vein. When fresh sample is collected lung and spleen it should be wrapped in plastic and placed into cold box with ice. To do serological test haemolysed or contaminated samples should not be used because it will give unreal result (Alder and spradbrow, 2001).

2.9.3.2. Virus isolation

Because of their extreme sensitivity and convenience, ten to twelve (10-12) day-old specific Pathogen free (SPF) embryonated eggs are readily used for cultivation of NDV. This is done by inoculation on to the chorioallantoic membrane or into the allantoic sac. Virulent ND viruses' can also be propagated in cell culture. Samples for isolation include antibiotic treated cloacal and tracheal swabs or trachea and bone marrow. For cultivation in eggs, finely ground tissues, organs and faecal samples treated with antibiotics are centrifuged at 1000 g for 10 minutes and 0.2 ml of the supernatant inoculated into the allantoic sac. The eggs are incubated at 37^{o}C and examined daily by candling. While eggs dying on the first day are discarded, those, which die thereafter up to day 7 of incubation, are chilled at 4^{o}C and the allantoamniotic fluid was harvested. The presence of the virus is detected by hemagglutination test (Alexander, 2003).

2.9.3.3. Serological technique

The reliability of any serological test depends to a large extent on the quality of the samples submitted. Haemolysed or contaminated samples give unreliable results. Blood from domestic chickens is usually collected from a wing vein. A separate needle should be used for each animal to avoid the risk of mechanically transmitting infectious agents from one animal to another. Blood samples for serology must not be frozen until the serum has been separated from the clot. Serum samples can be submitted frozen, provided that there are no blood cells present in the sample (Alder and Spradbrow, 2001).

2.9.3.4. Haemagglutination inhibition test

The haemagglutination inhibition (HI) test is used most widely in ND serology; its usefulness in diagnosis depends on the vaccinal immune status of the birds to be tested and on prevailing disease conditions. HI is done based on principle that the haemagglutinin on the viral envelope can bring about the clumping of red blood cells chicken and that this can be inhibited by specific antibodies (Aldous and Alexander *et al*., 2005). Sera from species other than chicken red blood cells can also cause clumping (agglutination), so it should be removed by adsorption of the serum with chicken RBCs determining these properties. This is done by adding 0.5 ml of antisera to 0.025 ml of packed chicken RBCs, shaking gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 g for 2-5 minutes and the adsorbed sera are decanted. Any pretreatment of the sera is unnecessary as Chicken sera can rarely give nonspecific positive reactions in the HI test. The results were recorded as $\log_2$ values of the highest reciprocal of the dilution that showed inhibition. HI titres ≥ 4 $\log_2$ were considered positive results for NDV (OIE, 2013).

2.9.3.5. Enzyme linked immunosorbent assay

The ELISA consists of a microtiter plate that has NDV antigen attached to the bottom of each well. Addition of serum containing anti-NDV antibodies creates antigen-antibody binding which is detected using antibodies produced in another species against chicken antibodies. An enzyme is conjugated to the anti-chicken antibodies so when anti-NDV antibodies are present and bound to the NDV antigen, the enzyme bound to the anti-chicken antibodies catalyzes a

color change in the well. This can be read by viewing the plate or quantitatively using a spectrophotometer. Serial dilution of the anti-NDV antibody test serum can be used to determine the titer (Saif *et al*., 2008).

2.9.3.6. Molecular techniques

Molecular techniques such as polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) have been used for rapid and sensitive detection for Newcastle disease (Ananth *et al*., 2018).

Polymerase chain reaction

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

Multiplex PCR tests have been developed to allow simultaneous detection and differentiation of several avian viruses including NDV. These techniques have also been used experimentally to differentiate between velogenic, mesogenic and lentogenic strains from chickens. It is applied simultaneously that required for avian infection including Newcastle disease for amplification and quantification of the virus (Tang *et al*., 2012). The primers that are specific for each virus are newly designated from the nucleoprotein gene of Newcastle disease virus. This technique helps mass amplification of the virus using common primers in the presence of fusion protein gene which increased the markedly sensitivity of the tests. At present, it should be noted that multiplexing RT-PCR assays aiming at broadening the range of virus detection frequently result in reduced sensitivity of the test compared with single target assays (Tang *et al*., 2012; Saba and Mardani, 2014).

Reverse transcription polymerase chain reaction

Molecular techniques like reverse transcription polymerase chain reaction (RT-PCR) have been frequently used all over the world to detect viruses from the field samples. Reverse transcription polymerase chain reaction (RT-PCR) is used to detect RNA virus which is negative and single stranded RNA virus. There are two different configurations of the RT-PCR assay. In the two step RT-PCR configuration, the cDNA is synthesized in a different tube before performing PCR assay. In contrast a one-step RT-PCR firstly 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 (Pfaffl, 2004).

This is rapidly becoming the assay of choice. Several important steps need to be considered in developing an RT-PCR protocol. The first aspect is the RNA extraction. This needs to be an efficient process that can extract RNA from the samples even when it's in low concentrations and eliminate contaminants that will degrade the RNA. 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. RT-PCR for the detection of NDV was first described by Jestin & Jestin (1991) and to date it has been successfully developed in different modifications e.g. using universal primers to detect all NDVs (Gohm *et al*., 2000), pathotype specific primers that enable rapid differentiation of the pathotype or nested PCR. Poorly design primers can result in mispriming and the amplification of nonspecific products or the formation of primer dimmer (AbdElsalam, 2003; Añez-Lingerfelt *et al*., 2017).

Sequencing

Other molecular diagnostic tests like nucleotide sequence analysis are also important in NDV diagnosis (Shabbir *et al.,* 2012). DNA sequencing is used in the diagnostic laboratory to analyze the virulence potential of APMV-1 isolates. Sequencing techniques originated with Sanger using the dideoxynucleotide triphosphate (ddNTP) mediated chain termination and Maxam and Gilbert using the chemical degradation method. Sequencing techniques have been rapidly improving since that time, and next-generation or automated sequencing techniques have become standard for laboratory analysis of gene sequences (Maxam and Gilbert, 1977; Sanger *et al*., 1977).

2.10. Prevention and Control

2.10.1. Management Strategies

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

2.10.2. Vaccination

The general approaches to the control of Newcastle disease are hygiene and vaccination, Hygiene includes measures such as cleaning, disinfection, limiting access to wild birds, and personal hygiene of the farm staff. Vaccination in combination with appropriate hygiene measures, this remains the most effective way of controlling ND (Abdisa and Tagesu, 2017). Vaccination against vND would result in immunity against infection and replication of the virus. Realistically, ND vaccination usually protects the bird from the more serious consequences of disease, but virus replication and shedding may still occur (Lee *et al*., 2008).

Live vaccine:

Live vaccines are relatively cheap, sold as freeze dried, easy to administer and can be used for mass vaccination. These have been divided in to mesogenic and lentogenic groups with their preferred mode of administration being eye drop, beak intranasal installation, or dipping for lentogenic vaccines while mesogenic vaccine requires intramuscular injection. Drinking water and aerosol administrations can also be used (Alexander, 2003). In most countries, Hitchner B1 and La Sota vaccines are used and are derived from the mesogenic strain of NDV (Alexander, 2003). Some mesogenic vaccines may cause disease; particularly in young birds, especially if there is a dual infection with exacerbating organisms. Because of heat liable the

live vaccines are also have disadvantage under village management system where transport and cold storage facilities are often inadequate (Otim *et al*., 2005).

Inactivated vaccine:

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

Conventional ND vaccines have stood the test of time by demonstrating track record of protective efficacy in the last 60 years. However, these vaccines are unable to block the replication and shedding of most of the currently circulating phylogenetically divergent virulent NDV isolates. Hence, rationally designed vaccines targeting the prevailing genotypes, the so-called genotype-matched vaccines, are highly needed to overcome these vaccinations related challenges. Among the recently evolving technologies for the development of genotype-matched vaccines, reverse genetics-based live attenuated vaccines obviously appeared to be the most promising candidates (Muhammad *et al*., 2018).

