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PATHOLOGICAL CHARACTERIZATION, VIRAL ISOLATION AND MOLECULAR DETECTION OF NEWCASTLE DISEASE VIRUS IN SMALL SCALE POULTRY FARMS IN BISHOFTU TOWN, CENTRAL ETHIOPIA.

MSc Thesis

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MASTERS OF SCIENCE IN VETERINARY PATHOLOGY

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BISHOFTU, ETHIOPIA

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A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in the Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Pathology

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College of Veterinary Medicine and Agriculture, Bishoftu

DEDICATION

I dedicate this work to my blessings, Mom and Dad. Your everyday prayers and your love made me stronger than I thought I could ever be.

TABLE OF CONTENTS PAGES

ACKNOWLEDGMENTS .... **viii**

LIST OF TABLE .... **ix**

LIST OF FIGURES .... **x**

LIST OF APPENDIXES .... **xi**

LIST OF ABBREVIATIONS .... **xii**

ABSTRACT .... **xiii**

1. INTRODUCTION **1**

2. LITERATURE REVIEW **4**

2.1. Etiology **4**

2.2. Clinical Signs of Newcastle disease **5**

2.3. Pathology **7**

2.3.1. Pathogenicity *7*

2.3.2. Gross and Histopathological lesions *8*

2.4. Epidemiology of Newcastle disease **11**

2.4.1. Transmission and source of infection *11*

2.4.2. Host range *12*

2.4.3. Global distribution of Newcastle disease *12*

2.5. Diagnosis of the Newcastle disease **12**

2.5.1. Immunohistochemistry examination *13*

2.5.2. Serological technique *13*

2.5.3. Virus isolation *14*

2.5.4. Molecular technique *14*

2.6. Differential diagnosis of Newcastle disease **15**

2.7. Prevention and control of Newcastle disease **15**

2.8. Economic importance of Newcastle Disease **16**

2.8.1. Seasonality of the Disease *17*

2.9. Newcastle diseases in Ethiopia **17**

3. MATERIALS AND METHODS 19

3.1. Study area **19**

3.2. Study population **19**
3.3. Study design **19**
3.4. Sample size and sampling technique **19**
3.5. Sample collections **20**
3.6. Sample Processing **21**
3.6.1. Histopathological examination *21*
3.6.2. Sample preparation and viral isolation *21*
3.6.3. Molecular detection *22*
3.7. Questionnaire survey **23**
3.8. Data management and Analysis **24**
3.9. Ethical clearance **24**
4. RESULTS 25
4.1. Clinical findings **25**
4.2. Pathological findings **27**
4.3. Molecular findings **38**
4.3.1. Isolation of Newcastle Disease Virus *38*
4.3.2. Reverse Transcriptase Real-Time Polymerase Chain Reaction result *39*
4.4. Questionnaire survey result **40**
5. **DISCUSSION** **42**
6. CONCLUSION AND RECOMMENDATIONS **47**
7. REFERENCES **48**
8. APPENDICES **58**

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LIST OF TABLE **PAGE**

Table 1: Biosecurity practices in the study households 40

LIST OF FIGURES PAGES

Figure 1: Category of observed clinical signs of Newcastle disease 25
Figure 2: Clinical signs of chickens infected by ND 26
Figure 3: Various gross pathological lesions found in lung and trachea 27
Figure 4: Microscopic lesions of lung and trachea 29
Figure 5: Gross pathological lesions in proventriculus. 29
Figure 6: Microscopic lesions of proventriculus. 30
Figure 7: Gross lesions in the intestine 31
Figure 8: Microscopic lesions in small intestine. 32
Figure 9: Gross and microscopic lesions in liver 33
Figure 10: Gross and microscopic lesions of cecal tonsil 34
Figure 11: Gross and microscopic lesions in the spleen 34
Figure 12: Gross and microscopic lesions in the bursa of fabricius 35
Figure 13: Gross and microscopic lesions in the heart 35
Figure 14: Gross and microscopic lesions of kidney 36
Figure 15: Gross and microscopic lesions of brain. 37
Figure 16: ND virus cytopathic effect on DF-1 cells. 38
Figure 17: Amplification plot result of rRT-PCR. 39

LIST OF APPENDIXES **PAGES**

Annex I: Ethical Approval Sheet 58
Annex II: Chicken Necropsy Protocols 59
Annex III: Histopathological procedures 63
Annex IV: Staining Procedures 63
Annex V: A questionnaire survey 64
Annex VI: Preparation of Clinical Specimens and Inoculation of Cell Lines 65
Annex VII: RNA Extraction Procedure 66
Annex VIII: Mixing Master Mixes 67

LIST OF ABBREVIATIONS

AHI	Animal health institute
APMV	Avian Paramyxovirus, Type 1
cDNA	Complementary DNA
CNS	Central Nervous System
CPE	Cytopathic Effect
DMEM	Dulbecco’s Modified Eagle’s Medium
ELISA	Enzyme Linked Immunosorbent Assay
F	Fusion
HB1	Hitchener's B1
HI	Hemagglutination Inhibition
HN	Hemagglutinin-Neuraminidase
ICPI	Intracerebral Pathogenicity Index
ICTV	International committee on taxonomy of viruses
IVPI	Intravenous Pathogenicity Index
loNDV	Newcastle Diseases Virus of Low Virulence
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NVI	National Veterinary Institute
NVND	Neurotropic Velogenic Newcastle disease
OIE	World Organization for Animal Health
PBS	phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-PCR	Real-Time Reverse Transcriptase Polymerase Chain Reaction
SNNPR	Southern Nation, Nationalities and Peoples Region
SSP	Small-scale poultry
STATA	Statistics and data
VTM	Viral transport media
VVND	Velogenic Virulent Newcastle Disease

ABSTRACT

Poultry diseases are among the major constraints of chicken production in Ethiopia. Of which Newcastle disease (ND) is still one of the most important devastating diseases of chicken. Cross sectional study was conducted from November 2022 to May 2023 on clinically sick chickens to characterize lesions due to Newcastle disease, and to isolate the Newcastle disease virus, in 53 small scale farms of Bishoftu town. Questionnaire survey was done to evaluate farm biosecurity in farms that have had clinical diseases. Necropsy was done on a total of 120 chickens. Viral isolation was done on cell culture and detection of the viral genome was done by reverse transcriptase PCR. A total of 53 pooled tracheal and cloacal swab and tissue specimens were sampled from sick chickens showing clinical signs of Newcastle disease and from apparently healthy chicken that have had contact with diseased chicken. Out of 53 collected pools of swab and tissue samples, 24 pools (45.3%) were positive for Newcastle disease virus by real-time PCR. Newcastle disease virus was isolated from suspected cases in all "kebele" or county with the proportions of isolation of 11.32% for Dembi kebele, 3.77% for Filtuu, 5.66% for Qurqura, 1.89% for Kata, 5.66% for Gorba, and 9.43% for Calalaka. Depression, greenish or white diarrhea, torticollis, labored breathing, respiratory rales and drop in egg production with soft-shelled egg were commonly observed signs. Hemorrhagic lesions in the proventriculus, intestine, cecal tonsils, and hemorrhagic tracheitis were the common grossly seen lesions. The spleens were enlarged, friable and dark red or mottled, while the kidneys were enlarged, congested and swollen. Among the microscopic lesions were necrosis of the proventriculus mucosal epithelium with mononuclear cell infiltration into the lamina properia; Cecal tonsil hemorrhages, and lymphoid depletion; tracheal mucosa epitheliums necrotized with loss of cilia and interstitial pneumonia. Bursa of fabricates showed lymphocyte necrosis, and cystic structure and inter-follicular epithelial hyperplasia. Heart showed myocarditis, and myocardial necrosis. Kidneys showed swelling, pale necrotic areas and microscopic lesions include hemorrhages into the interstitium and renal tubular epithelial cell necrosis. Brain grossly showed encephalitis and hemorrhage, microscopically excessive lymphocytes in and around brain capillaries and multi-focal hemorrhages. Based on findings, it could be concluded that Newcastle disease is causing death of chickens, reduced egg production and causes economic loss in the study area. Prevention

works both at farm level and by regulatory body should continuously be done to reduce the loss. The specific viral strain causing the outbreak should be studied and documented.

Keywords: *Bishoftu, Newcastle Disease, Pathology, Poultry*

1. INTRODUCTION

The worldwide poultry population is predicted to be at 16.2 billion, with 71.6 percent living in developing countries, producing 6.7 million tons of chicken meat and 5.8 million tons of hen eggs (Sime, 2022). Poultry, particularly chickens, is the world's most extensively kept and numerous livestock species (Nyoni *et al*., 2019). Poultry production contributes significantly to the livelihoods of farmers and to the national economic system (Assefa, 2019).

Poultry production contributes significantly to the livelihoods of farmers and to the national economic system (Assefa, 2019). Ethiopia's poultry sector can be divided into village or backyard, small scale and commercial poultry production systems (Bush, 2006). Backyard poultry production is the predominant system in Ethiopia and account for nearly 99% of the poultry population consisting mainly of local chicken breeds under individual farm household management and it is also common to find a few exotic breeds distributed through the extension programs (Wong *et al*., 2017).

Small scale poultry production system comprises a flock size ranging from 50 to 500 exotic breed operation commercial bases and outdoor with a low biosecurity level. Small-scale poultry (SSP) production systems have been integrated with human livelihoods for thousands of years, enhancing diet, income, and food and nutrition security of the rural poor (Lizin *et al.,* 2022). It is mostly found in rural, resource-poor areas that often also experience food insecurity (Alem, 2014, Asfaw *et al*., 2021).The Commercial poultry production system is a highly intensive production system that involves greater than 10,000 birds kept in door and heavily depends on imported breeds (Bush, 2006).

The most common cause of the high mortality rates observed in small-scale poultry (SSP) flocks, particularly in tropical countries, is Newcastle disease (ND) (Wong *et al*., 2017). ND is caused by the Newcastle disease virus (NDV) also known as avian *paramyxovirus-1* (AMPV-1) ND is among the serious viral diseases of poultry and virulent strains can cause up to 100% mortality annually (Samal, 2011). It is a highly contagious disease of domestic and wild birds worldwide (Joshi *et al.,* 2021, Nyoni *et al*., 2019).

In Ethiopia, ND, locally known as fengle, was first reported in 1971 from a small poultry farm in Asmera (the then part of Ethiopia), and spread to the country along transport routes (Molia *et al*., 2011). Outbreaks of the disease were recorded in Addis Ababa in 1972, Harer, Shola, and Bishoftu poultry farms in 1974 (NVI, 1974). ND is endemic in the village chicken population in Ethiopia and it is the major constraint of poultry production (Jarso, 2015).

A number of studies have been conducted to determine the prevalence of ND in different agro-ecology and season of Ethiopia. Mortality may be very high, often reaching 50 to 100 % (Jarso, 2015). The reported apparent seroprevalence of the disease ranged from 5.6% (Belayneh *et al.,* 2014) to 38.8% (Mulualem, 2017). ND outbreak was reported 205 times from 2011 up to 2015. Among this, many reports from Oromia region followed by Amhara, Addis Ababa, SNNP, Benishangul gumuz and Tigray region by percentage 74.6%, 11.7%, 10.5%, 6.3%, 3.9%, 2.9%, respectively but the rest regions have not report of ND outbreak (Gelana, 2017).

Newcastle disease virus (NDV) is identified as a major killer, largely contributing to economic losses for the poultry sector in Ethiopia, and it is usually the first disease suspected during disease outbreaks. In Ethiopia, the application of biosecurity measures is in small-scale poultry farms are limited where most small scale poultry farms located around Bishoftu town in the Oromia region and Addis Ababa (Dawit *et al*., 2011).

Hence, there could be a spillage effect in which small scale farms with poor biosecurity and management can serve as source of infection for the growing commercial poultry farms and village chickens in the study area. The presence and severity of lesions in organs due to ND virus-infected birds depends on the type of infectious virus, the host and other factors that influence disease severity (Suarez *et al*., 2020). A confirmation of the disease and a better understanding of the clinical signs, gross and histopathological changes associated with ND, as well as an understanding of individual risk factors, would aid in preventing disease transmission and formulating effective control strategies. Therefore, the current study describes the common clinical signs, gross and, microscopic lesions and molecular detection of NDV virus.

