

**MOLECULAR DETECTION OF NEWCASTLE DISEASE VIRUS FROM
OUTBREAK CASES IN COMMERCIAL POULTRY FARMS IN NEKEMTE TOWN
AND SELECTED DISTRICTS OF EAST WALLAGA ZONE, OROMIA REGION,
ETHIOPIA**



**WALLAGA UNIVERSITY
SCHOOL OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND
PUBLIC HEALTH**

MSc. THESIS

BY:

ELFINESH MITIKU GURMESA

**MAY, 2025
NEKEMTE, ETHIOPIA**

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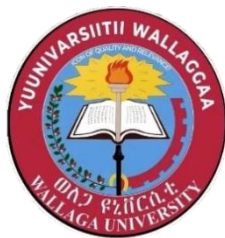
**A RESEARCH THESIS SUBMITTED TO THE SCHOOL OF VETERINARY
MEDICINE GRADUATE STUDIES IN PARCIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN
VETERINARY MICROBIOLOGY**

BY:

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**MAY, 2025
NEKEMTE, ETHIOPIA**



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APPROVAL SHEET FOR SUBMITTING FINAL THESIS

As members of the examining board of the final MSc thesis open defenses, we certify that we have read and evaluated the thesis prepared by **Elfinesh Mitiku Gurmesa**, entitled: **“Molecular Detection of Newcastle Disease Virus from Outbreak Cases in Commercial Poultry Farms in Nekemte town and Selected Districts of East Wallaga Zone, Oromia Region, Ethiopia”** and recommend it to be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology

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Certification of the final thesis

I hereby certify that all the corrections and recommendations suggested by the board of examiners are incorporated into the final thesis entitled: **“Molecular Detection of Newcastle Disease Virus from Outbreak Cases in Commercial Poultry Farms in Nekemte town and Selected Districts of East Wallaga Zone, Oromia Region, Ethiopia”** by **Elfinesh Mitiku Gurmesa**

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STATEMENT OF THE AUTHOR

I declare that this research thesis titled “**Molecular Detection of Newcastle Disease Virus from Outbreak Cases in Commercial Poultry Farms in Nekemte town and Selected Districts of East Wallaga Zone, Oromia Region, Ethiopia**” is my work and that all sources of materials used for this thesis have been appropriately acknowledged. This research thesis is submitted in partial fulfillment of the requirements for the Degree of Masters of in Veterinary Microbiology at Wallaga University. I seriously declare that this research is not submitted to any other institution anywhere for the award of any degree or diploma. The research deposited at the University Library to make available to borrowers under rules of the Wallaga University Library.

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DECLARATION

This is to certify that this thesis, entitled: **“Molecular Detection of Newcastle Disease Virus from Outbreak Cases in Commercial Poultry Farms in Nekemte town and Selected Districts of East Wallaga Zone, Oromia Region, Ethiopia”** was accepted in partial fulfillment of the requirements for the award of the degree of Master of Science by the School of Graduate studies, Wallaga University, through the School of Veterinary Medicine; done by Elfinesh Mitiku Gurmesa, was a genuine work carried out by her under my guidance. The assistance and help received during the course of this investigation have been duly acknowledged. Therefore, I recommend that it be accepted as fulfilling the research thesis requirements.

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LIST OF ABBREVIATIONS

AHI	Animal Health Institute
AOAV-1	Avian Orthoavulavirus type 1
APMV-1	Avian Paramyxoviruses type 1
CSA	Central Statics Agency
DIVA	Differentiation of Infected from Vaccinated Animals
DNA	Deoxy Nucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ICPI	Intracerebral Pathogenicity Index
ICTV	International Committee on Taxonomy of Viruses
IVPI	Intravenous Pathogenicity Index
LCR	Ligase Chain Reaction
NDV	Newcastle Disease Virus
OIE	Office International Des Epizootic
PCR	Polymerase Chain Reaction
RNA	Ribo Nucleic Acid
rRT-PCR	Real Time Reverse Transcriptase Polymerase Chain Reaction
SPSS	Statistical Package of Social Science
VTM	Virus Transport Medium
WAHID	World Animal Health Information Database
WOAH	World Organization Animal Health

ABSTRACT

Newcastle disease (ND) is a highly contagious and economically devastating viral disease affecting poultry worldwide, including Ethiopia. This study aimed to molecularly detect Newcastle disease virus (NDV) responsible for outbreaks in commercial poultry farms in Nekemte town and selected districts of East Wallaga zone, Oromia region, Ethiopia. A cross-sectional study was conducted from December 2024 to May 2025, focusing on farms reporting suspected ND outbreaks. A total of 20 pooled samples (4 pooled oropharyngeal swabs, 4 pooled cloacal swabs and 12 pooled tissue samples) were collected from clinically affected and freshly dead poultry. Real-time reverse transcriptase polymerase chain reaction (rRT-PCR) was employed to detect the NDV M gene. Results confirmed the presence of NDV in commercial poultry farms in Nekemte town and selected districts of East Wallaga zone, with an overall detection rate of 5%. The highest detection was observed in brain tissue samples (33.33%), suggesting viral tropism for neural tissues. The outbreak investigation revealed significant morbidity (15.18%), mortality (7.85%), and case fatality (51.72%) rates, underscoring NDV as a major cause of poultry losses in the areas. Affected poultry exhibited respiratory, gastrointestinal, and neurological symptoms, while postmortem examinations identified hemorrhagic ulcers and liver necrosis, consistent with velogenic NDV strains. Nekemte town (07) had the highest NDV detection rate (25%), while no positives were detected in other study sites, indicating localized outbreaks. The study confirms NDV as a significant threat to poultry farms in the study areas, highlighting the need for improved vaccination programs, biosecurity measures, and further research to characterize circulating strains.

Keywords: *Detection, East Wallaga zone, Ethiopia, Molecular, Newcastle disease, Outbreak.*

1. INTRODUCTION

In developing countries, raising poultry is crucial for producing revenue, maintaining food security, and reducing shortages (Attia *et al.*, 2022). Similar to this, Ethiopia's government is primarily focused on raising chickens in order to meet the country's demand for eggs and meat in an effort to maintain food security. There are an estimated 57.01 million poultry in the country, of which 78.85%, 12.02% and 9.11% indigenous, hybrids, and exotic poultry, respectively (Central Statistical Agency [CSA, 2021]). In spite of the abundance of poultry, the benefits and contributions to the country are still much below their potential because of diseases like Newcastle disease that affect poultry. Newcastle disease is a global problem that affects a wide variety of poultry. It has a significant negative impact on the global economy (Napit *et al.*, 2023). In many developing countries, particularly Ethiopia, it is widespread and has the potential to kill up to 80% of poultry farms. It poses major challenges to commercial poultry farming (Mesfin and Bihonegn, 2018).

Newcastle disease (ND) is caused by the virulent strain of *Avian Paramyxovirus type 1* (APMV-1), which belongs to the genus *Avulavirus* and the family *Paramyxoviridea* in the order *Mononegavirales*. It is a single-stranded, enveloped negative sense RNA virus with a genomic size of about 15 kilobases. Six key structural proteins are encoded by the six open reading frames found in the NDV genome: hemagglutinin-neuraminidase (HN), matrix protein (M), fusion protein (F), phosphoprotein (p), nucleoprotein (NP) and RNA-dependent RNA polymerase (L). W and V are also encoded from the P gene through a process known as RNA editing. The fusion protein (F) protein is a significant virulence marker (Nurzijah *et al.*, 2022; Tesfaye *et al.*, 2023).

Newcastle disease is a highly contagious and is transmitted to healthy poultry by infected droppings and respiratory secretions. Coughing, greenish diarrhea, and torticollis are the symptoms of these respiratory, gastrointestinal, and neurological disorders, respectively (Teskaye *et al.*, 2023). The severity and clinical manifestations of NDV can differ according to the virus strain. Based on their virulence and clinical manifestations the strains can be

categorized into three primary pathotypes: low virulent (lentogenic), moderate virulent (mesogenic), and high virulent (velogenic) (Worku *et al.*, 2022).

Newcastle Disease (ND) can be diagnosed through multiple approaches, including molecular assays, serological tests, necropsy examinations, clinical evaluations, and epidemiological history analysis. Its clinical presentation closely resembles highly pathogenic avian influenza (HPAI), necessitating rapid and accurate differentiation during outbreaks to implement effective containment measures. Viral isolation remains the gold standard for definitive diagnosis, while advanced techniques such as hemagglutination inhibition (HAI), viral neutralization (VN), Enzyme-linked immunosorbent assay (ELISA) and nucleic acid-based methods like RT-PCR and real-time RT-PCR (rRT-PCR) are widely employed for confirmation (Mossie and Abera, 2024). The M gene has been identified in clinical samples by using primers from the conserved matrix gene, rRT-PCR has been utilized to identify (Wise *et al.*, 2004; Cattolli *et al.*, 2009).

Newcastle disease is prevented and controlled by culling infected birds, tight biosecurity standards, and vaccination schedules. Strong vaccination programs are the most economical substitute, and these strategies are actually effective in places where they are appropriately applied (Mesfin and Bihonegn, 2018). However, a number of factors, including a lack of biosecurity, insufficient immunizations programs, antigenic variation, maternal antibodies impeding the effectiveness of live vaccines, a brief duration of the immune response, and immune suppression, have been linked to global outbreaks (Dimitrov *et al.*, 2017).

1.1. Statement of the Problem

In several countries across the world, Newcastle disease is currently endemic. Several ND outbreaks have occurred recently in countries including Nigeria, Egypt and Ethiopia that have large poultry industry (Amoia *et al.*, 2024).

In Ethiopia's poultry industry Newcastle disease is the most challenging poultry disease and causing significant financial losses (Mossie and Abera, 2024). Both lentogenic and velogenic

strains have been found in Ethiopia (Chaka *et al.*, 2013; Fentie *et al.*, 2014; Alemu *et al.*, 2024). A retrospective review of data over four years (2012-2015) revealed that 86 NDV outbreaks occurred in seven Ethiopian national and regional states. Of these outbreaks, 70.5% have been reported to have originated in the Oromia national regional state. As the most common infectious disease, it causes 80% of losses in commercial chicken and 43% in village chicken (Mesfin and Bihonegn, 2018). A few molecular tests are performed on poultry in some parts of the country; the percentage varies from 4.9% in the Eastern Shewa zone oromia region (Chaka *et al.*, 2013) to 50% in Addis Ababa (Habte *et al.*, 2022). On the other hand, Newcastle disease serological test results range from 5.6% in the Amara region (Belayneh *et al.*, 2014) to 68.9% in central and southwestern areas of Ethiopia (Emeru *et al.*, 2024). Even though the Newcastle disease virus is inflicting large losses all across the region and there have been multiple outbreaks of the disease in Ethiopian commercial poultry farms, the disease's present status has not been studied, and it is uncertain what types of virus is causing these outbreaks, especially in selected sites of East Wallaga zone of Oromia region Ethiopia. Thus, molecular detection of the virus is primary importance for filling in these knowledge gaps from Newcastle disease virus cases. The study was conducted in Nekemte town and selected districts of East Wallaga zone of Oromia region of Ethiopia in order to achieve the following objectives.