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

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted in selected Central Rift Valley areas (Bishoftu, Adama, Bote, Batu, Shashemene and ArsiNegelle) of East Shoa and West Arsi Zone of Oromia Regional State (Figure 8).

East Shoa zone is located about 98 km east of Addis Ababa that covers the total area of approximately 10241 Km^2 and Adama town is the capital of the zone. The Zone extends between 70'33'50" N – 90'08'56" N and from 380'24'10" E – 400' 05' 34" E. The temperature of the area ranges from 10^0C in uplands to over 30^0C in rift valley depressions with the mean temperature of 20^oC. Since the large portion of the zone is located along the rift valley system, rainfall varies from 600mm to 1000mm with mean annual rainfall of 816 mm. The livestock population of East Shoa zone is estimated to be 1,090,091 cattle, 319,598 sheep, 568,761 goats, 10644 horse, 7039 mules, 284, 583 donkey, 6818 camels, 14627 beehives and 1,250, 059 poultry. Out of a total 1,250,059 poultry, around 94% (1,169,710) are indigenous or village poultry whereas only 6% (69,562) are hybrids (CSA, 2013).

Both Arsi-Negelle and Shashemene are found in West Arsi zone of the Oromia Regional State. Arsi-Negelle is about 225 km south of Addis Ababa. Geographically, it is situated in the central rift valley system at 7^0 09'-70 41' N and 38^0 25'-38 ^{0} 54' E. The Zone covers three agro- ecological zones (low, mid and high land) based on temperature, rainfall, altitude and vegetation that range from 1500-2300 m.a.s.l. (ICRA, 2002). The high-altitude zone occupies the largest area followed by mid and low altitude climatic zones, respectively. Average annual temperature varies from 10-25 °C while annual rainfall varies between 500-1000mm (ORS, 2004). Shashemene is also 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 longitudes at an altitude ranging from 1900 to 1950 meters above sea level (m.a.s.l). Its annual average temperature ranges between 18^0C and 25 ^{0}C and has moderate annual rainfall ranging between 800 and 1300mm (BoFED, 2012). The livestock population of West Arsi Zone is estimated to be 1, 957, 066 cattle, 946, 595 sheep, 404, 118 goats, 214, 744 horses, 6, 304 mules, 210, 339 donkeys, 555 camels and 1, 105, 688 chicken (CSA, 2014).

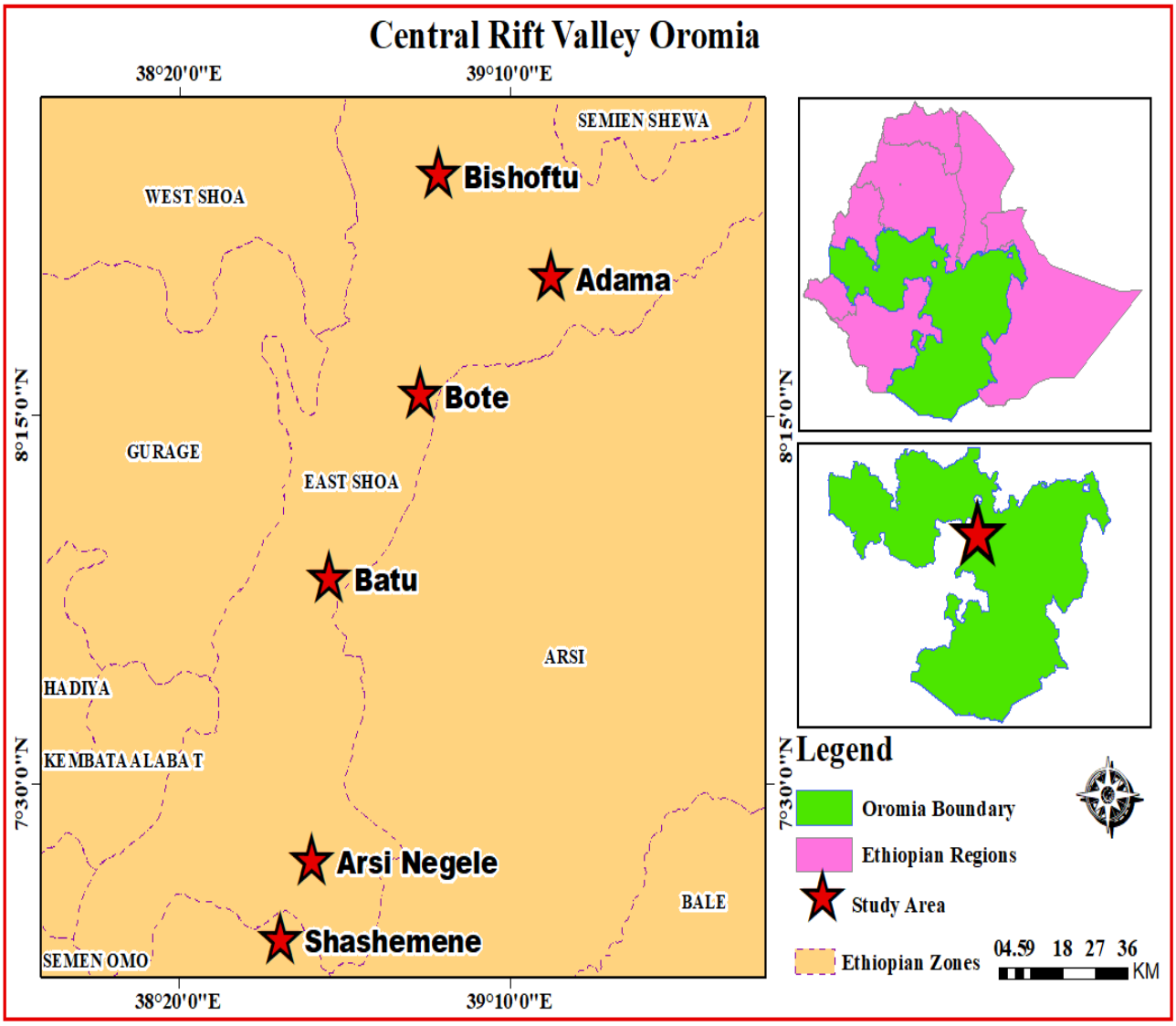

Figure 8: Map of study area, East Shoa and West Arsi Zones in Central Rift Valley of Oromia regional State, Ethiopia (by ArcGIS 10.4.1).

3.2. Study Populations

The study populations were non-vaccinated village chickens from East Shoa and West Arsi Zones in Central Rift Valley of Oromia regional State, Ethiopia.

3.3. Study Animals

Study animals were non-vaccinated village chickens from randomly selected districts in East Shoa and West Arsi zones. Village chickens were sampled in respective of districts, sex and age. The age was determined based on history from the owners (Belayneh *et al*., 2014) as young 3 - 6 months, adult 7 – 16 months and older more than 16 months.

3.4. Study Design

A cross-sectional study for Newcastle Disease was conducted in non-vaccinated village chickens found in the study areas from January to May, 2019.

3.5. Sample Size Determination

The sample size was calculated based on the prevalence of 38.4%, as previously reported by Terefe *et al*. (2015) using 95% confidence level and 5% marginal error. A formula according to Thrusfield (2005) was used to determine the required sample size as indicated below:

$$\mathrm{n} = \frac{z^2 \mathrm{Pexp}\ (1 - \mathrm{Pexp})}{d^2}$$

Where;

n = Sample size,

Z = Statistic for a level of confidence,

d^2 = Required absolute precision, and

P_{exp} = Expected prevalence

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

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

$$n = \frac{3.8416\mathrm{x}0.384\ (0.616)}{(0.05)^2}$$

$$n = \frac{0.909}{0.0025}$$

$$n = 364$$

Hence, a total of 364 non-vaccinated village chickens were required to be sampled. However, 420 domestic birds were randomly selected at poultry markets and villages in the study areas to increase the precision of the study. To calculate the prevalence the following formula was used:

$$\text{Prevalence} = \frac{\text{Number of Positive sample}}{\text{Total number of Sample}}\ x\ 100$$

3.6. Sampling and Method of Data Collection

3.6.1. Swab Sample Collection

A total of 420 non-vaccinated and healthy live chickens were sampled using random sampling technique from Adama, Arsi-negelle, Bote, Batu, Bishoftu, Shashemene Districts poultry market and villages by equal distribution in the study areas on consecutive market days. Tracheal and cloacal swab samples were collected from live chickens by inserting sterile cotton tipped swab into the trachea gently swabbed its wall and cloacal swab deeply into the vent and gently swabbing the wall of the vent. Both the tracheal and cloacal swab samples were placed in sterile separate cryovial (2 ml volume) containing 500 µl of freshly prepared viral transport media (VTM). During sampling, all the samples were labeled, placed in ice pack (4°C) and transported to molecular laboratory of National Animal Health Diagnostic and Investigation Center (NAHDIC); Sebeta, Ethiopia and stored at -80°C until processing. Sample collection and transportation were conducted according to the standard techniques recommended by OIE (OIE, 2008).