Objectives

- To isolate and detect (Molecular) Newcastle disease virus from clinical cases and those that have contact with clinical cases
- To characterize gross and microscopic lesions from clinical cases of Newcastle disease
- To assess biosecurity practice in farms with clinical diseases

2. LITERATURE REVIEW

2.1. Etiology

Newcastle disease (ND) is caused by virulent strains of avian *paramyxovirus type 1* (APMV-1), also known as Newcastle disease virus (NDV), of the genus Orthoavulavirus belonging to the family Paramyxoviridae. At present, there are 21 serotypes of avian paramyxoviruses designated APMV-1 to APMV-21(OIE, 2021). Strains of NDV that are of low virulence (loNDV) are often used as vaccines to prevent disease and death from ND (OIE, 2020).

The virions are pleomorphic in shape, and consist of single stranded, non-segmented, negative-sense RNA genomes. NDV virions are 100 nm or more in diameter, pleomorphic, but mostly spherical (Sohail *et al*., 2010). 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 spike-like projections of 8 nm lengths on the outer surface of the envelope (Samal, 2011, Waheed *et al*., 2013).

Based on the severity of the APMV-1 viruses in chickens, they have been divided into three or more pathotypes. The least virulent strains are lentogenic, followed by mesogenic and velogenic strains, which are all fairly pathogenic (Cattoli *et al*., 2011). The majorities of strains fall between the two virulence extremes and are either lentogenic or velogenic. Others believe these are lentogenic viruses, whereas some writers also identify an "asymptomatic enteric" category, Velogenic viruses may be split into two categories: strains that create a viscerotropic form with bleeding intestinal lesions, known as the viscerotropic form, and strains that generate a neurotropic form, generally linked with respiratory and neurological signs. These clinical types can overlap and aren't always distinct from one another (Miller and Koch, 2013).

Although a single system has recently been proposed, two distinct classification techniques have been utilized to categorize APMV-1 genotypes for epidemiological reasons. An APMV-1 isolate can have more than one label as a result of this. Both the unified system and one system divide APMV-1 isolates into the class I and class II clades. The genotypes of each of these groups are further broken down (Alhabeeb *et al*., 2013). Class II of APMV-1 strain, which includes both

extremely virulent and nonpathogenic variants contains the great majority of APMV-1 strains. Class I isolates are typically of low pathogenicity and are mostly found in marketplaces selling live birds and wild ducks. Some virulent APMV-1 genotypes are particularly significant, as they have spread widely and have been identified as possible panzootic viruses (Spickler, 2016).

2.2. Clinical Signs of Newcastle disease

Clinical signs are dependent on factors such as the virus strain, host species, age of the host, and route of infection, co-infection with other microorganisms, environmental stress, and immune status of the bird (Alhabeeb *et al*., 2013). Young birds have more severe and acute disease in comparison to older birds (Getabalew *et al.,* 2019). The clinical signs in chickens are recognizable at second day of the onset of the disease, 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 (Wakamatsu *et al*., 2006, Susta *et al*., 2011).

Incubation periods for APMV-1 infections in poultry can last anywhere between 2 and 15 days, while they typically last between 2 and 6 days in chickens infected with velogenic isolates. Depending on the bird species and the isolate's virulence, APMV-1 viruses can produce a variety of clinical signs (Cattoli *et al*., 2011). Lentogenic strains typically induce moderate respiratory diseases in chickens that manifest as coughing, gasping, sneezing, and rales. In chickens, illnesses brought on by mesogenic strains may be more severe. Although there may be neurological indications, decreased egg production, and respiratory signs, the mortality rate is often modest. If the flock is infected with other diseases in addition to lentogenic or mesogenic ND virus, the illness may be more severe (Hailu, 2012).

Although the clinical signs of velogenic strains can vary greatly, they produce severe, frequently deadly diseases in chickens. Lethargy, inappetence, ruffled feathers, and conjunctival reddening and edema are some of the early signs. Some birds experience enlargement of the tissues in the head and neck, respiratory signs (including cyanosis), and watery, greenish, or white diarrhea (Hadipour *et al.,* 2011). Egg production frequently experiences a sharp fall, and eggs may

become irregularly shaped, discolored, have rough or thin shells, or have watery albumen. It is also common to encounter sudden death with minimal or no clinical warning signals (Mohammed, 2018).

Torticollis, circling, paresis or paralysis of the wings and/or legs, and other neurological signs are frequent in some epidemics. Although CNS signs might appear simultaneously with other signs of sickness, they are typically detected later in the disease progression and the birds may appear bright and alert (Oladele *et al.,* 2005). The neurological health and/or egg production of any remaining chickens may be permanently compromised. Sometimes, flocks that have received vaccinations show clinical signs brought on by velogenic APMV-1 viruses; however these signs may not be as severe (Samal, 2011).

Other birds have comparable clinical signs; certain species may be more likely to exhibit neurological or respiratory signs than others. Although ND in turkeys is typically less severe than in hens, some strains can still result in serious illness. Game birds can occasionally get severe illnesses (Pansota *et al*., 2013). Neurological signs, diarrhea and/or respiratory signs, as well as nonspecific signs of illness, have been reported in pheasants. Guinea fowl may develop clinical signs, but they can also carry velogenic isolates sub-clinically. Ostriches and emus typically exhibit more respiratory signs than chickens; however these birds are typically less severely afflicted (Susta *et al*., 2011). Even with velogenic APMV-1 strains, geese and ducks often have subclinical infections; while there have been reports of clinical cases or epidemics. Reported clinical signs in waterfowl include nonspecific signs such as anorexia, neurological signs, diarrhea, ocular and nasal discharges, decreased egg production, and sudden death (Sahlu *et al*., 2015).

Acute ND outbreaks can result in the rapid death of flock members after an overnight incubation period with a particularly virulent strain of the virus. In poultry flocks, ND can occasionally only manifest as death (Susta *et al*., 2011). Temperature increase, drowsiness, thirst, anorexia, a wobbly stride, head shaking, and, in the final stages, paralysis of the wings and legs are frequently noted. In addition, birds develop lameness, drooping wings, lose their ability to fly, circle about, and elderly birds may molt (Abel, 2018).

2.3. Pathology

The ND virus strains differ significantly in their pathogenicity from host to host and/or within a host. Although turkeys, ducks, and geese can contract the disease and exhibit little to no clinical signs, even with strains that are fatal to chickens (Khalifeh *et al.,* 2009), chickens are the most susceptible species. However, the doses, mode of administration, age of the chicken and environmental factors all have an impact (Bello *et al*., 2018). In chickens, the pathogenicity of NDV is mostly determined by the strain of the virus. The disease generally becomes more severe the younger the chicken is. Younger chickens may die suddenly and without significant clinical signs when virulent viruses are present in the environment, whereas older birds may have a longer illness with recognizable clinical signs. Breed or genetic stock appears to have very little effect on the susceptibility of chickens to the disease (Fikre, 2003).

2.3.1. Pathogenicity

Natural routes of infection like nasal, oral and ocular appear to emphasize the respiratory nature of the disease while intramuscular, intravenous and intra cerebral routes appear to enhance the neurological (Khalifeh *et al.,* 2009). Molecular basis of the pathogenicity of ND virus is dependent on the post translation cleavage of fusion protein, for the progeny virus particles to be infective. The presence of additional basic amino acids in virulent strain fusion protein means that cleavage can be effected by proteases present in a wide range of cell types in different host tissues and organs For lentogenic viruses, cleavage can occur only with proteases recognising a single arginine (Brown *et al.,* 1999). Lentogenic viruses, therefore replicate only in cells where there are trypsin like enzymes, such as the respiratory and intestinal epithelia, whereas virulent viruses can replicate in cells located in a wide range of tissues and organs, resulting in a fatal systemic infection (Fikre, 2003, Bello *et al*., 2018). Accordingly, various pathological conditions of ND are discussed as follows.

2.3.2. Gross and Histopathological lesions

Velogenic Viscerotropic Newcastle Disease

Respiratory organs: On gross inspection the trachea demonstrates catarrhal tracheitis. The caudal pharynx and tracheal mucosa can occasionally show congestion or hemorrhages, and the oropharynx and trachea may develop diphtheritic membranes (Samour, 2014). The trachea exhibits edema, bleeding, mononuclear inflammatory cell infiltration, and congestion under a microscope (Saif *et al.,* 2008). Secondary bacterial infection is a serious problem that can result in thickened air sacs with catarrhal or caseous exudates. Within days of the onset of infection the respiratory tract's cilia can completely disappear. Lymphocyte and macrophage infiltration is frequent in the upper respiratory tract mucosa in the early stages of infection, associated with congestion and edema (Cattoli *et al*., 2011).

Circulatory organs: Gross myocardial degeneration is visible in the heart. It is associated with pericarditis in layer chicken. Microscopic examination of the heart reveals epicarditis and myocarditis, which are characterized by myocardial degeneration, necrosis, edema, and mononuclear cell infiltration (Etriwati *et al.,* 2017). Vascular disruption causes thrombosis in small vessels, necrosis of endothelial cells, hydropic degeneration of tunica media, hyalinization of capillaries and arterioles, and hemorrhages in addition to congestion, edema, and hemorrhages (Cappelle *et al.,* 2015).

Digestive organs: Catarrhal proventriculitis common and only samples from domestic chickens show hemorrhagic proventriculitis (Saif *et al*., 2008). Microscopic observation revealed proventriculitis, marked by desquamation of proventriculus epithelial surface and epithelial glands, hyperemia of the muscularis layer and infiltration of inflammatory cells to the submucosal layer of proventriculus glands (Miller *et al*., 2010). The cecal tonsils and lymphoid tissues of the intestinal wall (including Peyer's patches) frequently show hemorrhages, ulcers, edema, and/or necrosis; this lesion is particularly indicative of ND. Microscopic analysis of the cecal tonsil revealed bleeding, infiltration of inflammatory cells, and necrosis of the epithelial mucosal cells (Samour, 2014).

Domestic chicken livers exhibit degeneration and multifocal necrosis, whereas layer chicken livers exhibit perihepatitis (Sahlu *et al*., 2015). Degenerative alterations in the lymphopoietic system include regions of necrosis in the spleen, the loss of lymphoid tissue, and hyperplasia of mononuclear phagocytic cells in several organs, including the liver (Saif *et al.,* 2008).

Multifocal necrotic pancreatitis was common in the pancreas. When seen under a microscope, the acinar cells have inflammatory cell buildup and multifocal bleeding. Layer chickens had more severe liver and pancreatic lesions on macroscopic and microscopic examination than broiler and domestic chickens, which had milder lesions (Etriwati *et al*., 2017).

The distinction between VVND viruses and NVND viruses has been made using the presence of hemorrhagic lesions in the gut of infected chickens. Multifocal necrotic pancreatitis was widespread throughout the gastrointestinal system. The acinar cells exhibit multifocal hemorrhage and an accumulation of inflammatory cells beneath the microscope. On the macroscopic and microscopic level, layer chickens exhibited more severe liver and pancreatic lesions than broiler chickens, which had more moderate lesions. Virulent ND virus infections result in hemorrhages and necrosis of mucosal lymphoid tissue (Miller *et al*., 2010).

Urinary organs: Grossly the kidney may have nephritis, marked by swelling and fragility with multinecrotic foci. Microscopic analysis revealed interstitial bleeding, tubular epithelial cell necrosis, and inflammatory cell infiltration (Spickler, 2016).

Reproductive organs: Laying birds with VVND infection may have hemorrhaging of reproductive organs, including the ovarian follicles, and egg yolk in the abdominal cavity, as well as flaccid and degenerative ovarian follicles. Ovarian hemorrhages may be present, and the organs are frequently edematous. Egg yolk peritonitis affects chickens and turkeys infected with velogenic viruses during incubation (Cattoli *et al*., 2011).

Other: Petechiae of the wattle and combs and facial edema are commonly seen during infection VVND (Khalifeh *et al*., 2009). The head or periorbital region may be swollen, and the interstitial tissue of the neck can be edematous, especially near the thoracic inlet (Cappelle *et al*., 2015).