1.2. General Objectives

- ✓ To molecularly detect Newcastle disease virus responsible for occurrence of an outbreak in Nekemte town and selected districts of East Wallaga zone of Oromia region, Ethiopia.

1.3. Specific Objectives

- ✓ To determine Newcastle disease virus responsible for occurrence of an outbreak in Nekemte town and selected districts of East Wallaga zone of Oromia region, Ethiopia
- ✓ To detect the virus at molecular level from outbreak cases in the study areas.

2. NEWCASTLE DISEASE VIRUS

2.1. Definition of Newcastle Disease

Newcastle disease is an acute viral disease that spreads rapidly and affects domestic poultry as well as other birds. The disease is also known by other names, including pseudo poultry plagues, avian pseudoencephalitis, Ranikhet Disease, Pseudo-fowl pest, avian pest, and avian distemper (Sahlu *et al.*, 2015).

2.2. Taxonomy and Structure of Newcastle Disease Virus

Viral strains of *Avian orthoavulavirus 1* (AOAV-1), often referred to as *Avian paramyxovirus 1* (APMV-1) or NDV, are the cause of Newcastle disease. Previously, this virus was known as *Avian Avulavirus 1* (AAvV-1). Newcastle disease-causing viruses belong to the following members: realm *Riboviria*, kingdom *Orthornavireae*, Phylum *Negarnaviricota*, class *Monjiviricetes*, order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Avulavirinae*, and genus *Orthoavulavirus*. APMV-1 to APMV-21 is the 21 serotypes that comprise the isolated avian *paramyxoviruses*. Every virus falls under one of the several viral species that make up the genera *orthoavulavirus*, *paraavulavirus*, and *metaavulavirus* (International Committee on Taxonomy of Viruses [ICTV, 2019]).

Newcastle disease virus is an enclosed, pleomorphic virus with a diameter of around 200–300 nm. Its envelope is made of spicules that are 8–12 nm long and represent the F and HN glycoproteins. These spicules are inserted into the outer face of the infected cell's plasma membrane. The envelope's inside is lined by a matrix protein. Viral genomes are composed of single-stranded, non-segmented, negatively polarized RNA. The NDV RNA genome weighs between 5.2 and 5.7 X 10⁶ Daltons. A genome's nucleotide count ranges from 15186 to 15198 (Ali *et al.*, 2022). The genomic RNA is tightly attached to the nucleoprotein, phosphoprotein, and big protein, which when joined with the RNA form a tubular nucleocapsid with an 18 nm diameter (Mao *et al.*, 2022).

2.3. Viral Proteins

Six distinct proteins are encoded by the six genes that make up the NDV genome. Nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and large polymerase protein are by the genes organized in the 3'-NP-P-M-F-HN-L-5', respectively (Nurzijah *et al.*, 2022). The nucleoprotein is 55 kDaltons in molecular weight and 489 amino acids long. While phosphoprotein, which is composed of 395 amino acids and forms numerous bands with a molecular weight ranging from 50-55 kDaltons, is crucial for transcription and viral replication, it envelops genomic RNA and shields it from nucleases. A non-glycosylated protein connected to the envelope's inner surface is called matrix protein (M). It is approximately 40 kDaltons in molecular weight and includes 364 amino acids. It is crucial for the assembly of viruses and the stability of their envelopes. Glycoprotein (F), which has 553 amino acids, joins the target cell's membrane to the viral envelope. The polypeptide glycoprotein (HN) has a molecular weight of 74 kDaltons and a length of 577 amino acids. It is a multifunctional protein involved in sialic acid cleavage, viral fusion promotion, and virion attachment to target cell receptors. The large protein (L) is the largest in the viral genome consisting of 2204 amino acids and has a molecular weight of 250 kDaltons. This protein helps in the replication of genomic RNA and synthesizes viral mRNA (Fellahi and Boudouma, 2021). The P gene produces alternative mRNAs during P gene transcription, which are translated into the proteins known as accessory proteins V and W. With a molecular weight of kDaltona, the V protein is made up of 239 amino acids. It is an essential component of NDV virulence and is an interferon antagonist. Lastly, it is still unknown how the W protein functions in the NDV replication cycle (Nurzijah *et al.*, 2022).

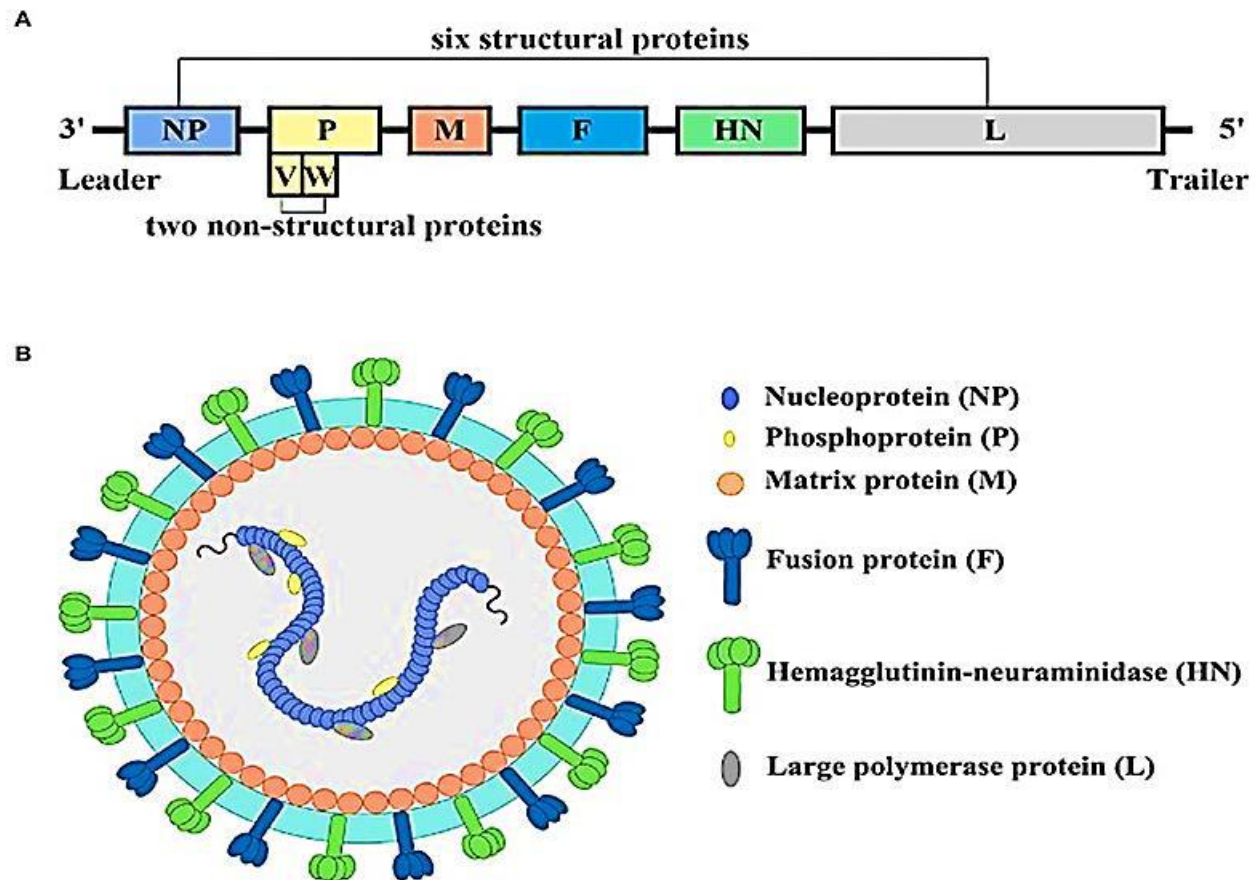


Figure 1: Schematic diagram of Newcastle disease virus genome and structure
Source: (Bello *et al.*, 2018)

2.4. Pathogenicity and Virulence of Newcastle Disease Virus

Newcastle disease virus has varied pathogenicity according on the strain of the virus. It is well recognized that the primary factor affecting virulence is the host cell proteases' activation of the fusion protein (F) (Ganar *et al.*, 2014). During replication, an inactivation precursor glycoprotein (F0) is created. For it to show virulence and become infectious, it needs to be broken down into F1 and F2 polypeptides (Worku and Tashome, 2020). The virus virulence is directly correlated with the F0 molecule's cleavability. On the other hand, the proteases found in the organs and tissues of the host may have an impact. The virulence of NDV isolates is primarily determined by the sequence of the F cleavage site between

positions 112 and 116, as per molecular analyses of the F0 glycoprotein site (Puro and Sen, 2022).

Based on their pathogenicity to chickens, NDVs are divided into three strains: low virulence (lentogenic), moderately virulent (mesogenic) and extremely virulent (velogenic). The lentogenic viruses have the 112G/E-K/R-G/E-R-L117 motif, while the amino acid sequence 112R/K-R-Q-R/K-R-F117 at the cleavage site is the velogenic and mesogenic strains. The pathogenicity of NDVs is mostly dependent on the two surface glycoproteins, the F and HN proteins. The primary virulence determinant is known to be the F protein, more specifically the amino acid sequence of its cleavage site. The HN protein contains the thermostability of NDV. All velogenic viruses and mesogenic strains have many (more than two) basic amino acid residues between positions 113 and 116 (113R-Q-K/R-R116), and the F protein contains phenylalanine at position 117. Conversely, lentogenic strains are identified by trypsin-like protease present in the respiratory and gastrointestinal systems, and include leucine at position 117 of the F protein together with a small number of basic amino acids 113 and 116 (113K/R-Q-G/E-R116) (Humayum, 2022). Based on the sequencing of their F genes, NDV strains are divided into two main classes (I and II). Class I viruses, which only comprise genotype I, are avirulent viruses that were identified from wild birds. Class II isolates include both virulent and avirulent isolated from domestic poultry and wild birds. Class II viruses (I to XXI) have at least 20 genotypes. Newly discovered NDV strains from all around the world are frequently reported (Amoia *et al.*, 2024).

2.5. Epidemiology of Newcastle Disease Virus

2.5.1. Geographical distribution of Newcastle disease virus

Newcastle disease was first reported to Indonesia in 1926 (Amoia *et al.*, 2021). The virus was identified as NDV by Doyle, who isolated it in Newcastle, England, in 1927 (Triosanti *et al.*, 2018). Then, a number of outbreaks of various bird species linked to virulent NDV were documented in Asia (China, Korea, India, Japan, Sri Lanka, Saudi Kazakhstan, Philippines, and Arabia), Europe (France, Italy, England, Scotland, Spain, and Russia), Africa (Kenya),

and Australia (Rahman *et al.*, 2018). Newcastle disease is present all over the global. In every country where poultry are raised and as shown in **Figure 2**, endemic NCDs are present in many countries in Asia, the Middle East, Africa, Central America, and South America (Sahlu *et al.*, 2015). Several ND outbreaks have also occurred recently in countries including Nigeria, Egypt and Ethiopia that have large poultry sectors (Amoia *et al.*, 2024).