3.6.2. Questionnaire Survey

A total of 180 (30 from each District) volunteer village chicken owners/individuals and traders were interviewed by using structured questionnaire (Appendix 1) to assess the socio-economic situations of village chicken producer, the risk factors and about awareness of the disease after the collection of swab samples.

3.7. RNA extraction

Viral RNA extractions from tracheal and cloacal swabs were conducted using Qiagen®viral RNA Mini kit according to manufacturer's instruction (Qiagen, 2014) as follows: - Swab samples

collected with viral transport medium were centrifuged briefly at 8000 rpm for 1 minute in order to get cell free supernatant. Then, 140 µl of the supernatant were lysed by adding 560 µl of buffer AVL containing carrier RNA. After ten minutes' incubation at room temperature, the sample was mixed and 560 µl of 96% ethanol was added to filtrate and again mixed thoroughly. Samples containing 630 µl of lysis buffer and absolute alcohol were transferred for binding to mini spin column and centrifuged at 8000 rpm for 1minute and old collection tube was replaced by a new collection tube. A 500µl wash buffer (AW1) was added and centrifuged at 8000 rpm for 1 minute and a new collection tube was used. Again, a 500 µl wash buffer (AW2) was added and centrifuged at full speed of 14,000 rpm for 3 minutes in order to remove any unwanted protein and others. Again, new collection tube was used and centrifuged at full speed of 14,000 rpm for 1 minute without adding anything. A new and sterile 1.5 µl of micro centrifuge tube was used followed by adding 60 µl of elution buffer (AVE) to mini spin column and incubated for 3 minutes at room temperature. Finally, the eluted RNA was kept at -80°C for storage.

3.8. Real –Time Polymerase chain reaction

One step real time PCR was performed using an Applied Biosystems 7500 fast thermocycler using the Forward Primer M+4100- 5'-AGT GAT GTG CTC GGA CCT TC-3', Reverse Primer M-4220- 5'CCT GAG GAG AGG CAT TTG CTA-3'. Probe M+4100- 5'FAM- TTC TCT AGC AGT GGG ACA GCC -TAMRA -3' were used to specifically detect pathogenic strains of NDV (IDT). For M-gene assay, the following amounts of reagents per 25 µl reaction were used: 5 µl of kit supplied PCR buffer (5x), 0.5 µl of each primer (10 pmol), 1 µl of probe (6 pmol), 0.8 µl of kit supplied deoxynucleoside triphosphates (dNTPs), 1.25 µl of 25 mM $MgCl_2$ and 0.5µl of 13.3 u/µl of RNase inhibitor and 1 µl Qiagen enzyme mix and 6.45 Rnase free water (QIAGEN). All the reagents in a single reaction tube (master mix) were mixed to ensure a homogenous distribution of the reagents. Aliquots of 17 µl of prepared reagents were distributed in to applied biosystem plate depending on number of samples to be tested. Finally, 8 µl of extracted RNA were added to each plate to get total volume of 25 µl reaction. For controls, NDV known positive field isolates and RNase free water as positive and negative controls, respectively, were used. The plates were sealed with a sealer in order to avoid evaporation and placed in to the thermal cycler or rRT-PCR machine that was connected to computer with its own software (Applied biosystems sequence detection

software version 1. 4. 0) and the program was adjusted for each specific reaction or set accordingly. The reverse transcription (RNA to cDNA) step was done for 30 min at 50°C, followed by 15 min at 95°C incubation. The cycling conditions for the M-gene assay were programmed at 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). The reporter dye (FAM) and quencher dye (TAMRA) signals were measured at the extension step of each cycle, and the threshold cycle (Ct.) for each sample was calculated. The samples that have a Ct. value <35 were considered as positive and >35 Ct. value considered as negative for M genes based on rRT-PCR (OIE, 2013).

3.9. Conventional Polymerase Chain Reaction

Conventional PCR was performed using primer sequences with forward primer NOH- 5'-TAC ACC TCA TCC CAG ACA GG-3' and reverse primer NOH- 5'-AGT CGG AGG ATG TTG GCA GC-3' (Europhins MWG operon) used to specifically detect pathogenic strains of NDV. The following amounts of reagents per 25 µl reaction were used: 5 µl of kit supplied PCR buffer (5x), 0.25 µl of each primer (20 µM), 0.5 µl of kit supplied deoxynucleoside triphosphates (dNTPs) of 10Mm, 0.1 µl of 40 U/µl of RNase inhibitor, 0.5 µl of one step RT-PCR enzyme mix and 13.4 µl Rnase free water (QIAGEN). All the reagents in a single reaction tube (master mix) were mixed and in to 20 µl of the mixed samples 5 µl of extracted RNA or template were added to get a total volume of 25 µl to each respective tube and were capped tightly in order to avoid evaporation and the tube were labeled. For controls, NDV known positive field isolates and RNase free water as positive and negative controls, respectively were used. After placing tubes, the thermal cycler or PCR machine was programmed for reverse transcription (RNA to cDNA) step and incubated for 30 min at 50°C, followed by 15 min at 94°C. The cycling conditions consisted 35 cycles and each cycle were having 30 second for denaturation at 94°C, 1 min for annealing at 55°C, and extension at 68°C for 1 min and additionally one cycle at 68°C for 7 min final extension (OIE, 2013).

3.10. Gel electrophoresis

A 2 µl of loading dye and 8 µl of amplified PCR products were mixed up and loaded in to separate lane (wells on the gel) and the gel was run for 45-60 minutes. The gel was placed under UV transilluminator or Gel doc machine that was connected to the computer with its respective

software (Image Lab software version 5.0) for imaging. Finally, the presence or absence of band were observed and the image was captured.

3.11. PCR product purification for sequencing

Column method of PCR purification was performed as per the manufacturer instruction. Briefly, 10 µl of membrane binding solution from DNA purification kit was added to equal volume of PCR product in to the sterile micro centrifuge tube. The mix was transferred to mini column and incubated at room temperature for 1 min to facilitate binding. Then, 700 µl membrane wash solution (ethanol added) was applied to a vacuum to pull solution through mini column and centrifuged at 10,000 rpm for 1 min. Again 500 µl membrane wash solution was added and centrifuged at 10,000 rpm for 5 min. Carefully mini column transferred to a clean 1.5 ml micro centrifuge tube and 50 µl of nuclease –free water was added to the mini column and incubated at room temperature for 1min. Again, it was centrifuged at 10,000 rpm for 1 minute. Finally, the products were stored at -20^{0}C before it was sent to Berlin, Germany (LGC Genomics) for sequencing (Appendix 4).

3.12. Sequence analysis of M-genes

DNA products were subjected to direct nucleotide sequencing using Sanger Sequencing Machine (Applied Biosystems, Berlin, Germany) and sequenced in a 96-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem). The data that comes from Germany were viewed by using Chromas and FinchTV softwares.

3.13. Data Management and Analysis

Data collected from questionnaire and laboratory test were filtered, edited and entered into Microsoft® Excel, Windows 2016. Descriptive statistics analysis was performed using IBM SPSS statistics 25 software (IBM SPSS, 2019). For sequenced data Chromas and FinchTV software's (https://softfamous.com) were used to view the results. Statistical tests with p-value of less than 0.05 were considered statistically significant at 95% level of confidence. Test agreement between conventional PCR and qPCR were assessed by using kappa statistic. Kappa values < 0 indicating

no agreement and 0-0.20 slight, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 substantial, and 0.81-1 almost perfect agreement (Landis and Koch, 1977).