Velogenic Neurotropic Newcastle Disease

Although animals killed in the early stages of the illness may exhibit splenic or proventricular congestion, gross lesions are frequently absent and the involvement of the visceral organs seems to be minor. The central nervous system is the primary site of microscopic histopathologic alterations in chickens infected with VNND strains (Miller *et al*., 2010).

On gross examination, the brain shows encephalitis with microscopic lesion showing hyperemia, perivascular cuffing, edema, gliosis (Etriwati *et al.*, 2017). Neurologic lesions comprise 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 but rarely in the cerebrum (Samour, 2014)

Mesogenic Newcastle Disease

Gross lesions are minimal with mesogenic strains. Chickens infected with mesogenic strains had mild splenomegaly and some degree of conjunctivitis when inoculated via eye-drop instillation. In the field, infection with mesogenic strains is often associated with secondary bacterial infections (Alexander, 2004).

A range of histological lesions are seen with mesogenic strains. The more virulent strains, those that cause a notable degree of clinical disease, consist mainly of non-suppurative encephalitis that has many similarities to the lesions caused by the VNND strains (i.e., perivascular cuffing and gliosis) (Susta *et al*., 2011).

Lentogenic Newcastle Disease

Grossly, lentogenic strains produce mild pulmonary hemorrhages and splenomegaly. Lentogenic strains of NDV have been isolated together with E. coli, and gross lesions consisted mainly of tracheal hemorrhages. When the same NDV isolate was experimentally inoculated into SPF chickens, no gross lesions were detected (Tang *et al.,* 2012).

Microscopic lesions of these strains are hyperplasia of the lymphoid follicles in spleen and air sacs, and proliferation of lymphoid follicles mainly in the lamina propria of the trachea. Aerosol delivery of the virus causes congestion, goblet cell hyperplasia, edema, and multifocal sub mucosal infiltration of scattered heterophils, lymphocytes, and plasma cells (Jordan *et al.,* 2005).

2.4. Epidemiology of Newcastle disease

2.4.1. Transmission and source of infection

Viral infection can occur through ingestion, inhalation, or conjunctival contact. The severity of the infection can range from asymptomatic, with minimal mortality, to severe illness, with 100% fatality. Even though young chickens are more sensitive and exhibit signs earlier than older ones, virulent NDV-infected birds may pass away without exhibiting any clinical signs of sickness (Ashraf and Shah 2014). Human agents are most likely the source of the village's greater ND spread. An outbreak of ND is unpredictable and discourage villager from paying proper attention to the husbandry and welfare of their chickens (Gelana, 2017).

The capacity of feral birds to infiltrate affected flocks and spread the disease will be significantly reduced in nations where poultry are maintained completely in bird proof housing, whereas birds kept on open range are more prone to contract strains carried by feral birds (Mazengia, 2012).When the temperature is just above freezing (1-2°C) the virus has been shown to survive on chicken skin for up to 160 days and in bone marrow for nearly 200 days (Carvallo *et al*., 2018). Some APMV-1 isolates can also be transmitted through the egg to hatching chicks. Although not common, egg-associated transmission of very virulent isolates is feasible because, unless the virus titer in the egg is low, the embryo normally perishes. Cracked or broken eggs as well as eggshells contaminated with feces are further sources of infection for newly hatched chicks. APMV-1 may be mechanically transmitted by flies (Spickler, 2016).

APMV-1 is easily spread via insects. In comparison to an inorganic surface (filter paper), survival is prolonged on eggshells and particularly in feces. Published data on the persistence of these viruses varies widely, likely as a result of the fact that many variables, including humidity, temperature, the suspending agent, exposure to light, and the method used to detect the viruses, can have an impact (Li *et al*., 2009). According to one study, APMV-1 could survive in

contaminated, uncleaned poultry houses for up to 7 days in the summer, up to 14 days in the spring, and up to 30 days in the winter. A different group reported virus isolation up to 16 days after the depopulation of an unvaccinated flock (Munir *et al*., 2012).

2.4.2. Host range

Host infected NDV infects a wide range of domestic and wild bird species worldwide. More commonly affected species include chickens, turkeys, ducks, pigeons, guinea fowl, Japanese quail and many wild birds of all ages. The most susceptible avian species to this disease are chickens and also some mammals like humans, cats and dogs (Zhang *et al.,* 2011). Class I NDVs are occasionally isolated from wild aquatic birds and domestic poultry and are mostly avirulent to chickens. 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 (Tang *et al.,* 2012).

2.4.3. Global distribution of Newcastle disease

Since its appearance in Newcastle, the disease has spread quickly throughout the world. At present, ND is present in all continents: Europe, Asia, Africa, and America. The continuous changes in the distribution of ND require the World Organization for Animal Health to provide weekly information on the disease and produce a biannual map of the situation of World Animal Health (Ashraf and Shah 2014).

2.5. Diagnosis of the Newcastle disease

Diagnosis should not be based on pathognomonic lesions or clinical signs because these types of signs and lesions are not specific to any strain of NDV (Getabalew *et al*., 2019). Some lesions may be seen with infection of low virulent strains, and signs may be similar to those seen with more virulent strains (Oladele *et al*., 2005).

History, clinical signs, and laboratory tests can all be used to diagnose ND. Due to the clinical similarities between ND and highly virulent avian influenza, an early and precise diagnosis is

crucial to contain an epidemic and stop the spread of the disease (Sohail *et al*., 2010). Viral isolation, serological tests, molecular tests (RT-PCR, and PCR), and virus isolation are all used in the laboratory to diagnose ND. Hemagglutination inhibition testing is the primary method for identifying ND. A definite diagnosis of ND is the isolation of the ND virus (Gelana, 2017).

2.5.1. Immunohistochemistry examination

Studies on antigen distribution are crucial in observing the presence of NDV within tissue because ND lesions are frequently mistaken for other poultry diseases. This makes it difficult to tell whether tissue lesions are caused by NDV infection or not. An explanation of the pathogenesis of ND in field cases can be provided by immunohistochemistry staining. An antibody that reacts to a particular antigen is used in immunohistochemistry staining to localize the antigen within the affected tissue, which improves the accuracy of a diagnosis by allowing the identification of antigens within tissues (Ramos-Vara *et al.,* 2014).

2.5.2. Serological technique

Hemagglutination inhibition test

The hemagglutination 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 hemagglutinin on the viral envelope can bring about the clumping of red blood cells chicken and that this can be inhibited by specific antibodies (Rauw *et al*., 2009, Shabbir *et al.*, 2012). 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 (OIE, 2020).

Enzyme linked immune sorbent 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 (Hadipour *et al*., 2011). 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. Viewing the plate or quantitatively using a spectrophotometer can read this. Serial dilution of the anti-NDV antibody test serum can be used to determine the titer (Sahlu *et al.,* 2015).

2.5.3. 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 (Hailu, 2012).

2.5.4. Molecular technique

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 ND (Abdisa and Tagesu, 2017).The duplex PCR is done based on principle that it has the ability to amplify and differentiate multiple specific nucleic acids using polymerase enzymes. 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. However, PCR requires DNA as a template and the target viruses in this study have RNA as their nucleic acid (Shaheen *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, monogenic and lentogenic strains from chickens (Samal, 2011). It is applied simultaneously that required for avian infection including ND for amplification and quantification of the virus. The primers that are specific for each virus are newly designated from the nucleoprotein gene of NDV. 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 (Tang *et al.,* 2012).

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 that is negative and single stranded RNA virus (Saif *et al*., 2008).

2.6. Differential diagnosis of Newcastle disease

During epidemiological diagnosis in the field and even after necropsy, misdiagnosis may occur and ND may be confused with several other pathologies because there are no pathognomonic signs to the disease. For this reason, the diseases with which ND shares the same signs are eliminated (OIE. 2013). The differential diagnosis for ND includes other causes of septicemia, enteritis, respiratory disease, and/or neurological disease (Abdisa and Tagesu 2017).

The clinical signs and the evolution of the signs caused by virulent strains may strongly resemble those of many diseases such as: Highly Pathogenic Avian Influenza, Infectious Bronchitis, Avian Cholera, Acute Infectious Laryngotracheitis, Diphtheria, Avian Smallpox, Septicaemic Infections, Acute Poisoning or mismanagement (deprivation of water, air, food). In pigeons it can be confused with salmonellosis or ornithosis. Despite the differential diagnosis, laboratory analyses are necessary for a definitive and accurate diagnosis (Terregino and Capua 2013).

2.7. Prevention and control of Newcastle disease

Newcastle disease may be prevented in chicken flocks with good biosecurity. Any pet birds, wild or feral birds, and farmed poultry with uncertain health status shouldn't be permitted to come into contact with flocks. Worker interaction with birds outside the farm should be avoided wherever feasible (Mohammed, 2018). A few biosecurity precautions include bird-proofing buildings, securing feed and water sources, limiting access to the site, and cleaning any entering vehicles and agricultural equipment. Additionally, pests like mice and insects need to be managed. Employees should take a shower and change into work-appropriate attire if at all feasible. Breeding that is all in or all out is also advised, as is disinfecting between groups (Desvaux *et al.,* 2011).

In areas where velogenic viruses are prevalent in chicken, vaccinations are frequently employed. Although certain viruses can spread and/or be sustained in some vaccinated flocks, vaccination can protect birds against clinical signs and may reduce viral shedding and transmission (Mazengia, 2012). There have been some questions regarding whether the currently accessible vaccinations properly protect birds against distantly similar APMV-1 genotypes, regardless of the fact that other variables have been involved in the field (Muhammadamin and Qubih, 2010). Hitchener's B1 (HB1) and La Sota, which are made at the National Veterinary Institute (NVI), Bishoftu, are the most widely used vaccines in Ethiopia. These vaccinations are administered in large quantities through spray, aerosol, or drinking water (Getabalew *et al.,* 2019).

Due to heat stress and water shortage, steroids are produced, which suppresses the immune system (Rauw *et al.,* 2009). Birds with impaired immune systems are also more likely to have ND, which has a high incidence and prevalence in chicks. A further risk factor for ND is a feed's lack of vital nutrients. High stocking densities, overcrowding, and intensive poultry production cause immunity to break down and are associated with severe ND outbreaks (Rasamoelina *et al.,* 2012). Immunological inhibited birds' immunological responses to ND are improved when immune booster items are used. Minerals, vitamin E, and selenium may be added to the meal to strengthen the birds' immune systems (Desvaux *et al*., 2011).

ND outbreaks can be stopped by quarantines, mobility restrictions, the depopulation of all exposed and sick birds, and a complete cleaning and disinfection of the area (Prosser *et al*., 2011). Farms must typically go without livestock for a few weeks before replenishing; the exact amount of time depends on the season, climate, and other variables. Governmental authorities may gather and analyze birds that pass away unexpectedly in any facility during various eradication campaigns. In order to identify new cases, this measure may be useful (Getabalew *et al.,* 2019).

2.8. Economic importance of Newcastle Disease

Despite the establishment of regular immunization programs, ND continues to be a sporadic epizootic, which poses a serious threat to the country. Low growth and production rates, high

costs for prevention and treatment, and a high death rate are the effects of the illness. The disease may spread quickly, and ND virus (NDV) infection, which is known to have infected more than 200 bird species, can cause 100% morbidity and mortality (Abah *et al.,* 2016).

The lentogenic type's causes broilers to lose weight more slowly, have more mortality, and are more likely to be condemned. Wherever commercial chickens are farmed, whether in developing or rich nations, outbreaks have happened often, resulting in enormous economic losses through trade losses and control attempts. For instance, in California during the most recent significant epidemic in the country in 2002–2003, more than 2,500 locations were depopulated (4 million birds) at a cost of US$162 million (Kryger *et al*., 2010).

2.8.1. Seasonality of the Disease

During the brief wet season, especially in April (66.8%) and May (31.4%), chicken mortality owing to illness breakout is greater. The NCD is one of the main infectious illnesses harming the production and survival of chicken in the central highlands of Ethiopia, according to another paper (Dzogbema *et al.,* 2021). ND was shown to be most widespread mostly during the rainy season, according to a different research from Benishangul-Gumuz, Western Ethiopia, conducted by (Alemayehu *et al.,* 2015). During the rainy season, chickens are more susceptible to illness due to a lack of supplemental nutrients (Mohammed, 2018).