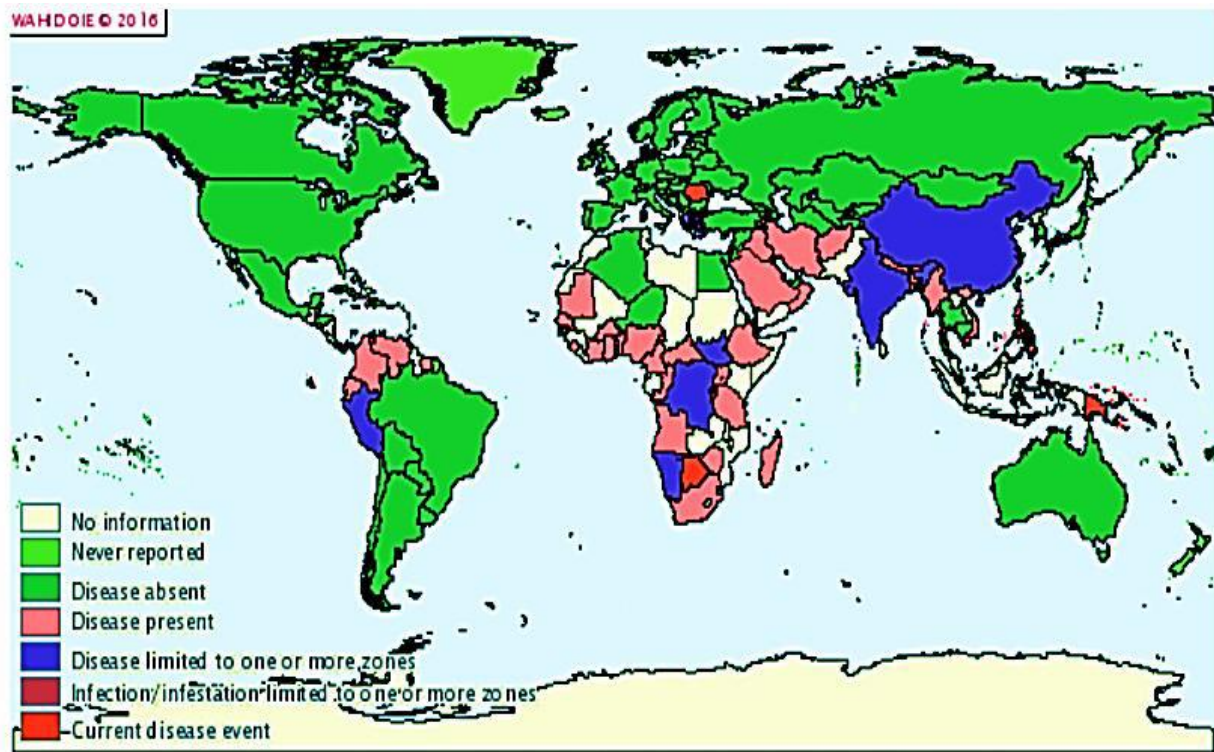


Figure 2: Global prevalence of Newcastle disease

Source: (WAHID Interface, 2015)

2.5.2. Species affected/ host range

NDV can affect over 241 domestic and wild bird species from 27 of the 50 bird orders, and it can infect a wide range of bird species either spontaneously or in a laboratory. Although birds of all ages are at risk, the disease primarily affects chickens. Given the type of virus, the host's susceptibility may vary greatly among bird species (Mossie and Abera, 2024). Although the severity of infection is highly based on the host species and viral strain, ND affects chickens more severely than turkeys, which often exhibit only mild signs. Quail,

guinea fowl, partridges, and pheasants are all under risk. . Even when they have the virus, ducks and geese (*Anseriformes*) may not exhibit clinical symptoms. Newcastle disease has been reported in camels, pigs, humans, sheep, mink, hamsters, mice, rabbits, and other animals. The most susceptible people are those who work in laboratory that handle poultry and make vaccinations (Moustapha *et al.*, 2023).

2.5.3. Sources of infection and route of transmission

Newcastle Disease Virus may be spread by contaminated food, water, equipment, and other domestic and wild birds, including infected chickens. Direct or indirect contact might spread the disease to healthy birds. Direct contact is mostly spread by ingesting or inhalation of secretions from an infected animal's mouth, respiratory tract, eyes and cloaca. During an amount of four to twelve months, birds who have received a Newcastle disease vaccinated can release viral particles. Contaminated feed, husbandry and transport equipment can spreads the disease indirectly. The virus can be spread by farm workers through their shoes and clothes, making them intermediates. It is also possible for the virus to spread by air from an infected poultry coop or from an affected location to a free area and the virus can spread up to 64 meters (Dzogbema *et al.*, 2021).

2.5.4. Risk factors and physicochemical nature of the virus

The risk factors that boost the frequency of infection are: age of the host, geographical location of the farm, distance between neighboring poultry farms, restocking poultry from market, poor biosecurity, presence of migratory wild birds, poor quarantine measures, and inadequate vaccination programs. The virus's environmental persistence varies greatly since it can be affected by a variety of factors, including temperature, humidity, the suspending agent, and light exposure. The virus is heat stable and can infect hens killed at 20°C for six months in their muscle and bone marrow. The disease's persistence and spread on farms have been influenced by each of these elements. Another noteworthy characteristic of the virus is that it is susceptible to disinfectants that contain detergents because of the lipid layer in its envelope. Formaldehyde, oxidizing agents, dyes, and lipid solvents are all harmful to the

virus (Mossie and Abera, 2024). At room temperature (18 to 23.5°C), the NDV can persist for long periods of time, especially in excrement (Getabalew *et al.*, 2019; Dzogbema *et al.*, 2021). Conversely, it stops working when the temperature is increased to 56°C for three hours or 60°C for half an hour. It is also inactivated at pH < 2 (Getabalew *et al.*, 2019).

2.6. Pathogenesis of Newcastle Disease Virus

Viral replication starts in the intestinal and respiratory tract epithelia and then spreads haematogenously to the bone marrow and spleen. The central nervous system, intestines, and lungs can become infected as a result of secondary viraemia. The amino acid sequence of the F glycoprotein determines the strain virulence, which is related to the degree of dissemination throughout the body. An infected cell produces the fusion (F) glycoprotein as a precursor molecule (F0), which host cell proteases cleave into the F1 and F2 subunits. In the absence of cleavage, non-infectious particles are created. Proteases can more easily cleave the F0 molecules of virulent strains of NDV because they contain basic amino acids in key locations. Conversely, the production of appropriate proteases in the intestinal and respiratory epithelia limits the reproduction of lentogenic strains (Quinn *et al.*, 2005).

2.7. Clinical Signs of Newcastle Disease Virus

After a natural exposure, the incubation time for ND might range from 2 to 15 days. The severity and degree of incubation of the disease may be depend on the virus strain, host age, stress, host species, secondary infections, host immune status, environmental factors, viral dosage, and exposure route (Getabalew *et al.*, 2019; World Organization Animal Health [WOAH, 2022]). In adult chickens, infections with lentogenic Newcastle disease virus strain can cause mild sickness or no noticeable respiratory or gastrointestinal symptoms. Although there is typically little mortality from mesogenic isolate infections, they can cause nonfatal respiratory diseases, decreased egg production, and uncommon neurological symptoms (Abdisa and Tagesu, 2017).

In especially susceptible flocks, infection with highly velogenic virus can result in up to 100% mortality (WOAH, 2022). The respiratory, gastrointestinal, and neurological problems are characterized by coughing, greenish diarrhea, and torticollis, respectively (Tesfaye *et al.*, 2023). Neurotropic velogenic strains of NDV primarily cause respiratory distress, which is followed by neurological illness and reduced egg production (Abdisa and Tagesu, 2017). Hemorrhages and ulcerations of the proventriculus and intestine are the main characteristics of lesions caused by the Newcastle disease virus (Rauw *et al.*, 2009). Viscerotropic velogenic NDV infection in chickens can cause severe clinical symptoms such as hemorrhagic lesions, necrotic ulcerations at the ends of proventricular glands, in the colon, caecal tonsils and spleen parenchyma, as well as dehydration and emaciation of the chicken carcass. Furthermore, congestive, edematous and hemorrhagic lesions may be seen in the lungs (Sonkusale *et al.*, 2023). Anatomical manifestations of NDV infection include progressive encephalitis, neuronal necrosis, neuronal phagocytosis, clusters of cells with microglial morphology in the brain's gray matter, the cerebellum, and the spinal cord, axonal degeneration, and demyelination lesions. These results suggest that the infection affects the central nervous system (Ecco *et al.*, 2011).

2.8. Diagnosis of Newcastle Disease Virus

For laboratory diagnosis, samples include postmortem like brain, lung, intestine, trachea, heart, liver, kidney and spleen and antemortem such as oronasal swab, cloacal swab and serum (Office International Des Epizootics [OIE, 2021]). Additionally, trachea or oropharyngeal swabs are often used as the specimens of choices because they are easy to process and usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. However, tissue and organ samples and even faeces have been used with some success. The system used for RNA extraction will also affect the success of RT-PCR specimens and even with commercial kits care should be taken in selecting the most appropriate or validated for the samples to be analyzed (OIE, 2012).

Laboratory confirmation should always be performed regardless of the possibility that the history, clinical symptoms, and lesions may indicate ND. However, direct detection of viral

antigens which can be accomplished by isolating the virus from swabs of living animals or organs removed from freshly dead chickens is the basis for a clear diagnosis of Newcastle disease. Virus culture is easily carried out on embryonated chicken eggs that are 9–11 days old or on a variety of cells, such as African green monkey kidney (Vero) cells, chicken embryo reticulum cells, duck fibroblast cell inoculation, chicken embryo fibroblast cells, and chicken embryo hepatocytes (WOAH, 2022). The best cells for NDV replication are primary and/or continuous avian cells, such as chicken embryo fibroblast cells, Duck fibroblast cells. To increase the chances of viral retrieval for low virulent isolates, trypsin is added to the medium, and virus replication is followed with cytopathic effects (OIE, 2018).

Serological tests that detect NDV include haemagglutination agglutination (HA), haemagglutination inhibition (HAI) and ELISA. HAI is used for detecting antibodies against Newcastle disease virus and ELISA to assess post-vaccination antibody titres. The serological tests are used in diagnostic laboratories but are not of value in surveillance and diagnosis because of the varied vaccines used in the field of poultry (OIE, 2021). The molecular tests known as real time reverse transcriptase polymerase chain reaction (rRT-PCR) and conventional reverse transcription-polymerase chain reaction (RT-PCR) are used to identify the circulating strains of the NDV (lentogenic, mesogenic and velogenic) by analyzing the virus's matrix gene and determining the strain's virulence by analyzing the NDV's fusion gene using particular primers. The test generates results in three hours and is highly sensitive. There is a significant chance that the test will contaminate the samples and the facility. The evolutionary links of diseases are mapped using phylogenetic analysis and gene sequencing (OIE, 2021). Furthermore, it is important to discuss nested PCR, light-extended fluorogenic primer (LUX), ligase chain reaction (LCR), intercalated DNA (SYBR Green), fluorogenic probe-based real-time RT-PCR, gel-based RT-PCR and restriction enzyme-based techniques. While the majority of these assays have demonstrated encouraging findings, it is still necessary to validate their capacity to identify various viral types (Cattoli *et al.*, 2011; Mao *et al.*, 2022).