3.14. Ethics Approval

Sampling from animals were carried out according to the experimental practice and standards approved by the Animal Welfare and Research Ethics Committee at National Animal Health Diagnostic and Investigation Center (NAHDIC) which is in accordance with the international guidelines for animal welfare.

4. RESULTS

4.1. rRT-PCR result

Out of the 84 pooled five samples tested by rRT-PCR (qPCR) for M gene, 14 (16.7%) samples were detected for NDV where 13 (15.5%) were identified as positive for NDV and 1 is having a Ct. value of 36.38 considered as negative for NDV in non-vaccinated village chickens. The current findings in village chickens for pooled five swab samples obtained in the study area with their Ct. values are presented in Table 3.

Table 3: M gene qPCR test Ct. values for NDV detection in non-vaccinated village chickens in Central Rift Valley of Oromia, Ethiopia.

Sample ID	Study areas	Swab type	Ct. value for M-gene
A1	Adama	Cloaca	24.82
A2	Adama	Cloaca	26.42
A3	Adama	Trachea	30.82
A4	Adama	Trachea	33.87
A5	Adama	Trachea	27.20
A6	Adama	Trachea	31.23
A7	Adama	Trachea	36.38
AN1	Arsi Negelle	Cloaca	23.41
AN2	Arsi Negelle	Cloaca	34.85
AN3	Arsi Negelle	Trachea	28.78
BA1	Batu	Trachea	31.50
S1	Shashemene	Trachea	28.96
S2	Shashemene	Trachea	24.39
S3	Shashemene	Trachea	23.68
Mean			28.41

The present findings of both positive and negative controls that are used for qPCR test (Figure 9) and non-vaccinated village chickens swab samples pooled five based on qPCR test for NDV was indicated (Figure 10) when read from Applied Biosystems 7500 fast real time PCR thermo cycler.

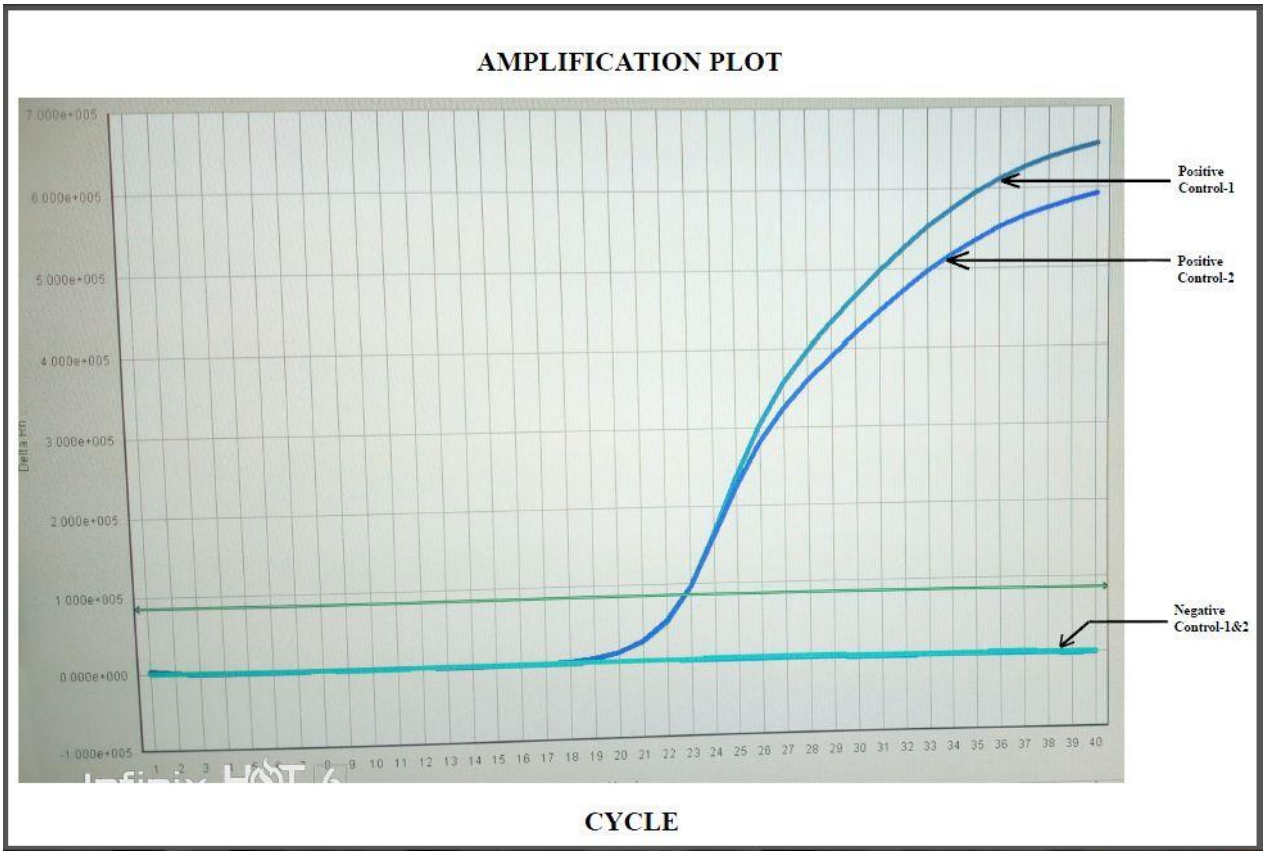

Figure 9: Positive and negative controls from qPCR.

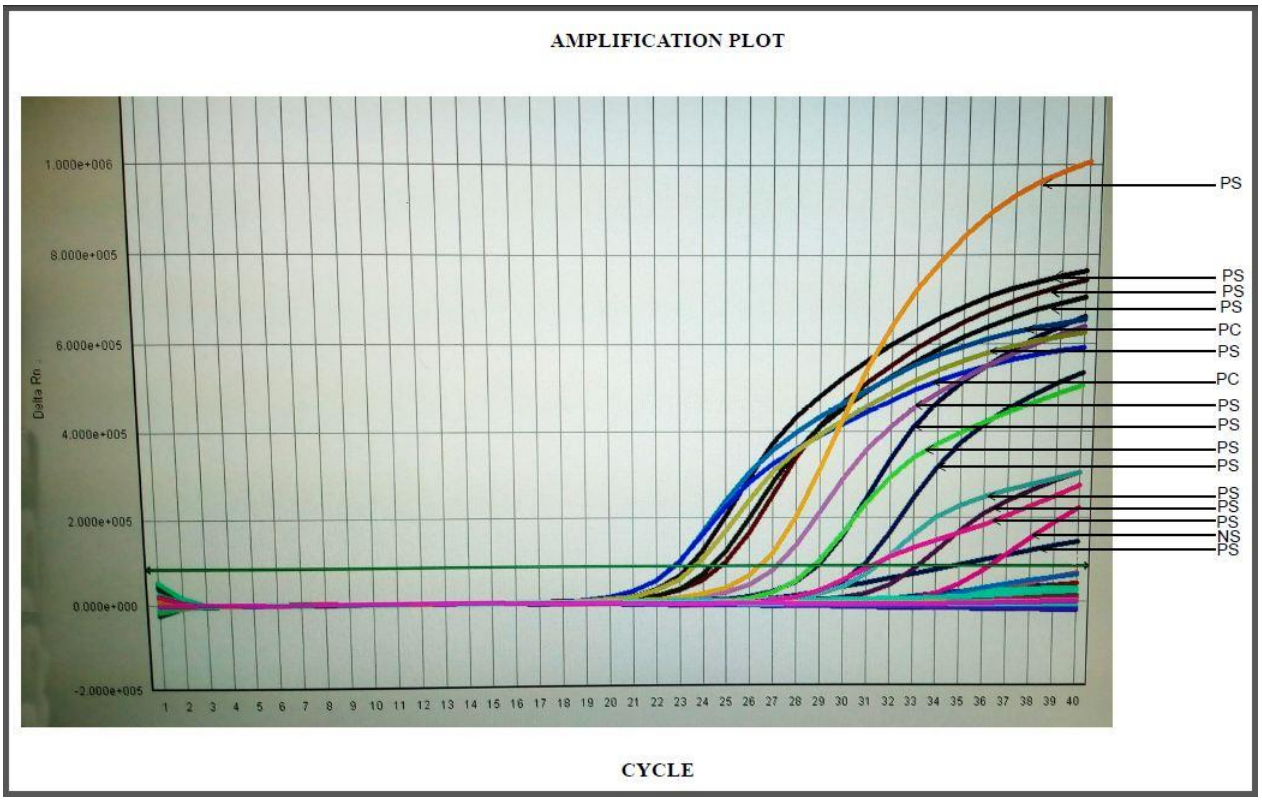

Figure 10: Amplification plot result of qPCR with their Ct. value where PS, PC, NS were positive sample, positive control and negative sample, respectively.