2.9. Newcastle diseases in Ethiopia

Newcastle disease is the most significant source of economic losses in Ethiopian chicken farming. The disease is known by many local names, the most frequent of which is "Yedoro Fengle" (Chaka *et al.,* 2012). Studies in Ethiopia have confirmed the presence of both velogenic and lentogenic causing upto100% mortality (Munir *et al*., 2012, Chaka *et al*., 2013, Fentie *et al*., 2014). It has become endemic in the village and commercial poultry population and recurs every year, which imposes heavy economic losses (Dessie and Jobre 2004).

To ascertain the frequency of ND in various Ethiopian agro-ecologies and seasons, several investigations have been carried out. In most regions of Ethiopia, it was cited as one of the most significant illness issues with chickens. Mortality rates might be quite high, frequently ranging

from 50% to 100% (Jarso, 2015). From 5.6% (Belayneh *et al.,* 2014) to 38.8% (Mulualem, 2017), the disease's apparent seroprevalence has been observed. From 2011 to 2015, 205 ND epidemic reports were made. Among them, there have been several complaints from the Oromia region, which is followed by the Amhara, Addis Abeba, SNNP, Benishangul-Gumuz, and Tigray regions by percentages of 74.6%, 11.7%, 10.5%, 6.3%, 3.9%, and 2.9%, respectively. However, there have been no reports from the remaining areas (Gelana, 2017).

3. MATERIALS AND METHODS

3.1. Study area

This study was carried out at Bishoftu Town, East Shewa Zone of Oromia Regional State. Bishoftu is located 45 kilometers southeast of Addis Ababa, at 9°N latitude and 40°E longitude and at an altitude of 1850 meters above sea level. The average annual rainfall is 1,150 mm, while the maximum and minimum temperature is 28.6°C and 12.9°C, respectively. It has a short rainy season from March to April and a long rainy season from June to September (CSA, 2009).

3.2. Study population

The study was conducted in 53 small scale (<500 chicken) poultry farms that have had outbreaks. The chickens kept by the farmers were Sasso, Cob500 and Bovans Brown breeds. Chickens showing clinical signs of ND and those which didn't show clinical signs but were in contact with the sick chickens were sampled.

3.3. Study design

A cross sectional study design was conducted to isolate and detect of NDV by cell culture and reverse transcriptase PCR, postmortem, examination, followed by histopathological examination and questionnaire in selected areas of Bishoftu town Ada'a District central Ethiopia (November 2022 to May 2023).

3.4. Sample size and sampling technique

A cross sectional study with purposive sampling method was used. From 53 small scale farms that had ND outbreak chickens showing clinical signs and apparently healthy chickens that have had contact with those that manifested ND clinical sign were sampled. Veterinarians working at agricultural offices and those that have veterinary drug vendors were pre-assigned to inform the occurrence of outbreak. Accordingly, Poultry disease outbreaks were reported from 7 kebeles (county) in Bishoftu Town. Which are from Dembi (9), Filtuu (9), from Qurqura, Kata, Gorba,

Calalaka and Dire Jitu 7 small scale poultry farms were investigated and from each farm 5 chicken were sampled. In this study, a total of 265 chickens under small scale poultry farm systems were involved. Among these, 195 were clinically sick and 70 apparently healthy Chickens of all age group, sex, breed and types (layers and broilers) were included as a study animal.

3.5. Sample collections

From a total of 265 chickens, 120 tracheal swabs (24 pool of five), 25cloacal swabs (5 pool of five), 40 brain tissue (8 pool of five), 40 cecal tonsils (8 pool of five), 40 proventriculus samples (8 pool of five).Thus, 53 pooled samples were collected in separate cryovial containing 2 ml from live and from euthanized chickens which were moribund and show clinical signs of ND. Tracheal and cloacal swabs 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. Additionally, tissue samples from the brain, proventriculus and cecal tonsil were placed in sterile separate cryovial containing 2 ml of freshly prepared viral transport media placed in ice pack for transportation and stored at -80C until processing.

Sick chickens were closely examined for general physical appearance including the gait and any nervous signs, the feces (Droppings), and presence of nasal or respiratory discharges were evaluated and recorded. Selected chicken with clinical ND were euthanized (Cervical dislocation) and any visible gross pathological changes/lesions in terms of organ color, consistency, shape, size and texture were recorded carefully according to the standard operational protocol in chicken necropsy (Corrie *et al*., 2008) (Annex II).

120 chickens showing suggestive clinical signs of ND were euthanized and sampled for histopathology. Representative tissue specimens from the brain, proventriculus, cecal tonsil, liver, intestine, spleen, lung, bursa, kidney and trachea were pooled and collected immediately by using bottle containing 10% formalin for histopathology examination. Additionally, representative tissue specimens from the same chickens were sampled for viral isolation and detection.

After sampling, the specimens both for histopathology and molecular procedures were labeled, and transported to the molecular and pathology laboratories at AHI Sebeta, Ethiopia. Sample collection and transportation were conducted according to the standard techniques recommended by OIE, (2004).

3.6. Sample Processing

3.6.1. Histopathological examination

A systematic post mortem examination was done on 120 chickens during the study period; gross lesions observed were recorded. Representative tissue specimens for histopathology were obtained from liver, spleen, cecal tonsils, lungs, trachea, segments of intestines, proventriculus, kidney, bursa and the brain and immediately fixed in 10% buffered formalin. Formalin fixed tissue samples were trimmed and processed in automated tissue processor following the protocol of Takulder, (2007) (Annex III).

In brief, after adequate fixation, the tissues were trimmed to about 1-2 mm thickness and dehydrated in ascending grades of ethyl alcohols (70, 95 and 100%), then cleared in xylene and embedded with melted paraffin wax (Melting point 58°C). After this the paraffin block was prepared and then the tissue was sectioned at about 5micrometer thickness by using semi-automatic microtome machine. The tissue ribbons were made to float on the warm water bath and collected on clean microscopic slides. The tissues were deparaffinized in xylene, rehydrated by passing through descending grades of ethyl alcohol (100, 95 and 70%) and finally stained by routine haematoxylin and eosin stain (H and E). The stained tissues were dehydrated in ascending grades of ethyl alcohols (70, 95 and 100%), cleared by xylene and the slides were mounted by canada balsam. After allowed to dry well, the slides were examined under microscope starting from the lower (10x) to higher magnification (100x) (Annex IV).

3.6.2. Sample preparation and viral isolation

The swab is vortexed, tissues were chopped and macerated well in the transport medium used for sample collection. The resulting suspension is then transferred to a centrifuge tube and centrifuged at 3000rpm for 10 minutes using refrigerated centrifuge. The supernatant is collected,

filtered using 0.45um Millipore syringe filters and ready to be inoculated in to the DF1cell lines or stored at – 20 °c until inoculation (Annex VI).

Cell culture preparation

Immortalized chicken embryo fibroblast cells (DF-1, passage 32) obtained from Deventer, Netherlands, were revived from liquid nitrogen and re-cultured in $25cm^2$ tissue culture flask. The confluent flask was then sub cultured to multiple $25cm^2$ TC flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2% Glutamax and 1% Antibiotic-Antimycotic solution at 37^oC in a humidified incubator at 5% CO^2. According to the number of samples to be isolated, cells were sub-cultured to 24-wells tissue culture plates until they become 80-90% confluent (Annex VI).

Virus isolation

The sample suspensions prepared and stored at -20^oC were thawed and 0.1ml of the samples inoculated in to each subconfluent DF-1 cell cultures in 24-wells. After 60min adsorption at 37^oC, maintenance medium is added to each well including two uninoculated wells as negative control and incubated at 37^oC in a humidified incubator at 5% CO^2. Cells were monitored every 24 h post-infection and inspected for cytopathic effects (CPEs) using an inverted microscope. On 5th day, the cultures were freeze-thawed and the resulting lysate was inoculated into fresh cultures until the third passage. Samples that did not show a cytopathic effect was continued up to the third passage (Annex VI).

3.6.3. Molecular detection

RNA extraction

Viral RNA extraction from 53 pools of tracheal, cloacal swabs and tissue pools were conducted using QIAamp viral RNA extraction kit according to manufacturer's instruction. The sample was centrifuged briefly in order to get cell free supernatant. The supernatant was lysed by adding 560

µl of prepared buffer AVL containing carrier RNA in to 1.5 micro centrifuge tube and 140 µl of sample was added to the buffer AVL carrier RNA in the microcentrifuge tube. The solutions were then mixed by pulse-vortexing and incubated at room temperature (15- 25°C) for 10 minutes. The tube was then briefly centrifuged to remove drops from inside the lid. Then equal 560µl of 96% ethanol was added to filtrate and mixed thoroughly and washed with 500µl washing 1 and 2 (500 µl buffer AW 1 and 500 µl AW 2), any unwanted protein and DNA were removed. Then, 60 µl of elute solution (AVE) was added to collect the RNA. Finally, the eluted RNA was kept at-20°C (QIAGEN Inc., Gaithersburg, MD, USA) (Annex VII).

Real-Time Polymerase Chain Reaction (RT-PCR)

After RNA was extracted the Master Mix preparations were carried out. The reaction mixes were prepared by pipetting all the components of the Master Mixture in to 2 ml PCR tube according to the kit protocol. The reaction components were template RNA, primer solutions, dNTP Mix and 5x QIAGEN One-step RT-PCR buffer, RNase-free water, probe and enzyme mix. Total RNA was extracted by scraping cells with RLT buffer and isolated according to manufacturers' instructions using the RNeasy Mini Kit (QIAGEN). The controls used were known isolate of NDV and RNase free water as positive and negative controls, respectively. Then Applied Bio-systems 7500 Fast Real-time PCR thermo cycler were used for amplification of the extracted RNA. A primer probe combination from a conserved region of the M gene APMVI F M+4100 5'-AGT GAT GTG CTC GGA CCT TC-3', APMV-I R M- 4220 5'-CCT GAG GAG AGG CATTTG CTA-3'and Probe APMV-1M+4169 5'-FAM TTCTCT AGC AGT GGG ACA GCC TGC TAMRA -3' was used to amplify all NDV samples (QIAGEN, Inc., Gaithersburg, MD, USA) (Annex VIII).

3.7. Questionnaire survey

Data on biosecurity practices were collected from farms with clinical disease while sampling for swab and tissue collection using semi structured questioner. fifty three poultry owners were interviewed to assess the owners educational level, experience of keeping chicken (year), isolation practice of sick chickens, disposal of dead carcass, regular vaccination of chickens, use

of foot bath, source of replacement practice and flock size, presences of nearby farm, contact of attendants with nearby farms and age in month (Annex V).

3.8. Data management and Analysis

Data obtained from questionnaire, laboratory test (RT PCR) and pathological lesions were stored into Microsoft Excel for Windows 2010. Analyses were performed using STATA software version 14. The data was analysed using simple descriptive statistics.

3.9. Ethical clearance

Ethical clearance was received (certificate Ref. No VM /ERC/03/01/15/2023) from Animal Research Ethics Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture. Chicken were handled according to the animal handling guidelines set by Animal Research Ethics Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture

4. RESULTS

4.1. Clinical findings

In the present study, total of 195 chickens showed clinical signs and 120 of them were confirmed to be ND (RT-PCR). Nervous, respiratory and enteric signs indicative of ND were observed in 26.47%, 41.18% and 32.35% of cases respectively (Fig. 1). Nervous signs observed were mild to severe depression with drooping wings, paralysis of leg and wings, and torticollis. Respiratory signs exhibited were labored breathing, increased rales, wheezing, and open-mouthed breathing with watery/tenacious mucus discharge from the nostrils. Greenish-white diarrhea (pasty vent) was the main enteric sign. Adult hens exhibited decreased egg production and eggs with watery albumin and thin shells.