Based on the mean time to death in embryos, the intravenous pathogenicity index (IVPI) in six week-old chickens, or the intracerebral pathogenicity index (ICPI) in day –old chicks. It is possible to classify the NDV isolates as lentogenic(less virulent), mesogenic (moderate virulent) and velogenic (virulent), But when compared to the other two methods, ICPI seems to be the most sensitive and accurate approach (Dortmans *et al.*, 2011). After intracerebral infection, the ICPI is calculated in day-old chicks; a score of 0 indicates a normal chick, 1 indicates a sick chick, and 2 indicate a dead chick. For eight days, each chick receives this score every day. Ultimately, the mean of the scores establishes the pathogenicity of the viral strain. When the ICPI score is higher than 1.5, the strains are velogenic. When the ICPI value falls between 0.7 and 1.5, they are mesogenic. Finally, if the ICPI value is less than 0.7, they are lentogenic. The six-week-old birds are injected intravenously, but the IVPI is computed in the same way manner as the ICPI. An IVPI of more than 2.5 indicates a virulent strain (Al-Shammari *et al.*, 2020). The average number of hours needed for all infected embryos to perish is known as the "mean time to death." When embryos perish within 60 hours, velogenic strains are likely present; when they survive past 90 hours, lentogenic strains are likely present (Cattoli *et al.*, 2011). The virulence of the viral strain influences the size and appearance of the plaques that NDV generates in vitro in embryonic fibroblast culture. NDV virulent isolates can also be distinguished by F protein cleavage site its amino acid sequence (Awad *et al.*, 2020).

2.9. Differential Diagnosis of Newcastle Disease Virus

Differential diagnosis of NDV refers to separating Newcastle disease from other illnesses that exhibit comparable symptoms or indications. The following illnesses have clinical signs that are comparable to Newcastle disease: Aspergillosis, Laryngotracheitis, Psittacosis (psittacine birds), Laryngopox (diphtheritic type), Mycoplasmosis, Infectious bronchitis, and Fowl cholera. In addition, mistakes in management such inadequate ventilation, shortage of feed, and deprivation of water might occur. Salmonellosis, adenovirus, various *Paramyxoviruses*, Pacheco's parrot illness (psittacine birds), Anomalies related to conformation, fowl cholera, and botulism in cormorants and other wild waterfowl (Sahlu *et al.*, 2015; Abdisa and Tagesu, 2017).

2.10. Public Health impact of Newcastle Disease Virus

Humans are among the many species that can be infected by NDV in addition to avian species. Human conjunctivitis can result from exposure to high concentrations of NDV. Those who work in laboratories and provide vaccinations are the most affected. The exposure of laboratory workers has decreased with the adoption of biological safety cabinets and personal protective equipment. Farm workers seldom get infected, and people who handle or eat poultry products don't seem to be at danger. Although the conjunctivitis normally goes away quickly, the virus will continue to manifest in the ocular secretions for four to seven days. Human reports of a mild, self-limiting influenza-like disease that manifests as fever and headache have also been made in certain instances. Human to bird transmission exists, despite the no data supporting human-to-human transmission (Pal *et al.*, 2021).

2.11. Economic Impact of Newcastle Disease Virus

In a human diet that is balanced, protein is crucial. Vegetables and animals are the two primary sources of protein. Several developing countries throughout the world started off as cottage industries producing poultry. 25.8% of the meat produced comes from farms and poultry. Because they are deadly illnesses, avian influenza and ND are serious issues in animal agriculture (Ge *et al.*, 2012).

Newcastle disease, which has a 100% fatality rate from subclinical infection in poultry, is the most difficult avian disease in Ethiopia and generates large financial losses for the poultry industry. High mortality, morbidity, disease containment strategies, outbreak eradication, and reduced egg production from breeder flocks are all linked to the financial losses. Frequent disease outbreaks have presented a serious risk to Ethiopia's developing chicken industry. Newcastle disease preventive and control measures must be strengthened in endemic countries (Mossie and Abera, 2024).

2.12. Status of Newcastle Disease in Ethiopia

Newcastle disease (ND) is the main cause of financial losses for Ethiopia's poultry industry. Although there are other regional names for the illness, "Yedoro Fengle" is the most often used (Chaka *et al.*, 2012). The velogenic form of the ND virus was responsible for about 80% of cases becoming deadly. Both lentogenic and velogenic strains are circulating in flocks of rural households in Ethiopia, according to recent studies (Fentie *et al.*, 2014; Alemu *et al.*, 2024).

Several studies were conducted in Ethiopia at different points in time to determine the limitations of village poultry under traditional management system. Although the outbreak's origins were difficult to identify, there is potential that factors such as nutrition, vaccination type, management and hygienic settings, and coexisting diseases may have contributed to the disease's onset, progression, and persistence (Mesfin and Bihonegn, 2018). The farms' multi-aged groups, close proximity and lack of separation between units, improper handling of dead birds, lack of an all-in, all-out system and continued housing of various bird species within the same farms all contributed to the outbreak and the disease's persistence there. Well-fed poultry can develop enough protective antibodies and react to vaccinations satisfactorily. Due to a weak antibody response following vaccination, poultry with nutritional deficiencies are more prone to illness (Nasser *et al.*, 2000). There are no effective control measures in place to prevent Newcastle disease in Ethiopian rural poultry, and the disease's epidemiology is poorly understood. This is partially caused by the country inadequate capability for illness monitoring (Terfa *et al.*, 2018).

In the Kersana-Kondalaity district of Ethiopia, Belayheh *et al.* (2014) studied 355 village chickens that were not vaccinated, and they found a 5.6% seroprevalence. Newcastle disease (ND) was found to be 27.9% prevalent in local scavenging chickens' production using a traditional management method in the Agarfa and Sinana areas of Ethiopia (Geresu *et al.*, 2016). In two districts of Ethiopia's Eastern Shewa zone, the overall seroprevalence of Newcastle disease was 5.9% during the dry season and 6.0% during the rainy season (Chaka *et al.*, 2012). The seroprevalence of Newcastle disease based on different test result varies

from 5.6% in Amara region (Belayneh *et al.*, 2014) up to 68. 9 % in in central and Southwestern areas of Ethiopia (Emeru *et al.*, 2024) as indicated in **Table1**.

Table 1: Summary on seroprevalence of Newcastle disease in different region of Ethiopia determined based on ELISA and HAI test

Study Region	Test	No. Examined	Prevalence (%)	Reference
Oromia	ELISA	234	14(5.9%)	Chaka <i>et al.</i> (2012)
Oromia	ELISA	216	13(6%)	Chaka <i>et al.</i> (2012)
Amhara	ELISA	355	20(5.6%)	Belayneh <i>et al.</i> (2014)
Addis Ababa	HAI	146	44(30.14%)	Delesa <i>et al.</i> (2014)
Oromia	ELISA	155	18(11.6%)	Terefe <i>et al.</i> (2015)
Tigray	ELISA	214	56(26.2%)	Getachew <i>et al.</i> (2016)
Amhara	ELISA	480	166(34.58%)	Haile <i>et al.</i> (2020)
Amhara	HAI	480	188(39.16%)	Haile <i>et al.</i> (2020)
Oromia	ELISA	480	144(30%)	Mamo and Yimer (2021)
Oromia	ELISA	311	121(38.91%)	Habte <i>et al.</i> (2022)
Southern	HAI	405	278 (68.64%)	Betela <i>et al.</i> (2023)
Central and Southwestern	ELISA	90	62(68.9%)	Emeru <i>et al.</i> (2024)

Retrospective data analysis from 2012 to 2015 revealed that 86 ND outbreaks occurred in seven national regional states of the country (Amhara, South nation and nationalities), Oromia, Tigray, Addis Ababa, Gambella, and Benshangul-Gumz. These reports, which came from all around the country, were based on clinical symptoms and fatality rates. The Oromia national regional state was the source of 70.5% of these outbreaks. The illness is the most common infectious disease, causing 80% and 43% of losses in commercial and village chickens, respectively (Mesfin and Bihonegn, 2018). Some regions of the nation do a few molecular tests, which ranges in chicken 4.9% (Chaka *et al.*,2013) in Eastern Shewa zone Oromia region to 50% Addis Ababa (Habte *et al.*, 2022)

Table 2: Summary on Molecular detection rate of Newcastle disease in different region of Ethiopia determined based on tissue and swab samples examination.

Study Area	Test	No. Examined	Detection rates (%)	Reference
Eastern Shewa zone, Oromia region Ethiopia	PCR	722	35(4.9%)	Chaka <i>et al.</i> (2013)
Eastern Shewa zone, Oromia region Ethiopia	PCR	275	105(38.2%)	Chaka <i>et al.</i> (2013)
Eastern Shewa zone, Oromia region Ethiopia	PCR	272	75(27.6%)	Chaka <i>et al.</i> (2013)
Northwest Amhara Ethiopia	PCR	240	15(6.25%)	Fentie <i>et al.</i> (2013)
Selected Rift-Valley areas Ethiopia	PCR	155	13 (8.38)	Terefe <i>et al.</i> (2015)
Selected Districts of Eastern Shewa zone Oromia Ethiopia	PCR	300	40 (13.33%)	Miressa <i>et al.</i> (2016)
East Shewa zone of Oromia region Ethiopia	PCR	15	1(6.66%)	Assefa <i>et al.</i> (2021)
Central Rift Valley of Ethiopia	PCR	84	13(15.47%)	Milkesa <i>et al.</i> (2021)
Central Area of Ethiopia	PCR	44	17(38.63%)	Worku <i>et al.</i> (2022)
Addis Ababa	PCR	18	9(50%)	Habte <i>et al.</i> (2022)
Central and Southwestern Areas of Ethiopia	PCR	239	59(24.6%)	Emeru <i>et al.</i> (2024)
Mid-Rift Valley and Central Part of Ethiopia	PCR	98	13(13.26%)	Alemu <i>et al.</i> (2024)

2.13. Prevention and Control of Newcastle Disease Virus

The two main strategies for preventing Newcastle disease are vaccination and good cleanliness. Vaccination is crucial in the management of ND, particularly in semi-intensive systems where birds are kept inside protected yards or houses. Cleaning, disinfecting, preventing access for wild birds, and maintaining farm employees' personal hygiene's are all examples of hygiene practices. Getting vaccinated and maintaining proper hygiene is still the best method to prevent non-communicable diseases. Immunity against viral infection and replication would arise from vaccination against the ND. In practice, ND vaccination protects the poultry against the most severe consequences of the disease, but viral replication and shedding may still occur (Abdisa and Tagesu, 2017). For many years, Inactivated and live vaccines have been an essential component of medical practice (Dimitrov *et al.*, 2017). However, many countries have recently accepted novel vaccine technologies, such as recombinant vaccines and antigen-matching vaccines, while other vaccine techniques are still in the experimental stage (Dimitrov *et al.*, 2017; Nurziah *et al.*, 2022).