4.2. Prevalence of Newcastle Disease Virus by Age and Sex

The prevalence of ND was higher in male (16.10%) than female (14.67%) chicken. There was statistically no significance difference ($p > 0.05$) by sex (Table 4). On the other hand, the prevalence of ND was higher in Adult (16.59%) but lower in old chicken (11.11%) as shown in Table 4.

Table 4: Prevalence of Newcastle disease by Sex and Age in the study area.

Variables	Number examined	Number positive	Prevalence %	p-value
Sex				
Male	236	38	16.1	> 0.05
Female	184	27	14.67	
Age				
Young	146	23	15.75	< 0.05
Adult	211	35	16.59	
Old	63	7	11.11	

4. 3. Prevalence of ND in village chickens in the study districts

The overall ND prevalence was 15.48% (13/84) in the study districts where the highest score was from Adama (42.86%) while no prevalence was detected in Bote and Bishoftu (Table 5). There was statistically no significance difference ($p > 0.05$) among the study districts.

Table 5: Prevalence of Newcastle disease in non-vaccinated village chicken in the study Districts, M-gene qPCR result.

Districts	No. (Examined pool of five)	Positive	Prevalence %	p-value
East Shoa Zone				
Adama	14	6	42.86	
Batu	14	1	7.14	< 0.05
Bishoftu	14	0	0	
Bote	14	0	0	
West Arsi Zone				
Arsi Negelle	14	3	21.43	> 0.05
Shashemene	14	3	21.43	
Total	84	13	15.48	

The current qPCR results also revealed a higher detection rate for M-gene from tracheal swab samples (TS), 21.43% (9/42), as compared to cloacal swab samples (CS), 9.52% (4/42), collected from village chicken from the study areas (Table 6).

Table 6: qPCR result for M-gene detection rate from swab samples from study area.

Swab type	No examined	No positive	Detection rate %	p-value
Trachea swab	42	9	21.43	> 0.05
Cloacal swab	42	4	9.52	
Total	84	13	15.47	

4.4. Conventional PCR- based molecular detection of NDV

Ten samples were tested by Conventional PCR assay for the detection of specific gene. Each band represents the extracted DNA from ten samples (Figure 11).

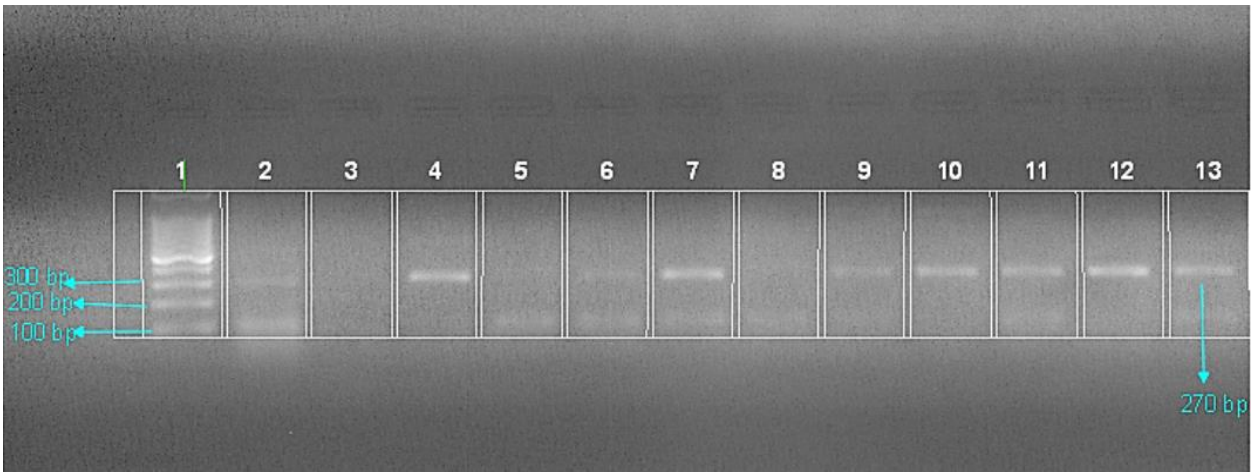

Figure 11: Gel electrophoresis in 2% Agarose gel for PCR amplified product of Newcastle disease virus (270bp) specific products. Lanes 4, 6, 7, 9, 10, 11, 12, 13 indicates positive for NDV and lane 5, 8 negative bands for NDV. Lane 1 for marker or ladder of 100bp; while lane 2 and 3 as positive and negative controls, respectively.

4.5. Test agreement result between qPCR and Conventional PCR detection technique

The kappa test agreement between qPCR and conventional PCR were having a value of 0.615 which indicates substantial result agreement between the two raters (Table 7).

Table 7: Kappa test agreement between qPCR and conventional PCR of Newcastle disease in the study area.

rRTPCR * cRTPCR Crosstabulation

			cRTPCR		
			Negative	Positive	Total
rRTPCR	Negative	Count	1	0	1
		% within rRTPCR	100.0%	0.0%	100.0%
		% within cRTPCR	50.0%	0.0%	10.0%
	Positive	Count	1	8	9
		% within rRTPCR	11.1%	88.9%	100.0%
		% within cRTPCR	50.0%	100.0%	90.0%
Total		Count	2	8	10
		% within rRTPCR	20.0%	80.0%	100.0%
		% within cRTPCR	100.0%	100.0%	100.0%

Symmetric Measures

		Value	Asymptotic Standard Error[a]	Approximate T[b]	Approximate Significance
Measure of Agreement	Kappa	.615	.337	2.108	.035
N of Valid Cases		10			

4.6. Sequencing result

The result from sequencing was obtained from LGC genomics company, Berlin, Germany. But when we check the result by using Chromas and FinchTV software's, we found that the result was very poor quality (Figure 12) due to DNA quality during purification and transportation to Germany. Hence, we could not proceed for further analysis.

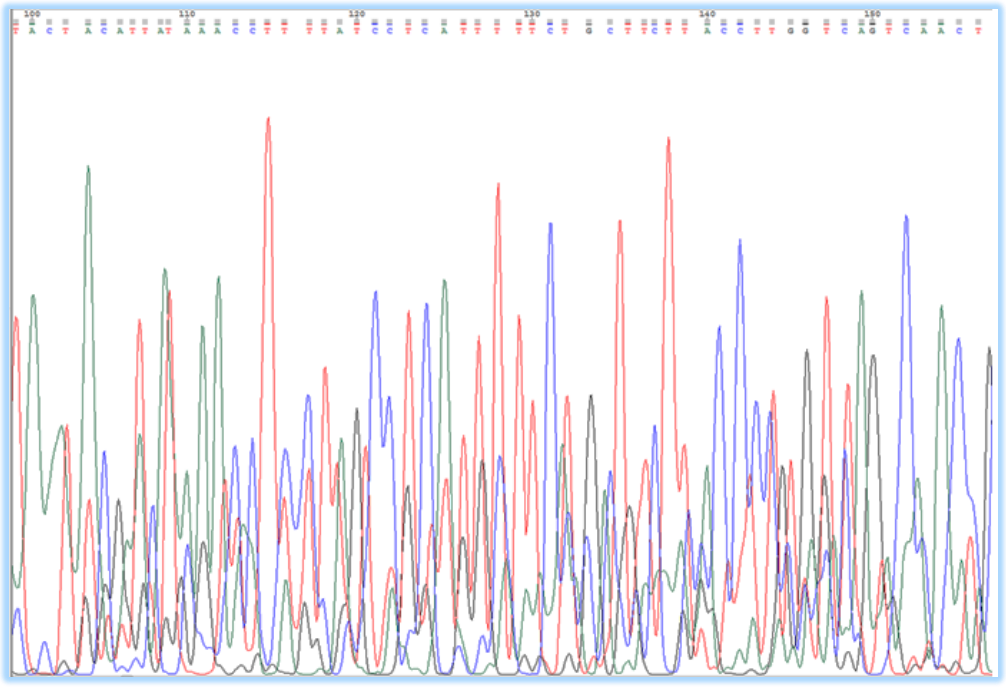

Figure 12: Chromatogram file sequenced data that came from LGC genomics Berlin, Germany.