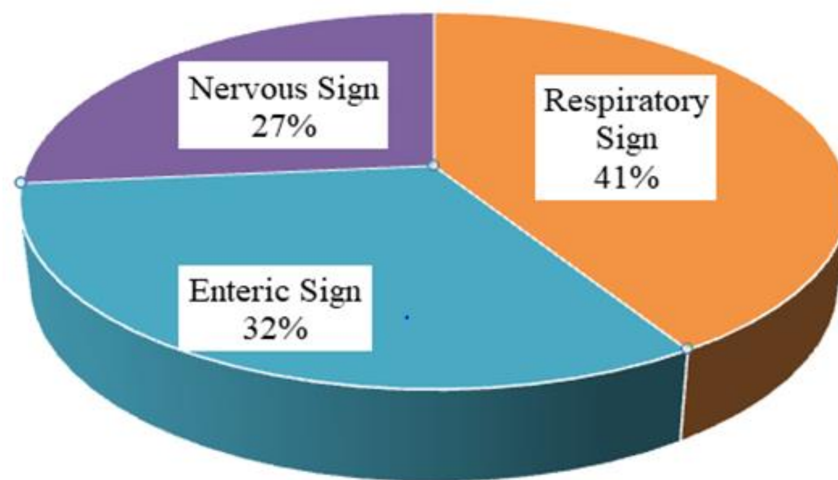


Figure 1: Category of observed clinical signs of Newcastle disease in percentage (N=120)

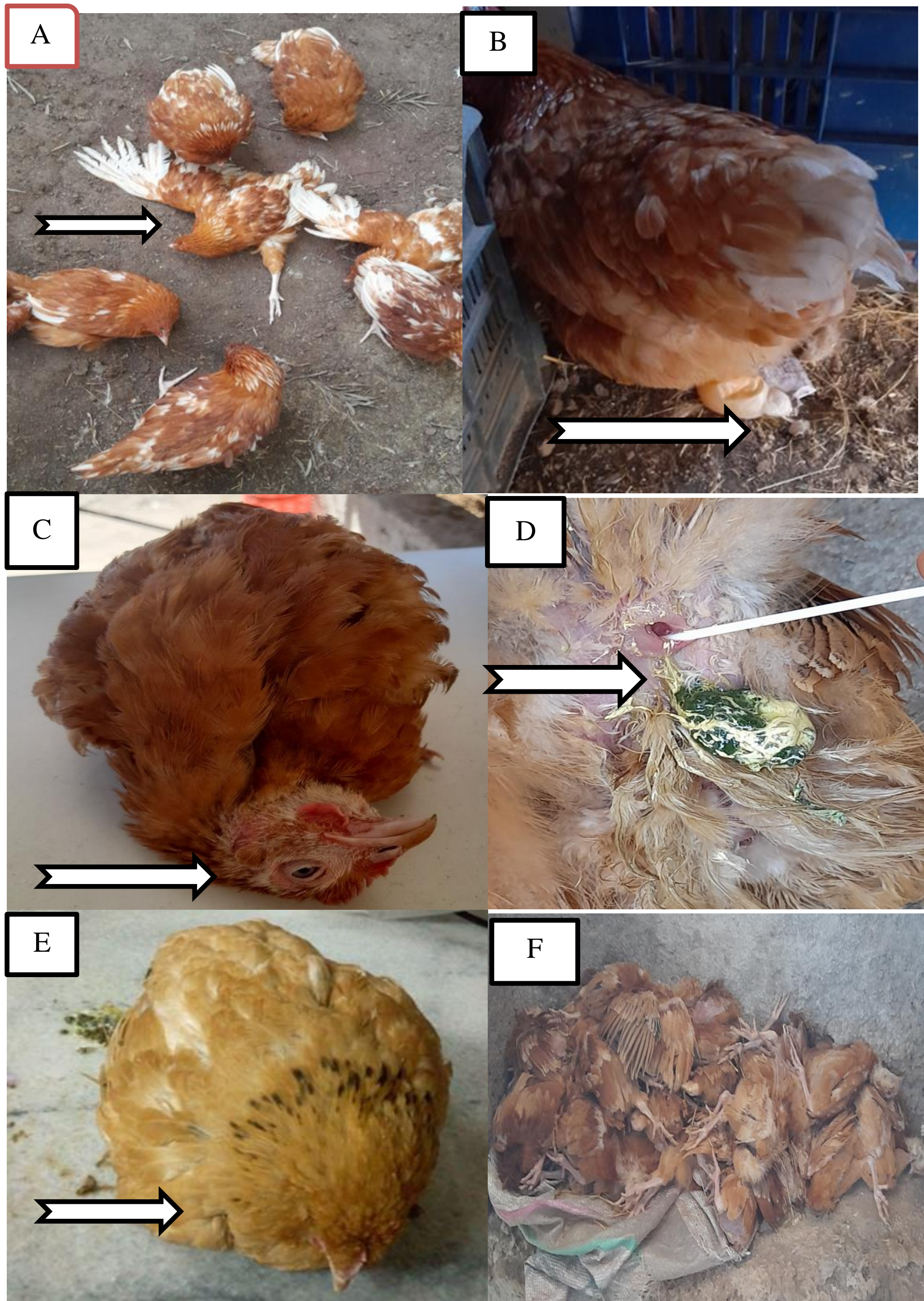


Figure 2: Clinical signs of chickens infected by ND, **(A)** Paralysis of wings and legs **(B)** Watery egg albumin and thin egg shell, **(C)** paralysis of the neck (torticollis), **(D)** Green diarrhea with pasting of vent, **(E)** Depression and **(F)** Dead chickens thrown in the same compound

4.2.Pathological findings

Gross lesion of trachea and lung

Sixty four percent of the lungs that were NDV positive showed black spot pigmentation, hemorrhages, Congestion and edema were among the gross lesions (Fig. 3 A & B). Sixty percent of tracheal showed diffuse hemorrhages (Fig. 3 C & D) and mucoid exudates in the lumen.

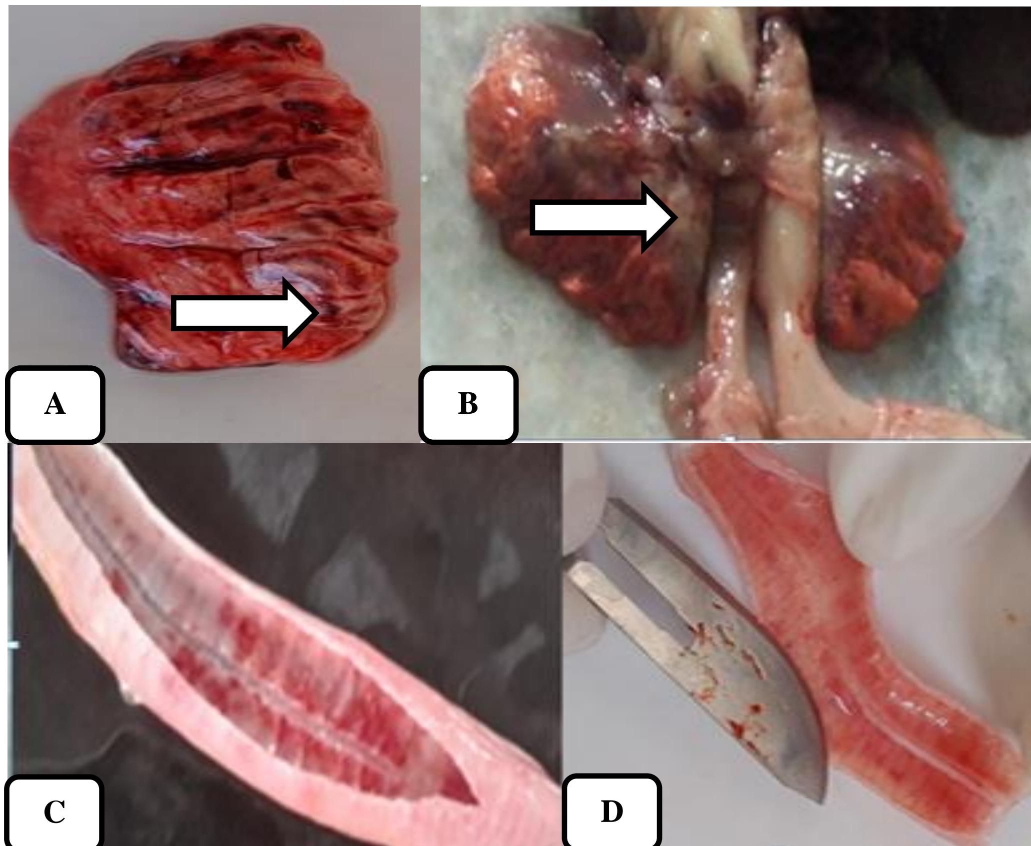
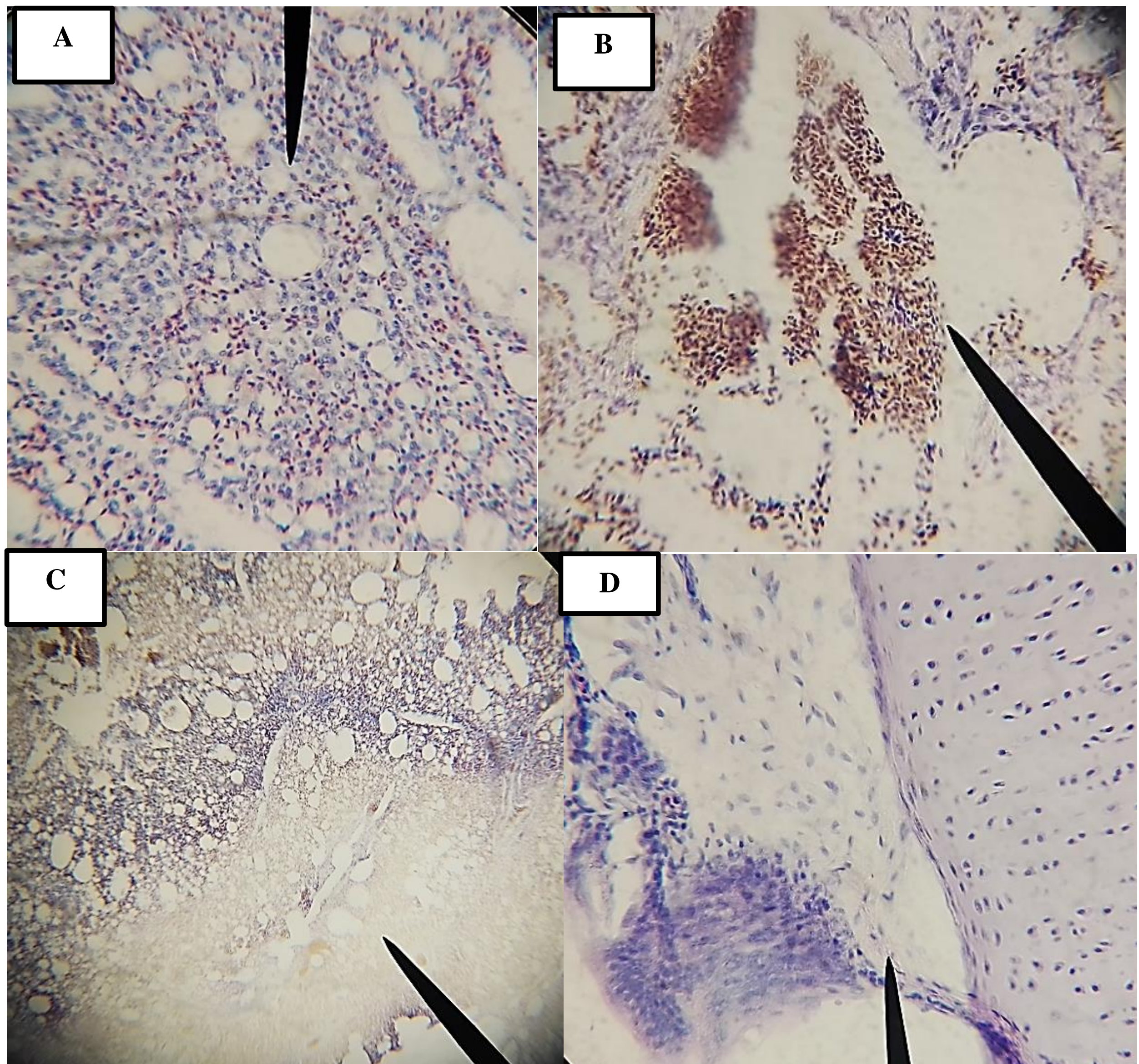


Figure 3: Various gross pathological lesions found in lung and trachea. **(A)** Hemorrhagic lung **(B)** severely hemorrhagic lung with black pigmentation **(C)** localized hemorrhages on tracheal mucosa**, (D)** Severe acute and diffuse hemorrhagic trachea.