The vaccine strains are based on naturally existing pathogenic, low pathogenic, or medium pathogenic strains (Dimitrov *et al.*, 2017). Bello *et al.* (2018) state that the following genotypes are linked to these vaccination strains: genotype I represents Ulster and V4, genotype II represents Lasota and B1, genotype III represents Mukteshwar, genotype IV represents Herts/33, UK, genotype V represents Anhinga, USA, genotype VIII represents AF22440. With the development of genetic engineering, NDV vaccine strains have become viable vectors for the development of multifunctional, effective vaccinations against human and animal infections (Choi, 2017). To protect chickens from a deadly challenge, for instance, an NDV virus-like particle based vaccine that express NDV F protein and influenza virus matrix 1 (M1) protein could be used. It can also be utilized as a Differentiation of Infected from Vaccinated Animals (DIVA) technique to differentiate between vaccinated and infected animals, and it dramatically reduces viral shedding (Park *et al.*, 2014). Izquierdo *et al.* (2019) found that vaccination strains in combination with wild type XII strains provided vaccinated animals with improved protection and significantly decreased viral shedding. A significant reduction in viral shedding and the ability to differentiate between vaccinated and

infected birds are two possible advantages of developing plant-derived vaccines over conventional ones (Nurzijah *et al.*, 2022).

2.13.1. Prevention and control of Newcastle disease in Ethiopia

The most practical and economical approach to controlling Newcastle disease among the several intervention techniques is vaccination, which guarantees successful chicken production by ensuring high levels of poultry health. Of these vaccine kinds, LaSota, HB1, and Thermostable I-2 are currently produced at the National Veterinary Institute (NVI) and are commercially available for a wider range of applications in Ethiopia's small and large scale commercial poultry farms. They are indigenous to Australia and belong to the class II genotype I virus (Mossie and Abera, 2024). It should be mentioned that while the inactive type of vaccine is administered by injection to individual birds, either subcutaneously at the back of the neck or intramuscularly in the breast muscle, the conventional vaccine is primarily administered to chickens orally or through the eyes (Mustafa and Ali, 2005). Good hygiene, avoiding concurrent infections, maintaining a healthy diet, quarantine, high management, appropriate vaccination, depopulation, and antibody monitoring following vaccination are all effective ways to control Newcastle disease (Bello *et al.*, 2018; Mossie and Abera, 2024).

3. MATERIALS AND METHODS

3.1 . Description of study areas

The study was conducted from December 2024 to May 2025 in commercial poultry farms with suspected NDV outbreaks in East wallaga zone Oromia region Ethiopia, at Nekemte town and four selected districts namely: Guto Gida, Diga, Wayu tuka and Sibu Sire. They have a diverse agro-ecology (**Figure 3**). Nekemte is found at 328 kilometers west of Addis Ababa, with latitude 9°04' 9571N, longitude 36°32'9281E, with altitudes ranging from 1960 to 2170 meters above sea level. The mean annual rainfall and average temperature range from 1500-2200 mm and 14-26°C, respectively. An estimated 925,144 bovines, 220,875 ovines, 146,775 caprines, 92,250 equines, and 794,484 poultry live in Nekemte town (Nekemte Town Agriculture Office, 2023).

Guto Gida district is found at a distance of 320 km south west of Addis Ababa, capital city of Ethiopia. The district surrounds the capital city of East Wallaga zone Nekemte town with livestock population of 13, 6005 bovines, 22,004 ovines, 23, 349 caprines and 837095 poultry as reported by Guto Gida Agriculture Office (2023).

Diga district is 12 km from Nekemte town and 343 km from Addis Ababa. About 80,099 people live there, together with 69,965 bovines, 16,424 ovines, 11,857 caprines, and 39730 poultry. It is bounded by Guto Gida district, West Wallaga zone and Benishangul Gumuz, North Sasiga district, and South Leka Dulecha, as reported by Diga Agriculture Office (2023).

Wayu Tuka district is located between 8°56'N and 9°7'N and 36°32'E and 36°48' E. It is situated in the East Wallaga zone of the Oromia national regional state, approximately 322 kilometers west of Addis Ababa. It is bordered to the north and East by Sibu Sire district, to the South by Leka Dulecha district and to West by Guto Gida district. The study's altitudes vary from 1300 to 3124 meters above sea level. The annual rainfall in this area ranges from

2158mm. There are about 1,008,968 bovines, 274,629 ovines, 189,644 caprines, 125,666 equines and 770,250 poultry (Wayu Tuka Agriculture Office, 2023).

Sibu Sire district is situated at a distance of about 280km from Addis Ababa and 50 km from the Zonal town, Nekemte. Sibu Sire district share boundary with Gobu Seyo district in the East, Wayu Tuka district in the West, Gudeya Bila and Guto Gida districts in the North, Wama Hagalo and Billo Boshe districts in the South. The district is between 800 and 2750 meters above sea level. The Sibu Sire district is located at latitude 9°4'N and longitude 36°49'E. Sibu Sire's mean annual minimum and maximum recorded air temperatures were 20°C and 26°C, respectively. The district received between 1000 and 1200 mm of rain on average each year, which falls between March and October. About 132, 4674 bovines, 45,895 ovines, 33,498 caprines, 32,348 equines, and 189,775 poultry are present (Sibu Sire Agriculture Office, 2023).

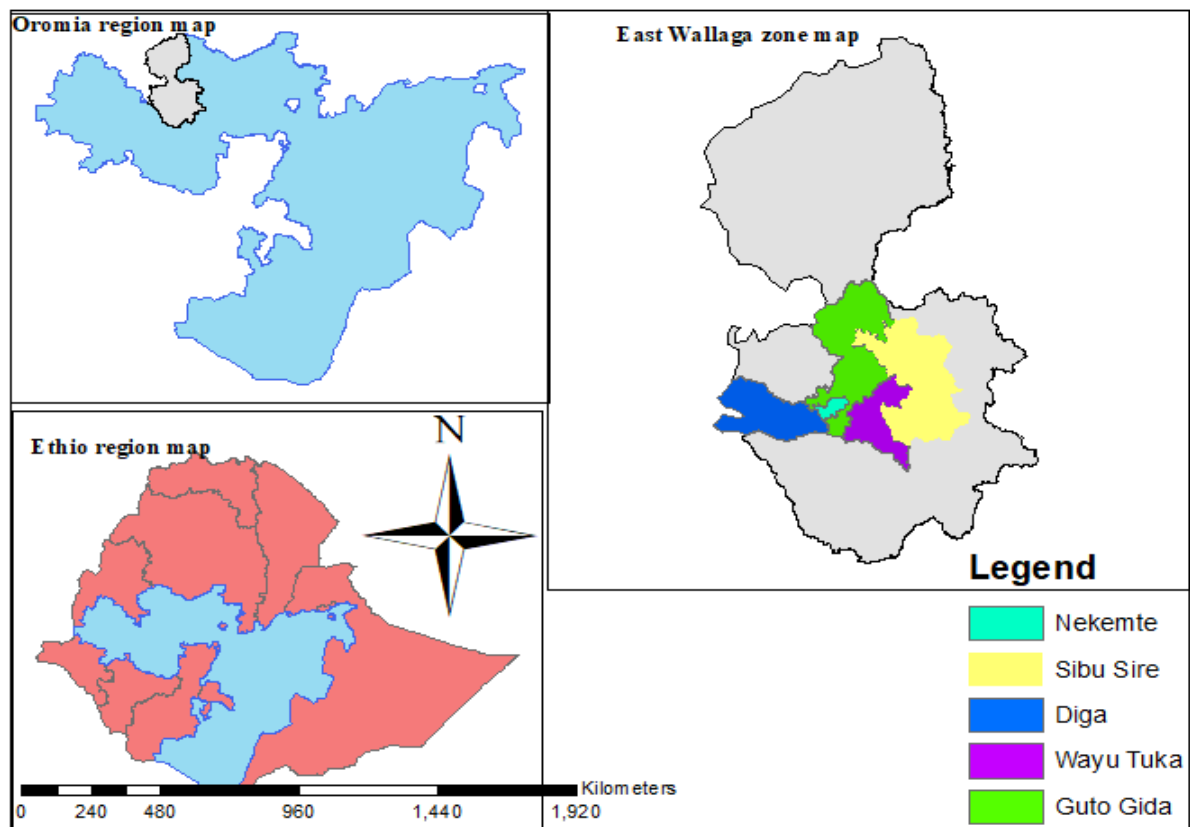


Figure 3: Map of study areas created using Arc GIS

3.2. Study Animals

The study was conducted in commercial poultry farms that are suspected outbreak cases of Newcastle disease and showing the clinical signs of the disease such as; decrease feed intake, coughing, sneezing, grasping for air, nasal discharge, greenish diarrhea, and paralysis of the extremities (torticollis) during outbreak investigation. Poultry of all breed, sex and age groups reared under commercial production poultry were included in the study.

3.3. Study Design

A cross section study design was performed during an active outbreak to detect NDV from suspected outbreak cases. The study focused on suspected cases of ND. Before the beginning of any outbreaks, proper information channels from concerned bodies regarding the outbreaks were collected through different contact addresses. Depending on the reported outbreak case of ND, a field investigation was conducted at the area of outbreaks, clinical information was recorded and appropriate samples were collected from poultry showing signs suggestive of infection.

3.4. Sample Size Determination and Sampling Technique

The sample size was determined based on the purpose of the study. Since the main objective of the study is to detect Newcastle disease virus circulating in the study areas. The study area, farms, and sampled chickens were purposively selected based on the report of active outbreak, accessibility and the willingness of the farm owners to allow sample collection and the presence of active clinical signs of ND. Newcastle disease outbreaks were investigated from December, 2024 to May, 2025 in Nekemte town and selected districts of East Wallaga zone of Oromia region Ethiopia. Due to logistic and financial reasons by investigating 7 outbreaks and from each outbreak simple random sampling method is used to select 5 representative samples, a total of 35 representative samples from 7 outbreaks were collected from all age group and priority was given for poultry's showing visible clinical sign to get live virus. Five samples should be taken from each affected farm in these kinds of

investigations, according to WOAHA (2021). Thus, from all seven farms investigated, 20 poultry sick showing clinical signs of ND were sampled and 15 freshly dead poultry. From each 20 poultry sick showing clinical signs of ND pairs of cloacal and oropharyngeal swab samples were collected in different cryovials and from each 15 freshly dead's poultry four tissue samples were collected in individual in different sample collection tube based on the clinical signs seen. In total, 20 cloacal swab, 20 oropharyngeal swab and 60 tissue samples were collected.