4.7. Questionnaire survey result on associated risk factor for ND

In this study, most of the respondents 71.1% (128/180) s indicated that "*Fengil in Amharic*" was the leading disease to cause mortality of their chicken in the village whereas, 25.6% (46/180) of the respondents indicated that locally known as "*Sombe in Afan Oromo*" and 2.2% indicated that locally known as "*Gunfan in Amharic*" (4/180) (Table 8). According to the respondents "*Fengil*" was manifested by frequent clinical symptoms such as greenish dropping, swelling of eyelid with abnormal accumulation of liquid, black comb and brachial vein, lowering the head down, paralysis and sudden death during disease outbreak. In line with this, 92.8% of the respondents replied they didn't vaccinate their chickens for the major disease in their areas.

Table 8: Questionnaire survey result on associated risk factors for ND in the study area.

Variables	Levels	Frequency	Percent
Where do your chickens spend during the day time?	Housed	48	26.7
	Scavenging	132	73.3
Is there any interaction of your chicken with other species of wild birds?	Yes	134	74.4
	No	44	24.4
	Fengil	128	71.1
Name of Major diseases (Local Name)	Sombe	46	25.6
	Gunfan	4	2.2
Affected Age	Young	22	12.2
	Old	1	0.6
	Adult	154	85.6
Affected Breed of Chicken	Local	75	41.7
	Exotic	63	35.0
	All	39	21.7
Season for disease occurrence	Rainy	177	98.3
	Dry	3	1.7
What are the major losses due to ND?	Egg Production	8	4.4
	Mortality	144	80.0
	Both	26	14.4
Do you vaccinate your chicken for these major diseases in your area?	Yes	13	7.2
	No	167	92.8

In relation to season of the year when the disease frequently occurs, most of the respondents, 98.3% (177/180) indicated, disease occurrence was higher during rainy season. However, 1.7% (3/180) of the respondents experienced high rate of disease occurrence during the dry season. To get insight about this situation correlation analysis was performed using monthly rainfall climatological data collected from Adama Metrological Agency and ND outbreak monthly annual report data from Adama District Livestock and Fishery Development Office. Accordingly, the highest correlation value of R= 0.62 was revealed showing the occurrence of the disease during rainy season (Figure 13).

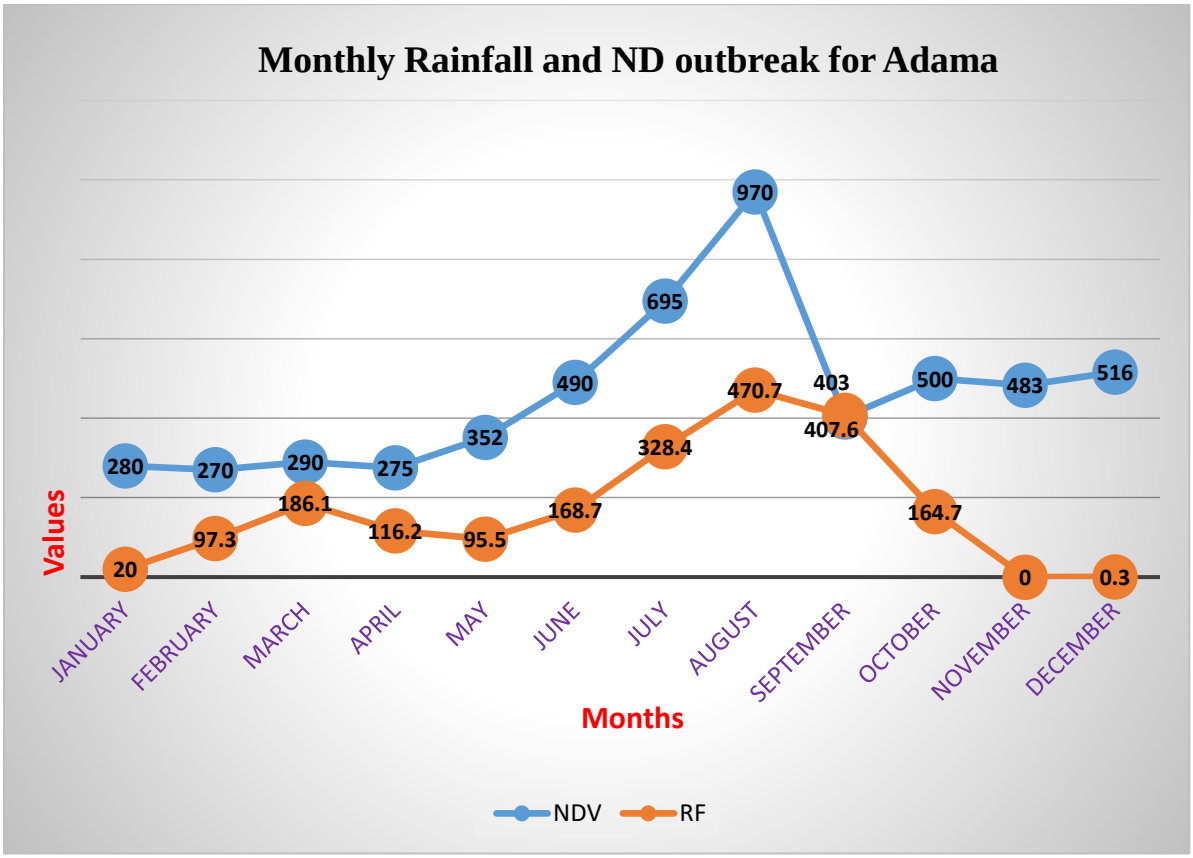

Figure 13: The graph of monthly rainfall and NDV outbreak for Adama District in 2018.

5. DISCUSSION

The present study on ND from non-vaccinated village chicken using qPCR revealed an overall prevalence of 15.48% in the study area ranging from 0% at Bishoftu and Bote to 42.86% at Adama. Even though, the distribution of the disease differs among the study areas in Eastern Shoa Zone using qPCR analysis with statistically significance difference ($p < 0.05$), Miressa *et al.* (2016) reported an overall prevalence of 26.7% from Adama and Bishoftu which is in a close resemblance with our current findings (21.42%) when it was analyzed only for Adama and Bishofitu. In addition, a qPCR study conducted by Terefe *et al.* (2015) in three selected districts (Batu, Bishoftu and TikurWuha) in rift valley areas revealed an overall prevalence of 8.4% which is less than the current finding and this could be due to the small area coverage. The negative results from the qPCR test in Bishoftu and Bote seem strange but a molecular and serological study conducted for NDV from Iran in non-vaccinated village chicken also showed negative result on qPCR but positive reaction on HI test (Rezaeianzadeh *et al*., 2011). They concluded that the serological finding may imply a circulation of NDV antibody on the native chickens in the area while molecularly, no virus was detected since there was no occurrence of active viral infection.