Microscopic lesion of trachea and lung

Microscopically, the trachea showed mononuclear inflammatory cell infiltration into the lamina propria, detachment of epithelium from basement membrane. The lungs showed interstitial pneumonia with mononuclear inflammatory cells infiltration into the interstitium. There were extensive thickening of the interstitium and large area of lung tissue necrosis with surrounding mononuclear inflammatory infiltration. In some lungs there were sever hemorrhage (Fig. 4).



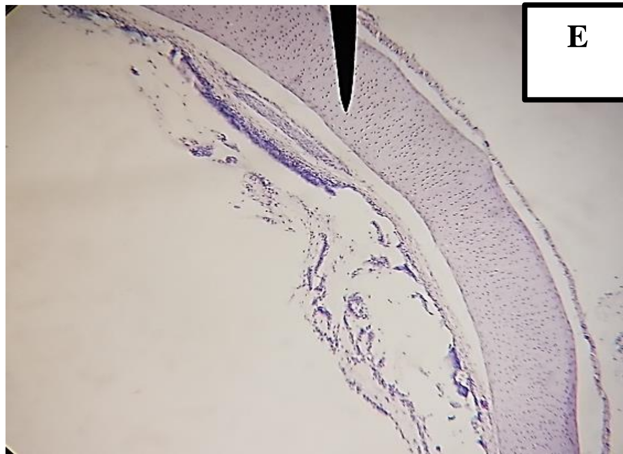


Figure 4: Microscopic lesions of lung and trachea**. (A)** Interstitial pneumonia with mononuclear inflammatory infiltration into the interstitium and extensive thickening of the interstitium, **(B)** sever hemorrhage, **(C)** large area of lung tissue necrosis with surrounding mononuclear inflammatory infiltration **(D)** trachea with detaching of lamina propria and mononuclear inflammatory cell infiltration and **(E)** detachment of epithelium from basement membrane (microscopic arrows).

Gross and microscopic lesion in digestive organs

Seventy one percent of sampled proventriculus show petechial hemorrhage and other showed severe diffuse hemorrhagic proventriculitis (Fig. 5).

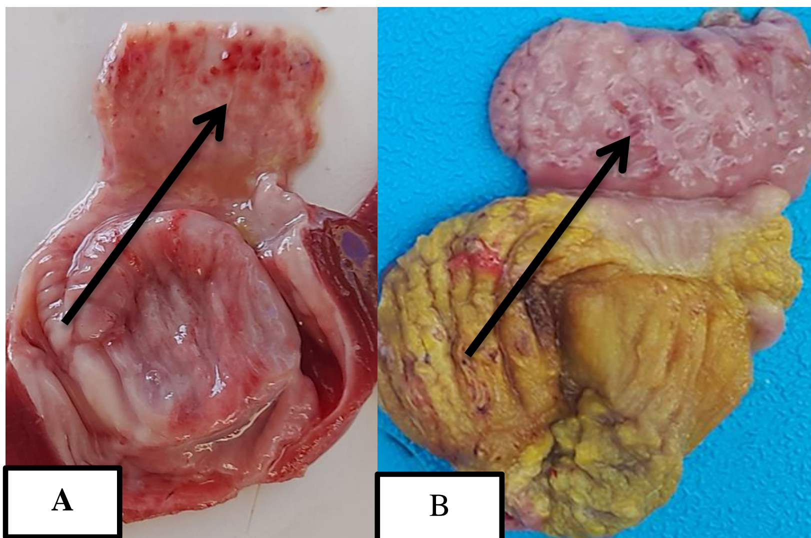


Figure 5: Gross pathological lesions in proventriculus. **(A)** Acute severe diffuse hemorrhage proventriculitis. **(B)** Petechial hemorrhagic proventriculus (arrows).

Microscopically, there were proventriculitis, marked by necrosis and desquamation of periventricular mucosal epithelium and proventricular gland epithelial or the secretory cells. In some there was a total loss of glandular epithelium leaving only lamina proprial connective tissue There were hyperemia and hemorrhage in the muscularis layer and infiltration of inflammatory cells into the sub-mucosal layer of proventriculus glands (Fig. 6).

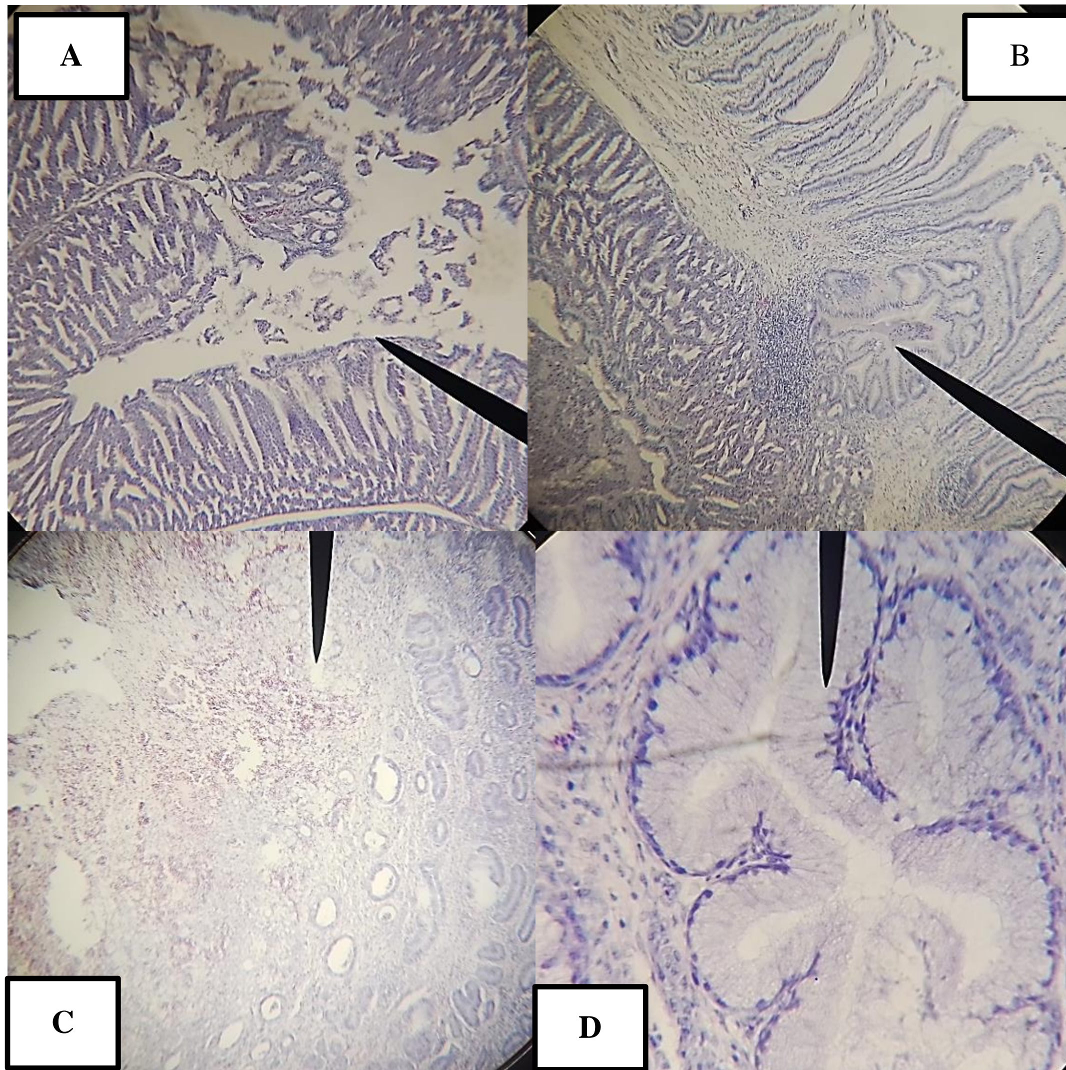


Figure 6: Microscopic lesions of proventriculus**.** **(A)** epithelial necrosis and sloughing off, **(B)** periventricular glands are short and collapsed the epithelium are necrotized in between the collapsed epithelium, **(C)** widespread hemorrhage and **(D)** proventricular gland epithelial are necrotized and lost leaving only the basement membrane (microscope arrows)

Eighty five percent of samples of intestine showed hemorrhagic lesions grossly.

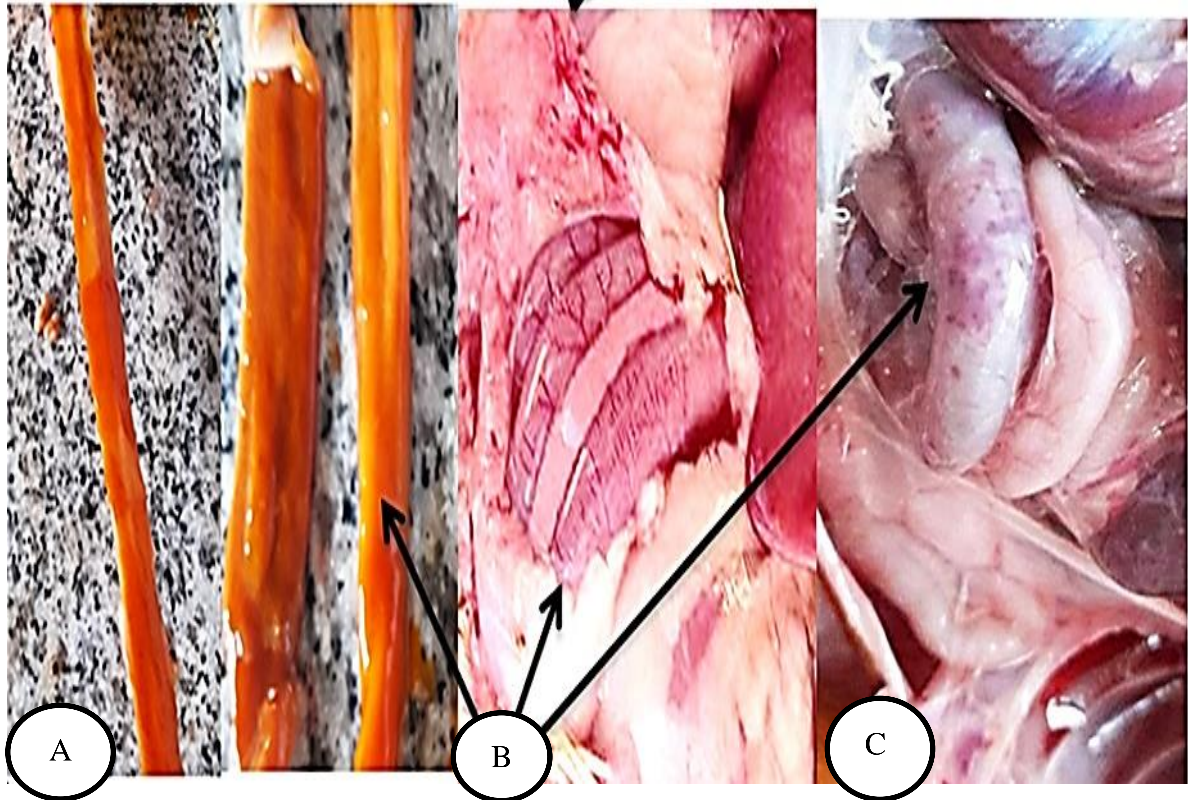


Figure 7: Gross lesions in the intestine. **(A & B)** severely hemorrhagic duodenum and, **(C)** pin point hemorrhage

Microscopically, segments of intestines were similarly characterized by presence of sloughed mucosal epithelium, moderate to severe infiltration of leucocytes which is mostly mononuclear cells and goblet cell hyperplasia (Fig. 8)