3.5. Data Collection and Study Methods

The data was collected through clinical assessment and laboratory tests. Throughout field investigation, information about the disease was gathered by history taking from poultry farm owners and animal health workers of each outbreak site. The information collected was recorded on data collection sheet, and then poultry were clinically examined for presence of Newcastle disease with clinical signs of the disease, postmortem findings and specimens were collected for diagnostic testing. In additionally, the information collected was recorded on data collection sheet included: about the farm name, location, date of the outbreak, breed, age, sex, flock size, management system, vaccination history, clinical signs, morbidity, mortality, and associated economic impacts. The format used to document the outbreak investigations is available (**Annex 8.1**).

3.6. Sample collection, Sampling procedures, Transportation and storage

During the study period, total 35 poultry suspected cases of ND were aseptically collected based on the clinical signs and postmortem findings observed in each the small scale commercial poultry farms from different outbreaks in East Wallaga zone. Physical examinations were performed on the poultry to look for any symptoms that could indicate an infection with Newcastle disease, such as nasal or ocular discharge, green diarrhea, respiratory distress, or nervous indications (torticollis). The swabs were collected in pairs (oropharyngeal and cloacal swab were always collected from the same poultry and premise) with clinical cases and post mortem tissues from freshly dead's chickens. Post mortem

samples including brain, lung and trachea, intestine and pool tissues samples (liver, heart, kidney and spleen) were collected from individual poultry and put in 2-3 ml virus transport medium (VTM), labeled and transported to the laboratory for detection of the virus. Oropharyngeal and cloacal swabs were taken from the same live chicken and the same premise in a different cryovial that contained newly made 2 ml of viral transport media (VTM) using a disposable sterile Dry swab®, standard plastic (Dacron bud) (Medical Wire and Equipment, England, UK). By carefully swabbing the oropharyngeal region and oral cleft, the choanal and oropharyngeal swabs were obtained. The process for collecting cloacal swabs involves gently pressing on the mucosal surface while swabbing in circular motions with a sterile swab inserted into the cloaca (Hy-Line, 2016). Each sample was labeled legibly and accompanied by necessary identification information (**Annex 8.2**). Following sampling, all the samples were promptly packed in an ice box with ice packs transported to the Guto Gida Veterinary Clinic maintaining +4°C on ice and stored at –20°C until transported to the Animal Health Institute in Sebeta, Ethiopia for laboratory examination. Finally, all samples were transported under chilled conditions by ice boxes and they were transported to the Animal Health Institute in Sebeta, Ethiopia and stored at –80°C until further processing. The procedures for sample collection and transportation were following the industry standards as advised by the WOAHA (2021).

3.7. Sample Preparation for Molecular Detection of Newcastle Disease Virus

3.7.1. Tissue and swab samples preparation for RNA extraction

The frozen tissue samples stored at –80°C were thawed at room temperature and processed for virus detection as indicated in the WOAHA (2021). Brain, lung, trachea, intestine and pool tissues (spleen, kidney, liver, and heart tissue) from outbreaks cases were pooled to increase viral concentrations. To increase viral loads, use five pooled tissue samples per test tube for the same samples, poultry and premises. Twelve pool tissue samples always collected from the same poultry and premise (five pooled brain per one test tube, five pooled lung and trachea per one test tube, five pooled intestine per one test tube and five pooled pool of tissues from heart, liver, kidney, and spleen samples per one test tube) collected from 15

poultry during disease outbreaks in 5 poultry flocks in 3 different small scales commercial poultry farm of the East Wallaga zone were used in this study. Approximately 1 g of pooled tissue samples was minced into small pieces using sterile scissors and sandy ground with a sterile mortar and pestle in a class II biosafety cabinet. Tissue suspensions were prepared by adding VTM containing antibiotics and antifungals. The suspension was transferred to a 1.5 ml micro centrifuge tube and the supernatant fluids were obtained through clarification using centrifugation at 10000xg for 5 minutes at +4°C. One ml of supernatant fluid was harvested and aliquoted into a sterile 1.5ml Eppendorf for use in the next.

Frozen swab samples, initially stored at –80°C, were thawed to room temperature for viral detection. To enhance viral load recovery, a pooled sampling strategy was employed. To increase viral loads, use five pooled swab samples per test tube collected from the same poultry farms. Four paired swabs (oropharyngeal and cloacal) collected from 20 poultry across five flocks during disease outbreaks in four small-scale commercial farms (East Wallaga zone) were combined for analysis.

Extraction Workflow:

1. Vortex Homogenization: Cryovial tubes containing swabs in viral transport medium were vortexed at maximum speed for 1 minute to release viral particles.
2. Liquid Phase Separation: A 1 mL aliquot of the homogenized suspension was transferred to a sterile 1.5 mL Eppendorf tube.
3. Centrifugation: Samples were centrifuged at 12,000 rpm for 3 minutes to pellet cellular debris and particulate matter.
4. Supernatant Collection: The clarified supernatant (1 mL) was carefully aspirated and aliquoted into fresh 1.5 mL Eppendorf tubes for downstream applications (e.g., RNA extraction or PCR).

3.7.2. RNA extraction from samples

All samples underwent initial processing to eliminate grass particles and extraneous debris prior to extraction. One milliliter of the clean supernatant was carefully aspirated and aliquoted into new 1.5 milliliter Eppendorf tubes used for the next steps in order to extract the RNA from each tissue samples, cloacal swabs and oropharyngeal swabs that were clinically suspected. 140µL of total RNA was extracted from each clinically suspected oropharyngeal swab, cloacal swab, and tissue sample using the Qiagen ® Rneasy Mini kit, following the manufacturer's instructions as indicated by Wise *et al.* (2004). Lastly, the RNA was stored for future use at a freezer at -80°C. Laboratory activities were carried out at the Animal Health Institute (AHI) in Sebeta's molecular laboratory (**Annex 8.3**).

3.8. Molecular Detection of Newcastle disease virus

The quantitative one-step real-time polymerase chain reactions (rRT-PCR) for the conserved region of the M-gene target were conducted on 20 five pooled samples (12 five pooled tissue samples, 4 five pooled oropharyngeal and 4 five pooled cloacal swabs) obtained from clinical cases of recently dead or alive chickens using an Applied Biosystems 7500 rapid thermocycler. All samples obtained from clinical cases of living or freshly dead's poultry (tissue samples, oropharyngeal, and cloacal swabs) were subjected to viral RNA extraction using the Qiagen ® Rneasy Mini kit in accordance with the manufacturer's instructions. In order to detect Newcastle disease virus (NDV) in the RNA extracted samples, the M-gene was specifically designed. To detect M-gene based NDV, specific Forward primer M+4100- 5'-AGC GAT GTG CTC GGA CCT TC-3', Reverse Primer M-4220- 5' CCT GAG GAG AGG CAT TTG CTA-3' and Probe M+4169-5' FAM-TTC TCT AGC AGT GGG ACA GCC TGC – TAMR -3' were used. Master mix reagents per 25 µl reaction were used from Qiagen one-step RT-PCR kit: 5 µL of kit supplied PCR buffer (5X), 0.5 µl (20pmol/ µl) forward primer, 0.5 µl (20pmol/µl) reverse primer, 1 µl (6 pmol/µl) of probe, 0.8 µl of kit supplied deoxynucleoside triphosphates(dNTPs) (10mM each), 1.25 µl of 25 mM MgCl₂, 0.5 µl of 13.3 u/µl of RNase inhibitor and 1 µl one step Qiagen enzyme mix and 6.45 RNase free water (QIAGEN). Depending on the number of samples to be analyzed, aliquots of 17 µl

of prepared master mix were dispersed into an Applied Biosystem plate. To guarantee a uniform distribution of the reagents, all of the chemicals were combined in a single reaction tube (master mix). Each plate was then filled with 8 µl of extracted RNA, resulting in a total reaction volume of 25 µl. NDV known positive field isolates and RNase-free water were used as positive and negative controls, respectively. In order to prevent evaporation, the plates were sealed with a sealer and put into the rRT-PCR machine or heat cycler that was connected to a computer running its own software (Applied Biosystems Sequence Detection Software, version 1.4.0), and the program was set or modified for each individual reaction. The RNA to cDNA (reverse transcription) stage was carried out for 30 minutes at 50°C and then incubated for 15 minutes at 95°C. 40 cycles of 10 seconds of denaturation at 94°C, 30 seconds of annealing at 52°C, and 25 seconds of extension at 72°C comprised the cycling conditions for the M-gene assay (Wise *et al.*, 2004). The threshold cycle (Ct.) for every sample was determined by measuring the quencher dye (TAMRA) and reporter dye (FAM) signals at the extension step of each cycle. Samples with a Ct value < 35 were considered positive for M genes using qRT-PCR, while samples with a Ct value > 35 were considered negative (OIE, 2013).

3.9. Data Management and Analysis

Collected data was encoded on Microsoft Excel 2010 spread sheet and stored in a computer before it is transferred to Statistical Package of Social Sciences (SPSS) Version 20 for statistical analysis. Data was managed and analyzed by using SPSS version 20 software to summarize the results obtained from rRT-PCR. The outcome was presented using descriptive statistics including frequencies and percentages were used and results summarized using tables. Several variable categories were used to estimate the rates of morbidity, mortality, and case fatality.

4. RESULTS

4.1. Outbreak investigations

A total of seven outbreaks in different sites of East Wallaga zone were identified during the study period. In the course of the outbreak investigation, 382 poultry raised in small-scale commercial poultry farms were suspected for ND. Among 382 poultry examined, 58 poultry exhibited clinical signs suggestive of ND and 30 poultry had died. Overall rates of 15.18%, 7.85% and 51.72% morbidity, mortality and case fatality, respectively were observed in the study area. Among the study areas, Guto Gida district (41.67%) had a slightly higher morbidity whereas Mortality rate is higher relatively in Wayu Tuka District (30%). The information about outbreaks, including rates of morbidity, mortality, and case fatalities, is shown in **Table 3** below.

Table 3: Summary of outbreak data in the study areas

Site of study	N _{o.} Clinically Examined poultry	N _{o.} Clinically affected poultry	N _{o.} Died poultry	Morbidity rate (%)	Mortality rate (%)	Case fatality rate (%)
Nekemte town (07)	270	22	14	8.15	5.19	63.63
Guto Gida District	12	5	1	41.67	8.33	20
Nekemte town (04)	18	5	2	27.78	11.11	40
Diga District	17	6	5	29.41	17.64	83.33
Sibu Sire District	25	8	1	32	4	12.5
Nekemte Town (03)	20	5	1	25	5	20
Wayu Tuka District	20	7	6	35	30	85.71
Overall	382	58	30	15.18%	7.85%	51.72%

***Nekemte town (07) = Nekemte town, 07 Kebele; **Nekemte town (04) = Nekemte town, 04 Kebele; *Nekemte town (03) = Nekemte town, 03 Kebele

4.2. Clinical Findings

The predominant clinical signs observed in this study included reduced feed intake, torticollis (head twisting), depression, wing and leg paralysis, respiratory distress (coughing and gasping), and greenish diarrhea (**Figure 4**). These findings are consistent with typical manifestations of Newcastle disease (ND).