The result of the present study (15.48%) was higher than the findings of other researches done by serological detection method in Eastern Shoa and Kersana Kondaltity district with an overall sero-prevalence of 5.9% and 5.6%, respectively (Chaka *et al*., 2012; Regasa *et al*., 2007). In another serological study conducted in selected areas in rift valley an overall sero-prevalence of 5.6% was reported (Belayneh *et al*., 2014) and 11.6% (Terefe *et al*., 2015) from which one can discern out that the results under the present study, molecular tool, outweighs the results of the former studies by serological (ELISA) method. On the other hand, the findings under the present study was considerably lower than the previous outbreak reports by Zeleke *et al.* (2005) and Tadesse *et al.* (2005), in which sero-prevalence of 19.8% and 32.2% were reported in rift valley areas and central part of Ethiopia, respectively. The overall discrepancy of the findings might be due to variations on management system that may serve as a stress factor and favor infection or the diagnostic tool they used.

The present higher proportion (42.86%) of qPCR positive result for NDV in chickens from Adama district might indicate chickens have been previously infected by the non-virulent strains or

survived outbreaks of the virulent NDV strains (Abdullsattar, 2004). Moreover, the detected high prevalence of ND in this study may mean that natural infection could have occurred since all the chickens were non-vaccinated. However, clinical signs of ND were not observed on the examined chickens during sample collection. Furthermore, the higher prevalence recorded in Adama district cannot be attributed to more chicken had been sampled (70 chicken sampled) compared to other study districts (70 chicken sampled), because as the number of sample size increase the probability of having higher prevalence's also increase. In addition, according to the report by Zeleke *et al.* (2005) and Tadesse *et al.* (2005) low altitudes do have higher sero-prevalence than the high altitude in their study and they were investigated as there were few chickens in the highland area. Hence, chicken population number is a factor for the transmission of the disease in their study. The present study also revealed significant variation with almost closer agro-ecology between East Shoa and West Arsi Zones.

The findings of the present study also found out to be higher than the results reported from North Eastern Coast and Amazon Biome of Brazil from 1022 cloacal/tracheal samples tested by qPCR targeting the M gene segment of APMV-1, 7 (0.7%) were positive (Thomazelli *et al*., 2012). Correspondingly, sero-prevalence in backyard poultry in Mauritania, 4.8%, were reported (Bell *et al*., 1990), 4.8% in California (McBride *et al*., 1991), and 5% in South Africa (Thekisoe *et al*., 2003), 2.2% in Mexico (Gutierrez-Ruiz *et al*., 2000). This could be due to differences in study settings or by exposure to mild virus strains that induced immunity but did not kill many chickens (Sagild and Haresnape, 1987; Martin, 1992; Chaka *et al*., 2012).

The prevalence under the present study was far lower than reports from Ecuador (97%; Sonia *et al.*, 2006), Tanzania (46.1%; Yongolo *et al*., 2001), Zambia (36.9%; Alders *et al*., 1994), Zimbabwe (27%; Kelly *et al*., 1994), and Bangladesh (88%; Biswas *et al*., 2009). These variations could have been attributed to seasonal differences during sampling periods and methods employed for the studies. The variation in sero-prevalence of the disease might be due to differences in geographical location and seasonal variability. According to Awan *et al.* (1994), low ND prevalence is suggestive of an interepidemic phase and this could partly explain the high proportion of negative results on qPCR in village chickens in the present study.

In the present study a higher detection rate was obtained from pooled tracheal 21.43% (9/42) than cloacal 9.52% (4/42) swab samples with statistically insignificance difference ($p > 0.05$). This is

in agreement with the finding of Chaka *et al.* (2012) who reported higher positivity of tracheal than cloacal pooled swab samples speculating the presence of viscerotropic velogenic virulent NDV in the study areas. On the other hand, Haque and his colleagues detected NDV from pooled oropharyngeal and cloacal swabs and suggested that the chickens are in the early or advanced stage of the disease since NDV is transmitted through aerosol and ingestion or the chickens are on the shedding stage of the infection (Haque *et al*., 2010).

The difference in the prevalence between adult, young and old age was statistically significant ($p< 0.05$), which agrees with the finding of Vui *et al.* (2002) who stated that the young had a significantly lower ND prevalence than the adult. Furthermore, the result of the current study corroborates with the findings of Getachew *et al.* (2014) from Alamata District, Southern Tigray, Ethiopia with sero-prevalence of 17.3% and 29.7% for young and adult, respectively. They hypothesized that, more frequent exposure of Adult birds to field virus, which might have survived the disease at an earlier age.

The present study revealed a higher prevalence rate of ND among the male 16.1 % (38/236) compared to female chickens 14.67% (27/184) with statistically insignificance difference ($p > 0.05$). This finding corroborates with the study reported by Zeleke *et al.* (2005) in the Southern and Rift Valley districts of Ethiopia where a higher sero-prevalence rate among males (21.74%) than females (19.16%) was observed. In contrary to this finding, Tadesse *et al.* (2005) reported a slightly higher sero-prevalence among female (32.63%) chickens when compared with male (31.63%) chickens in Ethiopia. The higher prevalence of the disease observed in the present study could be due to higher number of male chickens involved by chance in the study area during sampling than females.

From the questionnaire interview, almost none of the chicken owner had ever vaccinated their chicken (92.8%). In this study 71.1% of the respondents indicated that the decreased flock size was due to diseases. This finding is in agreement with Chaka (2012) and Nega (2012) who reported 71.7% and 77.5% of the respondents indicated their flock size decreased due to diseases. These indicated that households had lost their chickens, possibly due to mainly incidence of diseases in their flocks, among other factors. The majority of the respondents (71.1%) indicated ND locally known as "*Fengil*" was the leading disease to cause mortality of chicken in the village. This was corroborated by qPCR test positivity during the sampling period. Several researchers also

confirmed this result: Nega (2012), Selam and Kelay (2013) and Chaka (2012) reported 93%, 86% and 60.5% of the respondents, respectively, indicated diseases, mostly ND, was the most important cause for chicken mortality in their village. Furthermore, the result also agrees with the findings of Dessie and Ogle (2001) who reported disease as the most important factor for the death of chickens.

On the other hand, 98.3% of the respondents indicated that disease occurrence specifically ND was higher during rainy season. This result supports with the findings of Selam and Kelay (2013) who reported 77.8% of the respondents indicated disease occurrence was higher at short and long rainy season. Even from the correlation analysis result (R= 0.62) between the monthly rainfall and disease outbreak data from Adama District indicates that ND was prevalent during rainy season, which also strongly support the finding from the questionnaire interview in the study area.

6. CONCLUSIONS AND RECOMMENDATIONS

In general, the present study revealed that Newcastle disease is endemic in village chicken of East Shewa and West Arsi Zones of Oromia region with an overall prevalence of 15.2% by qPCR indicating molecular tools are useful techniques used to assess the status of NDV infection in an area. In addition, this study provides important information on epidemiology of NDV in Ethiopia and highlights the importance of implementing surveillances and biosecurity practices in live poultry markets and village chickens. Furthermore, there could be a spillage over effect in which village chickens can serve as source of infection for the growing small-scale poultry farms in the country. Several circulating virus strains are believed to be the causes for the periodic outbreaks of the disease, with a tendency towards higher incidence during the rainy season. This may also hold true for many other parts of Africa, or elsewhere in the world, where similar agroecological conditions exist. The presence of NDV in household chickens could pose a significant threat to the development of the emerging small and medium commercial poultry production sector in Ethiopia.

Therefore, based on the current findings the following recommendations were forwarded:

- It is paramount that the regular vaccination to prevent Newcastle Disease Virus should be given for village chickens to decrease the loss associated with mortality. The strict biosecurity measures have to be taken in poultry farms to prevent further spreading of the NDV.
- Even though, an attempt was made to sequence M-gene of NDV but the quality of sequence score is poor. Therefore, further molecular epidemiology study is necessary to better understand and characterize ND virus strains circulating in the Oromia region and also the rest of Ethiopia.
- Further continuous studies should be conducted in other areas including the present study area in order to assess the re-occurrence of NDV, to get the national picture about the disease.
- Additional study is required to understand the interaction between the infectious diseases, to estimate their impact on the backyard poultry production system.

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8. APPENDICES

Appendix 1: Questionnaire format used.