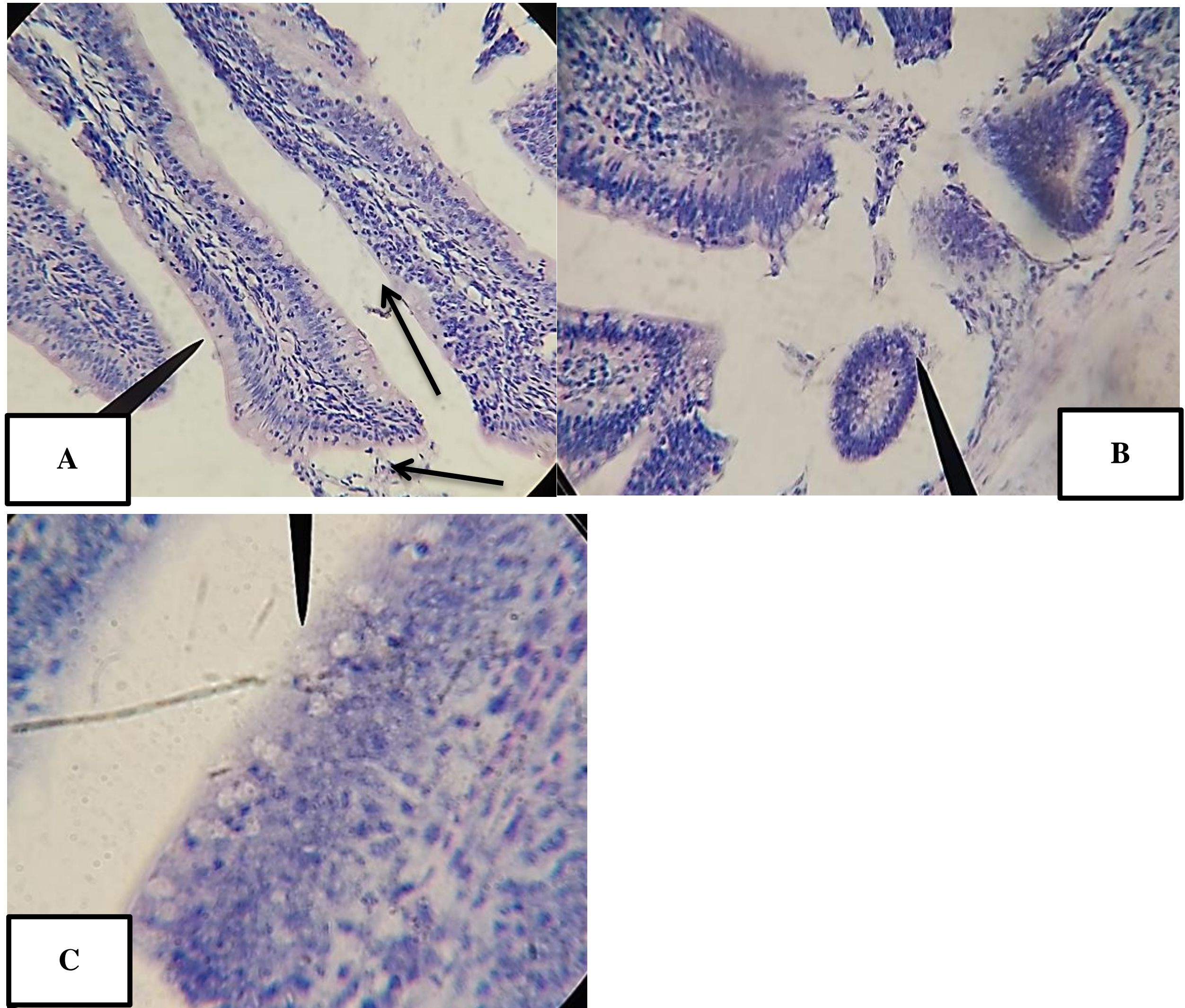


Figure 8: Microscopic lesions in small intestine**. (A)** Normal epithelium villi and microvilli on some part (microscope arrow) but necrosis of epithelium on other part sloughed (arrow), **(B)** sever epithelial necrosis including the crypts (microscope arrow), **(C)** Goblet cell hyperplasia and inflammatory cells infiltration into the lamina propria, **(D)** The necrotized epithelium are sloughed into the lumen

Of the sampled liver, 41% grossly showed sever diffuse areas of paleness which indicates hepatic necrosis. There were also areas with wide spread hemorrhages. Microscopically the hepatocytes were necrotized; lymphocytes and macrophages infiltration, were infiltrated into necrotized region hemorrhage and necrosis were observed (Fig.9)

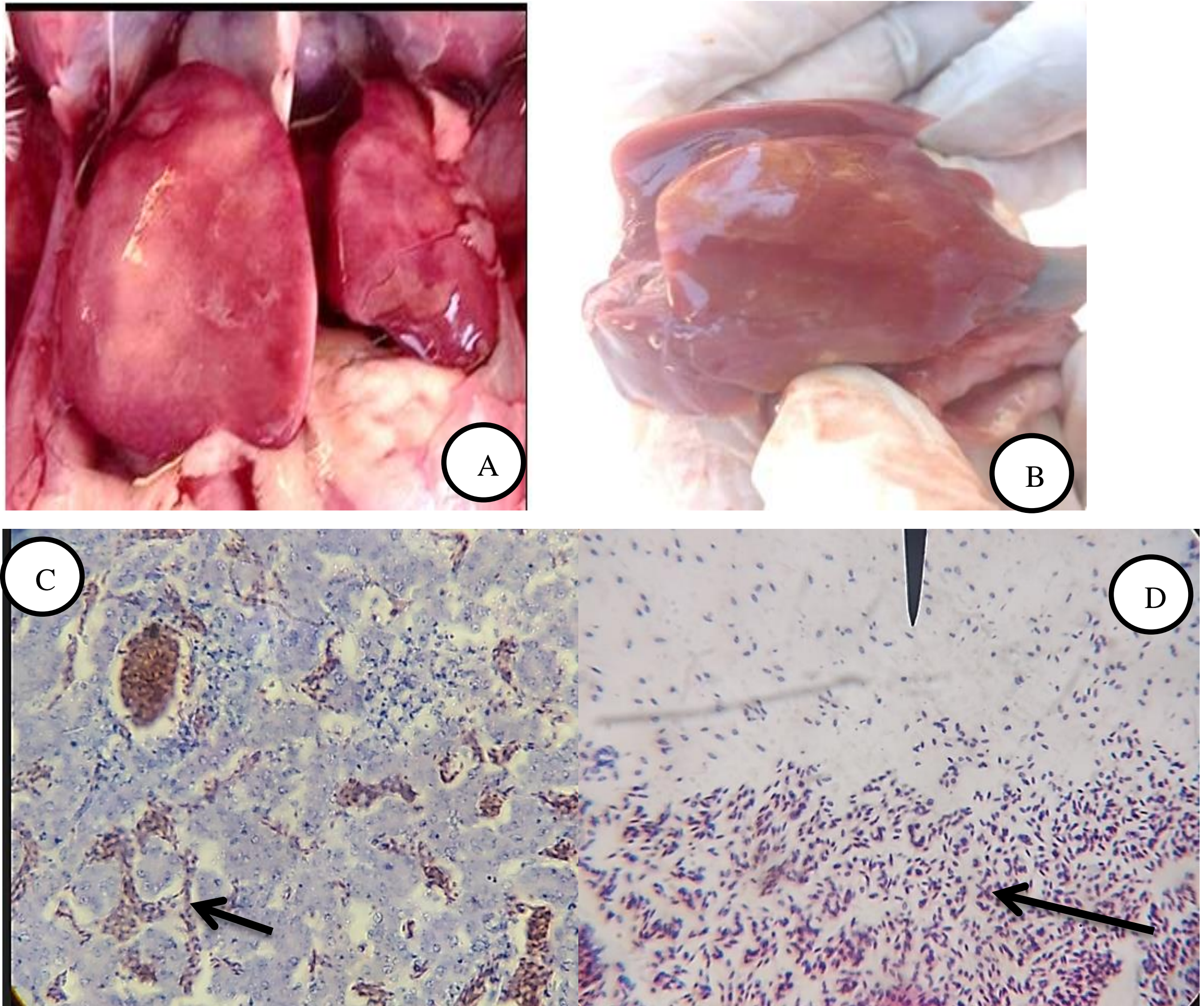


Figure 9: Gross and microscopic lesions in liver. **(A)** congestion **(B)** diffused pale necrotic foci, **(C)** Severe multiple hemorrhages, **(D)** Locally extensive hepato necrosis with infiltration of inflammatory cells into the area (microscope arrow) and massive hemorrhage just next to necrotic areas (drawn arrows)

Gross and microscopic lesion in lymphoid organs

Of cecal tonsil, 75% showed severe hemorrhages and were enlarged /swollen in size. Microscopic lesion of cecal tonsil includes necrosis of lymphoid cells, and hemorrhage (Fig. 10)**.**

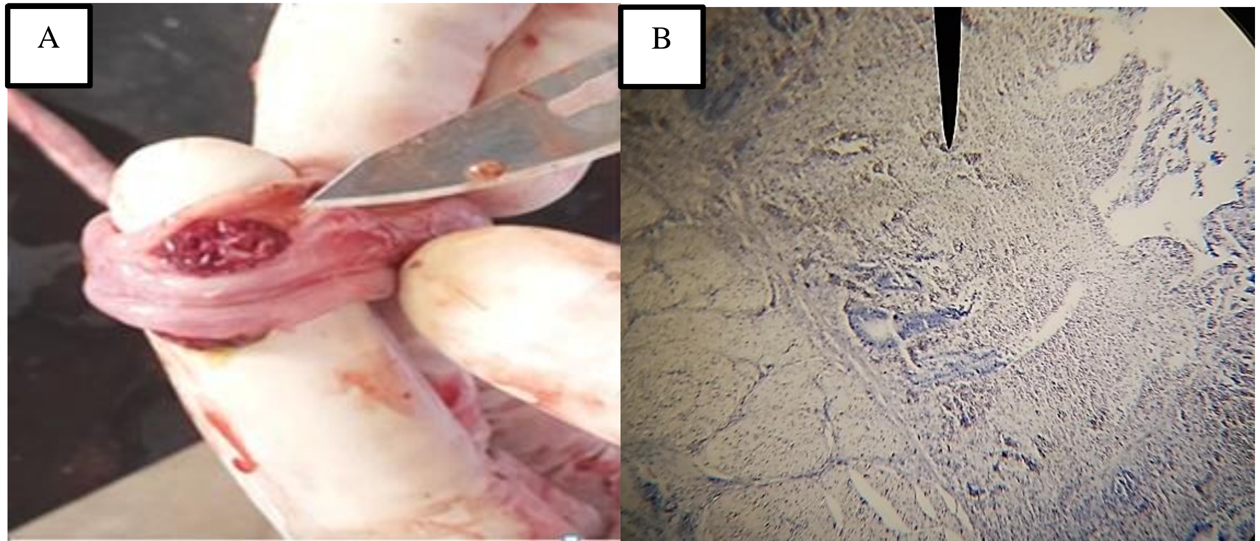


Figure 10: Gross and microscopic lesions of cecal tonsil. **(A)** Swollen and hemorrhagic cecal tonsils**, (B)** Sever necrosis of the lymphocytes (microscope arrow)

Of the sampled spleens, 33% showed gross lesions that include congestion, swollen, and hemorrhagic. Microscopic lesions include severe necrosis of lymphoid follicles and hemorrhage (Fig. 11).

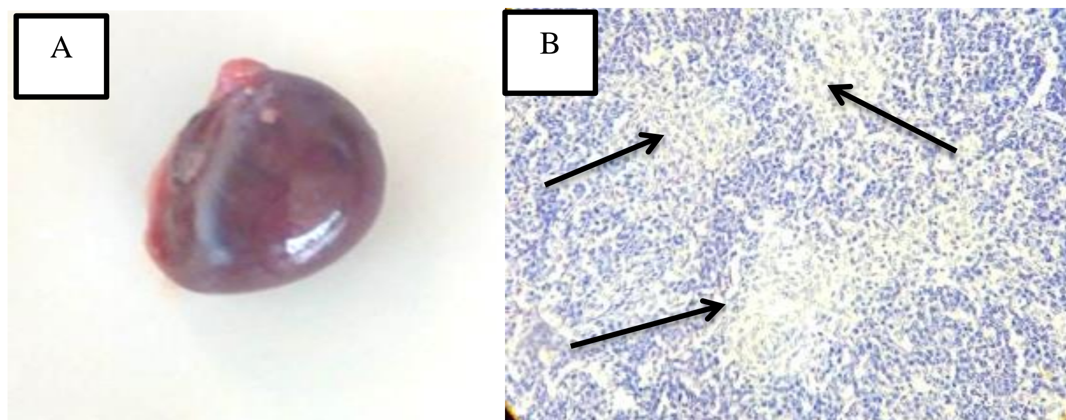


Figure 11: Gross and microscopic lesions in the spleen. **(A)** Hemorrhagic and enlarged spleen and **(B)** severe multifocal necrosis of the lymphoid follicles (arrows)

Bursa of fabricius: The common gross bursa lesions were swellings and hemorrhages. But in 5% of collected bursa there were atrophies of the bursa. The microscopic lesions of bursa were sever necrosis of lymphocyte giving a cystic structure that contain necrotic debris There were infiltration of hetrophils into the interfollicular connective tissue. (Fig. 12).