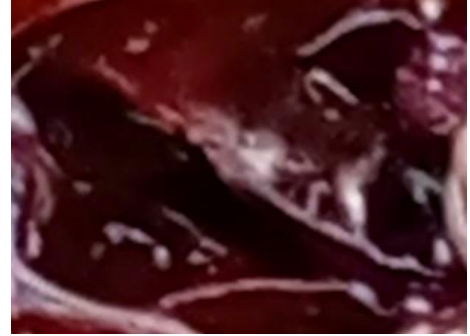
Figure 4: Clinically affected poultry suspected Newcastle disease virus infection.

4.3. Postmortem Findings

Postmortem examinations were conducted on poultry suspected to be infected with Newcastle disease virus (NDV) to evaluate gross pathological lesions. Systematic necropsy revealed characteristic pathological changes including severe hemorrhagic ulcers along the intestinal mucosa (**Figure 5A**) and distinct hepatic lesions exhibiting diffuse degeneration with multifocal necrotic foci (**Figure 5B**).



(A)



(B)

Figure 5: Postmortem findings of Newcastle disease virus infected poultry

4.4. Overall Detection of Newcastle Disease Virus

A total of 20 pooled samples (comprising 12 tissue pools, 4 oropharyngeal swab pools, and 4 cloacal swab pools; each pool containing five samples) were screened for Newcastle disease virus (NDV) using matrix (M) gene-based real-time reverse transcriptase polymerase chain reaction (rRT-PCR). Among these, one pooled sample tested positive for NDV. For further confirmation, the positive sample with the highest viral load (Ct value 15.37) and other samples were subjected to additional rRT-PCR analysis. The assay controls performed as expected: the positive control showed a Ct value of 16.17, laboratory-positive samples exhibited Ct values ranging from 19.25 to 34.23, and no amplification was observed in the negative control. All reactions were performed using an Applied Biosystems 7500 real-time PCR system. The samples and control rRT-PCR amplification curve are indicated in **Figure 6 A and B**.

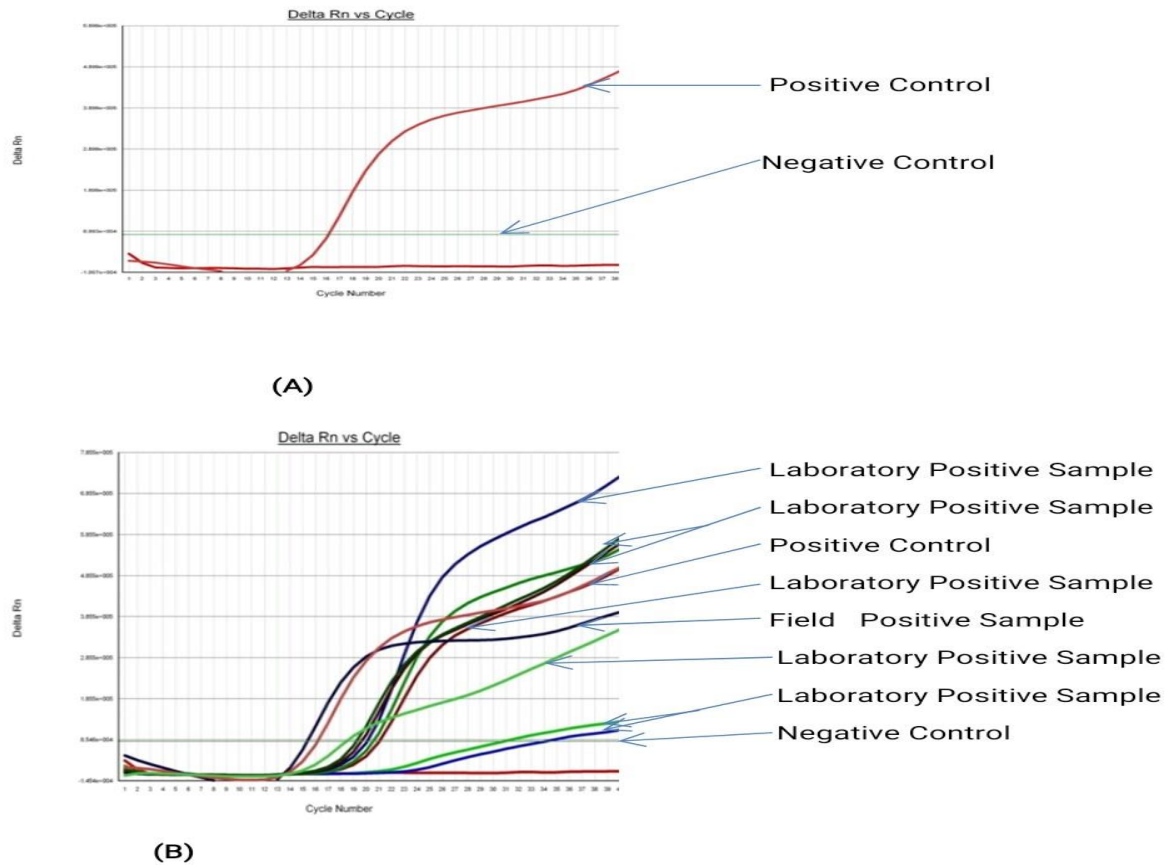


Figure 6: Amplification plot result of rRT- PCR test for Newcastle disease virus M-gene with their Ct. Value

4.5. Detection of Newcastle Disease Virus Based on Site of Study Area

The overall Newcastle disease virus (NDV) detection rate in the study area was 5% (1/20 pooled samples). The analysis revealed that Nekemte town (07) had the highest detection rate (25%), while no NDV was detected in samples from Wayu Tuka district, Diga district, Sibbu Sire district, Guto Gida district, Nekemte town (04) and Nekemte town (03) (**Table 4**).

Table 4: Detection rates of Newcastle disease virus based study sites

Study sites	Type of Sample	No. of Pooled five samples	No. Positive	Detection rates (%)
Wayu Tuka District	Tissue	4	0	0%
Diga District	Tissue	4	0	0%
Nekemte town (07)	Tissue	4	1	25%
Sibu Sire District	Swab	2	0	0%
Guto Gida District	Swab	2	0	0%
Nekemte town(04)	Swab	2	0	0%
Nekemte town (03)	Swab	2	0	0%
Overall		20	1	5%

*****Nekemte town (07) = Nekemte town, 07 Kebele; *****Nekemte town (04) = Nekemte town, 04 Kebele; ****Nekemte town (03) = Nekemte town, 03 Kebele; **Pooled five tissues = pooled five brain tissue samples per one test tube, pooled five Lung and Trachea tissue samples per one test tube, pooled five Intestine tissue samples per one test tube and pooled five pools Tissues (Kidney, Liver, Heart and Spleen per one test tube); *Pooled five swab samples = pooled five Claocal swab samples per one test tube and pooled five Oropharyngeal swab samples per one test tube

4.6. Detection of Newcastle Disease Virus Based on Types of Samples

Among 12 pooled five tissue samples (pooled five tissues per one test tube), one pooled five tissues tested positive for NDV (8.33% detection rate), while all 8 pooled five swab samples were negative. The overall NDV detection rate was 5%, with the highest rate in brain tissue pools (33.33%). No other tissues or swabs were positive (**Table 5**).

Table 5: Newcastle disease virus detection rates based on types of samples

Samples types		No. of pooled five samples		Detection rate of M-gene (%)
		Examined	Positive	
Tissues sample	Brain	3	1	33.33
	Lung and Trachea	3	0	0
	Intestine	3	0	0
	Pool tissues	3	0	0
	Total	12	1	8.33
Swab samples	Cloacal swab	4	0	0
	Oropharyngeal swab	4	0	0
	Total	8	0	0
Overall		20	1	5

*****Pooled five Brain tissue samples = pooled five brain tissue samples collected from the same farm per one test tube; ***** Pooled five Lung and Trachea tissue samples = pooled five Lung and Trachea tissue samples collected from the same farm per one test tube; ***** Pooled five Intestine tissue samples = pooled five Intestine tissue samples collected from the same farm per one test tube; ***Pooled five pools tissue samples = pooled five pools Tissues (Kidney, Liver, Heart and Spleen) collected from the same farm per one test tube; **Pooled five claoal swab samples = pooled five Claocal swab samples collected from the same farm per one test tube; *Pooled five Oropharnygeal swab samples = pooled five Oropharnygeal swab samples collected from the same farm per one test tube

5. DISCUSSION

Newcastle disease is a serious viral infection that affects poultry farms all around the world, including Ethiopia. The current study was carried out to identify the virus at the molecular level from active outbreaks. The outbreak on these farms resulted in overall morbidity, mortality, and case fatality rates of 15.18%, 7.85% and 51.72% % respectively. This result is closely related to the findings of Worku *et al.* (2022), who found that in a particular area of central Ethiopia, the overall rates of morbidity, mortality and case fatality were 18.7%, 9.5%, and 50.5%, respectively. Mortality of the NDV outbreak during the present study was 7.85% out of the investigated commercial poultry. This current study Mortality rate of NDV lower than previous study reported 55% in Chad (Antipas *et al.*, 2012), 21.1% in India (Khorajiya *et al.*, 2015), 97.7% in Eritrea (Bereket *et al.*, 2017) and 16.3% in Central Ethiopia (Yadeta *et al.*, 2024). This difference may be related to the age and immune status of the poultry, coexisting illnesses, poultry husbandry and viral strain. This may also have to do with the differences in antigen and genetic makeup between the vaccine strains and the circulating field strains.

Clinical indicators of NDV infection were noted in this study, including decreased feed intake, head twisting (torticollis), depression, paralysis of the wings and legs, coughing, gasping for air, and greenish diarrhea were prominent. This agreed with studies conducted in India (Khorajiya *et al.*, 2015), in Eritrea (Bereket *et al.*, 2017), in Jimma zone of Oromia region Ethiopia (Tsfaye *et al.*, 2023) and Iraq's Thi-Qar province (Kadhim *et al.*, 2024). This finding is slightly different from a previous report (Wise *et al.*, 2004) that showed diarrhea, dizziness, loss of appetite, and ocular enlargement and inflammation. This variation may be due to the virus strains' virulence and tropism, immune status, route of infection, age of poultry, and environmental factors. The upper respiratory system of the infected host is affected by aerosolization of a high viral dosage, while neurologic symptoms are caused by intravenous inoculation.

Postmortem findings were performed on NDV infected poultry and gross pathological alterations were observed. Hemorrhagic ulcers in the intestinal wall and liver degeneration and multifocal necrosis were observed in postmortem NDV-infected poultry. These findings agreed with previous reports (Murree *et al.*, 2016; Bereket *et al.*, 2017; Worku *et al.*, 2022). Hemorrhagic laryngotracheitis, lung congestion, and edema have been reported (Dodovski *et al.*, 2015), but these results are not consistent with the present study. This difference might result from variation NDV strains that can affect various organs of the poultry.