Part I: General Information

Date_____

Region Oromia, Zone _____, District _____,

Name of the respondent_____

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. Who is responsible for the attendance of the chicken? _____

4. Where do your chickens spend the day time (Housed/Scavenging)?

5. How far do your chickens move for scavenging and watering? _____

6. Is there any interaction of the chicken with other species of wild birds? Yes/No

7. Did you encounter health problem in your chicken/Farm? _____

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

8. What are the major health problem affecting your chicken?

9. Which disease-causing high losses in your chicken/Farm? _____

10. What are the major losses of the disease (Production status/Morbidity/Mortality)?

11. In which types of diseases and chicken the mortality is more serious? _____

12. At what season do you frequently encounter disease problem?

13. Do you vaccinate your chicken for these major diseases in your area? (Yes/No). If yes which one? Source of vaccine? _____

14. What do you think about other predisposing factor that facilitate for the occurrence of major disease? _____

Appendix 2: Pictures during sample collection and questionnaire interview in each District.

Figure 14: Sample collection and questionnaire survey in Adama District.

Figure 15: Sample collection and questionnaire survey in Bote District.

Figure 16: Sample collection and questionnaire survey in Arsi Negelle District.

Figure 17: Sample collection and questionnaire survey in Bishoftu District.

Figure 18: Sample collection and questionnaire survey in Batu District.

Figure 19: Sample collection and questionnaire survey in Shashemene District

Appendix 3: Master mix and sample preparation for RT-PCR.

Figure 20: Master mix preparation.

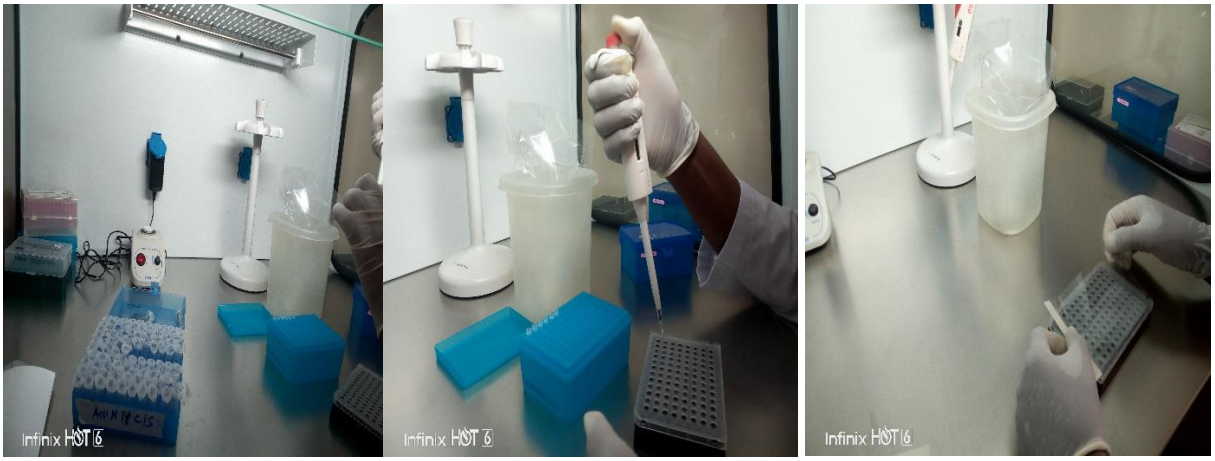
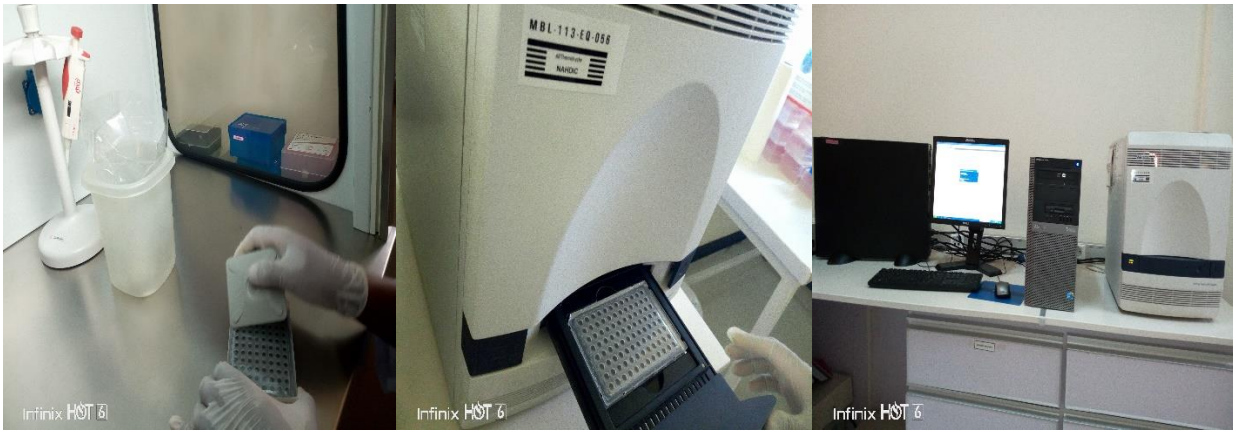


Figure 21: Sample preparation for RT-PCR.



Appendix 4: DHL tracking Result during sending documents to Berlin, Germany for sequencing.

Table 9: DHL result summary of the progress during sending sample to Germany.

Result Summary

	Waybill: 4822148656 Signed for by: BACKHAUS › Get Signature Proof of Delivery	**Wednesday, May 15, 2019 at 08:36** **Origin Service Area:** › ADDIS ABABA - ADDIS ABABA - ETHIOPIA **Destination Service Area:** › BERLIN - BERLIN - GERMANY	1 Piece

Wednesday, May 15, 2019		**Location**	**Time**	**Piece**
18	Delivered - Signed for by: BACKHAUS	BERLIN	08:36	1 Piece
17	With delivery courier	BERLIN - GERMANY	07:52	1 Piece
16	Arrived at Delivery Facility in BERLIN - GERMANY	BERLIN - GERMANY	05:15	1 Piece
15	Departed Facility in LEIPZIG - GERMANY	LEIPZIG - GERMANY	02:24	1 Piece
14	Processed at LEIPZIG - GERMANY	LEIPZIG - GERMANY	01:05	1 Piece
Tuesday, May 14, 2019		**Location**	**Time**	**Piece**
13	Customs status updated	LEIPZIG - GERMANY	23:51	
12	Clearance processing complete at LEIPZIG - GERMANY	LEIPZIG - GERMANY	23:51	
Sunday, May 12, 2019		**Location**	**Time**	**Piece**
11	Processed for clearance at LEIPZIG - GERMANY	LEIPZIG - GERMANY	21:03	1 Piece
Saturday, May 11, 2019		**Location**	**Time**	**Piece**
10	Arrived at Sort Facility LEIPZIG - GERMANY	LEIPZIG - GERMANY	02:16	1 Piece
9	Customs status updated	LEIPZIG - GERMANY	00:32	
8	Departed Facility in BRUSSELS - BELGIUM	BRUSSELS - BELGIUM	00:12	1 Piece
Friday, May 10, 2019		**Location**	**Time**	**Piece**
7	Processed at BRUSSELS - BELGIUM	BRUSSELS - BELGIUM	21:15	1 Piece
6	Arrived at Sort Facility BRUSSELS - BELGIUM	BRUSSELS - BELGIUM	07:21	1 Piece
Thursday, May 09, 2019		**Location**	**Time**	**Piece**
5	Departed Facility in ADDIS ABABA - ETHIOPIA	ADDIS ABABA - ETHIOPIA	21:53	1 Piece
4	Transferred through ADDIS ABABA - ETHIOPIA	ADDIS ABABA - ETHIOPIA	21:52	1 Piece
3	Processed at ADDIS ABABA - ETHIOPIA	ADDIS ABABA - ETHIOPIA	20:42	1 Piece
2	Shipment information received	ADDIS ABABA - ETHIOPIA	15:43	
1	Shipment Accepted	ADDIS ABABA - ETHIOPIA	16:26	1 Piece

Source: https://www.dhl.com/en/express/tracking.html?AWB=4822148656&brand=DHL.