There are only a few researches for comparison, and less molecular studies are carried out in Ethiopia to detect NDV than serological studies. The current work used rRT-PCR for M gene test to detect NDV from a total of 20 pooled five samples collected from 12 pooled five necropsy specimens and 8 pooled five swabs examinations. In this study, the RT-PCR detection of the M-gene (**Figure 6**) indicated that the poultry were infected with the virus. Additionally, one positive qRT-PCR sample with a high viral load concentration (15.37 Ct. value) was quantified out of 20 pooled five samples. This current study was higher viral load concentration (15.37 Ct. value) than in the previous study, M-gene Ct. values ranged from 18 to 31 in Selected Rift-Valley Areas, Ethiopia (Terefe *et al.*, 2015), Ct. value ranged from 20.7-34 in central Ethiopia (Worku *et al.*, 2022) and Ct. value ranged from 23.41- 36.38 in live poultry markets Ethiopia (Milkesa *et al.*, 2021). The Ct value variation may be due to varying viral loads in samples, rRT-PCR sensitivity, RNA extraction efficiency and sample types.

However, in suspected outbreaks cases in small scale commercial poultry farms from in Nekemte town and selected districts of East Wallaga zone of Oromia region Ethiopia, the current study found an overall molecular detection rate of 1/20 (5%) for NDV strain. The current study's findings were consistent with the 4.9% F gene discovery from the East Shewa Zone of Ethiopia reported by Chaka *et al.* (2013), 6.25% in northwest Ethiopia (Fentie *et al.*, 2014), 1/15 (6.66%) M gene found in the East Shewa zone of Oromia region Ethiopia (Assefa *et al.*, 2021) and 4.2% M gene discovery in Kerala, India (Ravishankar *et al.*, 2022). The current result of study (5%) was lower than the finding of other researcher done by M gene overall detection rate 8.4% in selected Rift Valley Areas of Ethiopia (Terefe *et al.*,

2015), 21.1% in India (Khorajiya *et al.*, 2015), 13.33% in Adama and Bishoftu (Miressa *et al.*, 2016), 40%, in Bishoftu (Gelana, 2017), 22.55% in Assam India (Deka *et al.*, 2022), 50% in central Ethiopia (Habte *et al.*, 2022), 38.63% in Central Ethiopia (Worku *et al.* 2022), 13.26% in Mid-Rift Valley and Central Ethiopia (Alemu *et al.*, 2024) and 24.6% in Central and Southwestern areas of Ethiopia (Emeru *et al.*, 2024). This difference may result from variations in the study population and primer used for detection. Our study also revealed in the study sites where the highest score was from Nekemte town (07) (25%) while none was detected in Wayu Tuka district, Diga district, Sibu Sire district, Guto Gida district, Nekemte town (04) and Nekemte town (03) (**Table 4**). This study similarly, previous reported by Milkessa *et al.*, (2021) in the subject area 42.86 % in Adama while none was detected in Bishoftu and Bote. This negative result may suggest that there is no virus present in the study area because there isn't an active viral infection.

On the other hand, the virus was molecularly identified in the current investigation from several organs of infected poultry samples, with 33.33% (1/3) found in the pooled five brain tissue and none found in the pooled five lung and trachea, pooled five intestine, pooled five tissue collected from liver, kidney, spleen and heart and pooled five swab samples (**Table 5**). The higher rate of NDV detection from brain tissue in the present study may indicate a virulent form of the virus invading the brain or central nervous system. Brain tissue samples can also show the potential spread of virulent strains in outbreak cases and are appropriate for molecular NDV diagnosis. Based on sample type, this study were different from previous reports in Central Ethiopia; 54.54 % (6/11) of lung and trachea samples, 54.54 % (6/11) brain tissue samples, 36.36% (4/11) of intestinal samples and 9% (1/11) of pooled tissue samples were positive (Worku *et al.*, 2022). The current study's brain samples had a 33.33% (1/3) positive test, which was lower than the previously reported 54.54% (6/11) of positive brain samples from Central Ethiopia (Worku *et al.*, 2022). This difference may be due to the stage of infection, tissue tropism, immune status and age of poultry. This might be also due to virulent strains of NDV and high sensitivity, high specificity, efficiency of rRT-PCR.

6. CONCLUSIONS AND RECOMMEDATIONS

Newcastle disease virus (NDV) is a highly contagious and economically significant avian pathogen affecting poultry production worldwide, including Ethiopia. This study detected NDV in samples collected from active outbreaks in small-scale commercial poultry farms in Nekemte town and selected districts of the East Wallaga zone, Oromia region, Ethiopia using M-gene-based rRT-PCR. The overall NDV detection rate was 5%, with Nekemte town (07) showing a relatively higher rate (25%), while no positive cases were identified in other surveyed areas. Notably, brain tissue samples exhibited the highest viral load suggesting their suitability for molecular NDV diagnosis and indicating the potential circulation of virulent strains in outbreak cases. The study recorded morbidity (15.18%), mortality (7.85%), and case fatality rates (51.72%), highlighting ND as a leading cause of poultry losses in the study areas. Given its substantial economic impact on small-scale farms, NDV remains a critical threat requiring targeted intervention. To mitigate ND outbreaks and enhance poultry health management in the study area, the following measures are recommended:

- Implement routine vaccination programs tailored to circulating NDV strains.
- Strengthen biosecurity measures and conduct farmer awareness campaigns on ND risks and prevention.
- Conduct genotypic characterization of local NDV strains to identify prevalent genotypes and inform vaccine selection.

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8. ANNEXES

Annex 8.1: Data collection format for clinical examination during outbreak investigation

Date of sample Collection _____

Region Oromia Zone East Wallaga District _____ Farmers Association _____

Geo Reference:

Altitude _____ Longitude _____

Latitude _____

Purpose of Sample collection: Molecular Detection of Newcastle Disease Virus in commercial Poultry Farms from Outbreak cases in selected sites of East Wallaga zone of Oromia Ethiopia

Bio data regarding Poultry sampled									
Sample Code	Sex	Age	Breed	Biosecurity	Vaccination History	N ^o . of animal examined	N ^o . of Clinically affected animal	Clinical signs	N ^o . of animal death

Annex 8. 2: Sample collection format

Sample code	Site of outbreak			Sex	Age	Breed	Type of Sample swabs							Date of collection	Result
	Zone	District	Kebele				OP	CL	B	T	L	I	PT		
S1															
S2															

Keywords: S1(Sample 1), S2(Sample 2), OP (Oro-pharyngeal), CL (Cloacal) B (Brain), T (Tracheal), L (Lung), Intestine (I), PT (Pool Tissue: liver, heart, spleen, kidney)

Annex 8. 3. Standard Operating Procedure (SOP) for RNA Extraction

Recommendations for working with RNA

1. Workspace Setup

Pre-PCR Zone: Dedicated equipment, biosafety cabinet, and lab coats

Post-PCR Zone: Separate room with exclusive equipment (no cross-use)

2. Contamination Control

Wear gloves; replace after contact with skin/common surfaces

Use RNA-specific pipettors with filter tips and RNase-free tubes

Clean surfaces/tools with 70% ethanol + RNaseZap

UV-treat workspaces (15 min) to eliminate DNA

3. Reagents & Materials

RNase-free chemicals (QIAamp Viral RNA Mini Kit, VTM)

95-100% ethanol (AW1/AW2 buffer prep) + 70% ethanol (cleaning)

Nuclease-free tubes, RNaseZap, PPE (gloves/lab coats)

4. Equipment

Class II BSC with UV, -20°C freezer, microcentrifuge

Micropipettors (filter tips), vortex, 37°C water bath

Kit Preparation

Carrier RNA Activation: Add 310 µL AVE buffer to lyophilized RNA; vortex. Aliquot and store at -20°C (≤3 freeze-thaw cycles).

Buffer Prep:

AW1: Add 25 mL 96-100% ethanol (per bottle instructions)

AW2: Add 30 mL 96-100% ethanol (per bottle instructions)

Note: Maintain strict separation of pre/post-PCR workflows to ensure RNA integrity.

RNA extraction Procedures

I. Buffer Preparation

AVL Buffer Activation

- Heat AVL buffer in 37°C water bath until fully dissolved (no crystals). Vortex thoroughly.

AVL-Carrier RNA Mix:

- In a RNA-designated biosafety cabinet (BSC), combine AVL buffer and carrier RNA at 560:5.6 µL ratio (e.g., 11.2 mL AVL + 112 µL carrier RNA for 20 samples).

Note: Prepare 10% excess to account for pipetting losses. Include an extraction control.

II. Sample Lysis**Lysis Setup**

- Transfer 560 µL AVL-carrier RNA mix to a 1.5 mL nuclease-free microcentrifuge tube.
- Add 140 µL samples (e.g., viral transport medium) to the tube.
- Mix: Pulse-vortex for 15 sec to homogenize.

Incubation

- Incubate at 15–25°C for 10 min.
- Briefly centrifuge (5–10 sec) to collect droplets from the lid.

III. Ethanol Precipitation**Binding Solution**

- Add 560 µL ethanol (96 –100%) to the lysate.
- Pulse-vortex for 15 sec, then centrifuge briefly (5–10 sec).

IV. Column Binding & Washing**Initial Binding**

- Transfer 630 µL lysate-ethanol mix to a QIAamp Mini Spin Column (in 2 mL collection tube).
- Centrifuge at 6,000 ×g (8,000 rpm) for 1 min. Discard flow-through; reuse collection tube.

Repeat Binding

- Reapply remaining lysate to the same column.

- Centrifuge again at 6,000 ×g for 1 min. Discard flow-through; replace with a new collection tube.

Wash Buffers

- AW1 Wash: Add 500 µL AW1 buffer to the column. Centrifuge at 6,000 ×g for 1 min. Discard flow-through.
- AW2 Wash: Add 500 µL AW2 buffer. Centrifuge at 20,000 ×g (14,000 rpm) for 3 min. Discard flow-through.

Optional Dry Spin

- Transfer column to a new 2 mL tube. Centrifuge at 20,000 ×g for 1 min to remove residual ethanol.

V. RNA Elution

Elution

- Transfer column to a clean 1.5 mL tube.
- Add 60 µL AVE buffer (room temperature) directly onto the membrane.
- Incubate at room temperature for 1 min, then centrifuge at 6,000 ×g for 1 min.

VI. Storage

RNA Handling

- Discard the column. Store eluted RNA in the 1.5 mL tube:
- Immediate use: Keep on ice.
- Long-term: Store at -20°C (short-term) or -80°C (long-term).

Critical Notes:

- Perform all pre-PCR steps in an RNA-designated BSC with RNase-free consumables.
- Use filter tips and avoid cross-contamination between pre-/post-PCR zones.
- Validate extraction controls for quality assurance.

Protocol adapted from QIAamp Viral RNA Mini Kit guidelines.

Mezene Woyessa

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