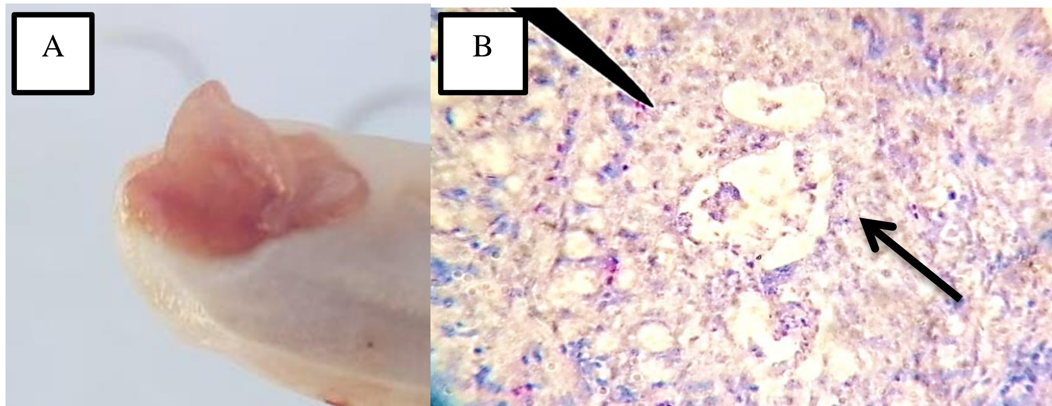


Figure 12: Gross and microscopic lesions in the bursa of fabricius. **(A)** Hemorrhagic bursa s and **(B)** depletion of lymphoid follicle and cyst formation (drawn arrow)

Of sampled heart, 21% grossly showed gray to white small nodules mainly around and over apex of the heart. Microscopic heart lesions include myocarditis marked by myocardial degeneration, and necrosis, with mononuclear cell infiltration (Fig. 13).

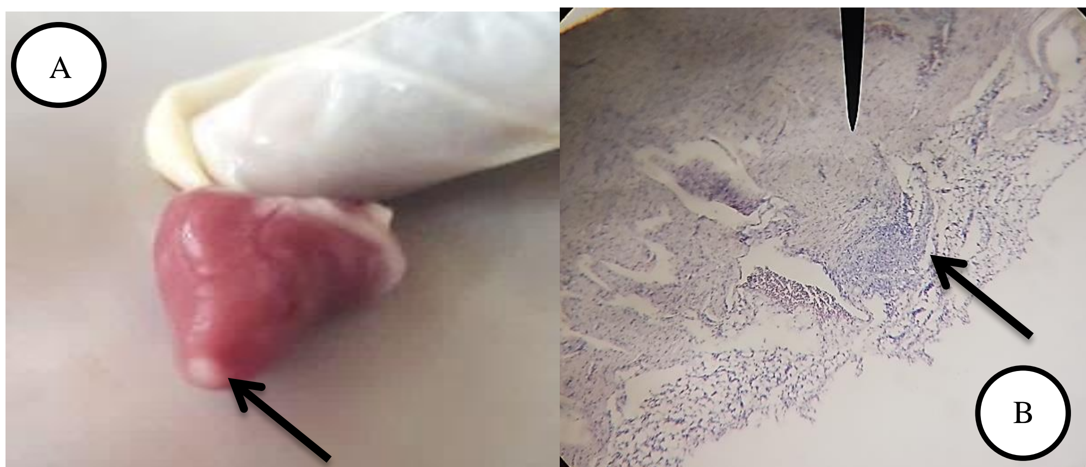


Figure 13: Gross and microscopic lesions in the heart **(A)** Gross pale nodule found on the apex of heart and, **(B)** sever necrosis of the myocardium (microscope arrow) with heavy infiltration of inflammatory ells mainly lymphocytes (drawn arrow)

Of the sampled kidneys, 24% grossly showed marked swelling, fragile and show multifoci necrotic pale areas. Microscopic lesions include widely distributed hemorrhages in the interstitium, sever tubular epithelial cell necrosis including necrosis of the basement membrane in many tubules and inflammatory cell infiltration in the interstitium (Fig. 14)**.**

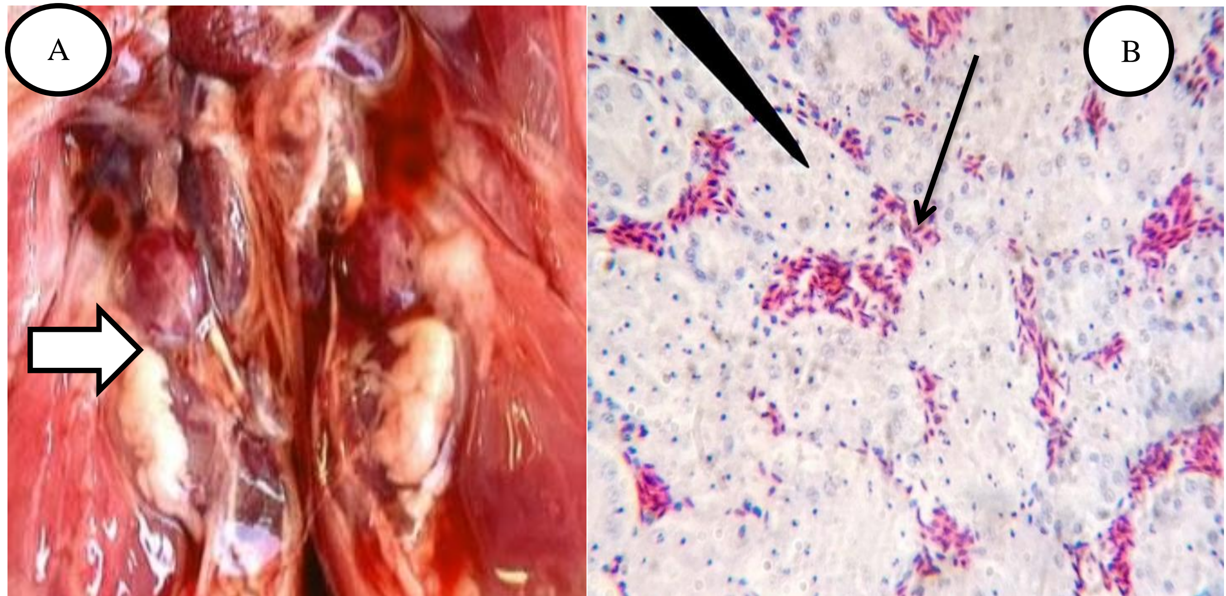


Figure 14: Gross and microscopic lesions of kidney **(A)** Swollen kidney with multiple necrotic foci and hemorrhage, **(B)** Total necrosis of the renal tubular epithelium (microscope arrow) and hemorrhage into interstitial area (drawn arrow)

Of the sampled brain, 38% the brain grossly suffered encephalitis and hemorrhage in the cerebellum (Fig. 15 A). Results of the microscopic examination showed that there was excessive lymphocyte in the brain capillaries and multi focal hemorrhages (Fig. 15)

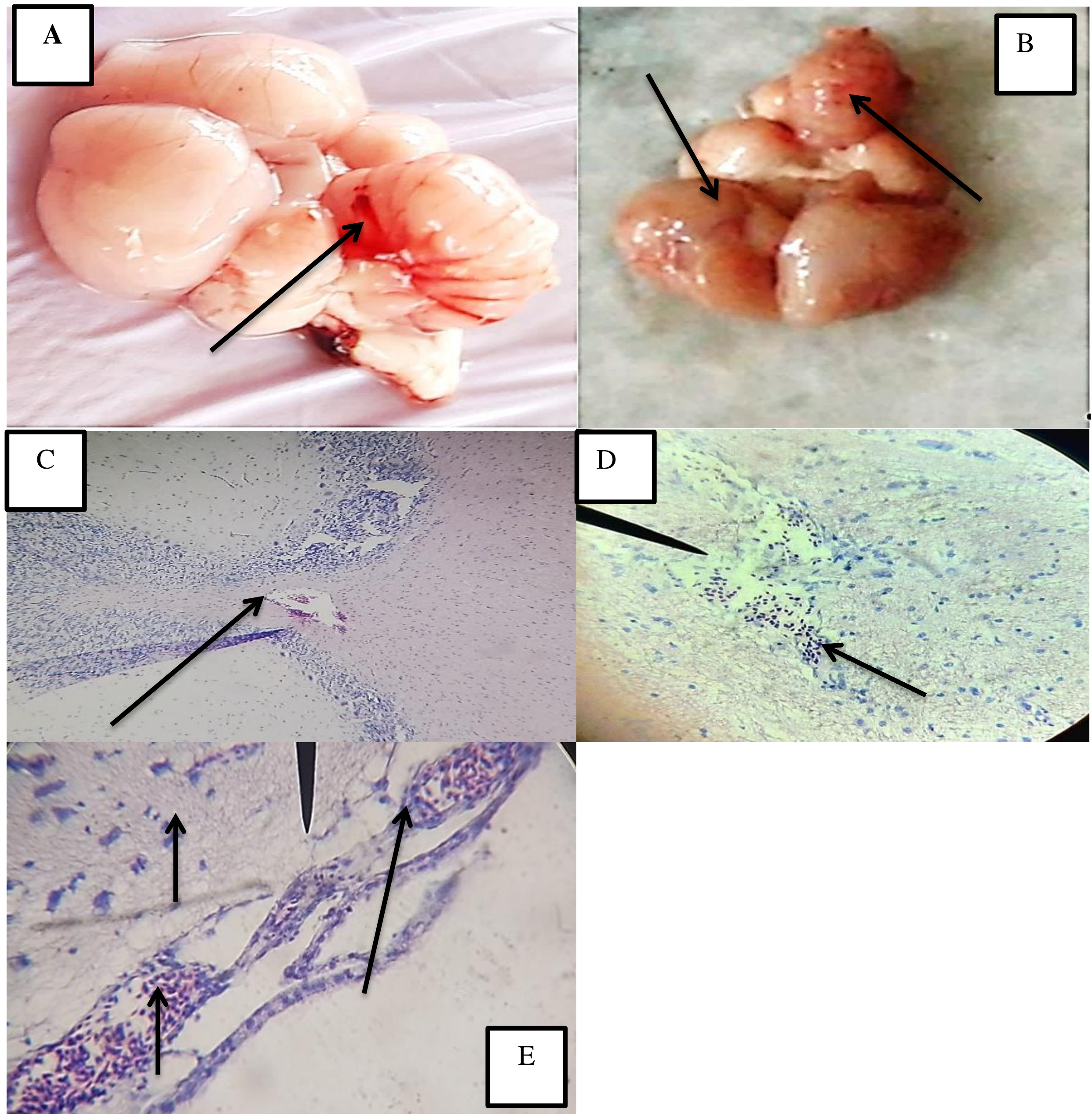


Figure 15: Gross and microscopic lesions of brain**. (A)** Hemorrhage in the cerebellum, **(B)** hemorrhage on wide parts of the brain**, (C& D)** heavy lymphocytic infiltration into brain **(E)** Meningitis with inflammatory cells infiltration. Meningeal capillaries are dilated and are full of RBC and**,** brains just to meninges contain lymphocytic infiltration (drawn arrow).

4.3. Molecular findings

4.3.1. Isolation of Newcastle Disease Virus

Out of the 24 pooled (120 individual) positive samples by RT–PCR, 12 pools (1 pooled brain, 2 pooled cecal tonsil, 8 trachial swab and 1 cloacal swab samples) were cultured and isolated on DF-1 cell lines. Viral multiplication was evident by development of visible cytopathic effect of different degree on DF-1 cell lines which was characterized by cell swelling, rounding, detachment &cells floating and Vacuolation (Fig. 16).

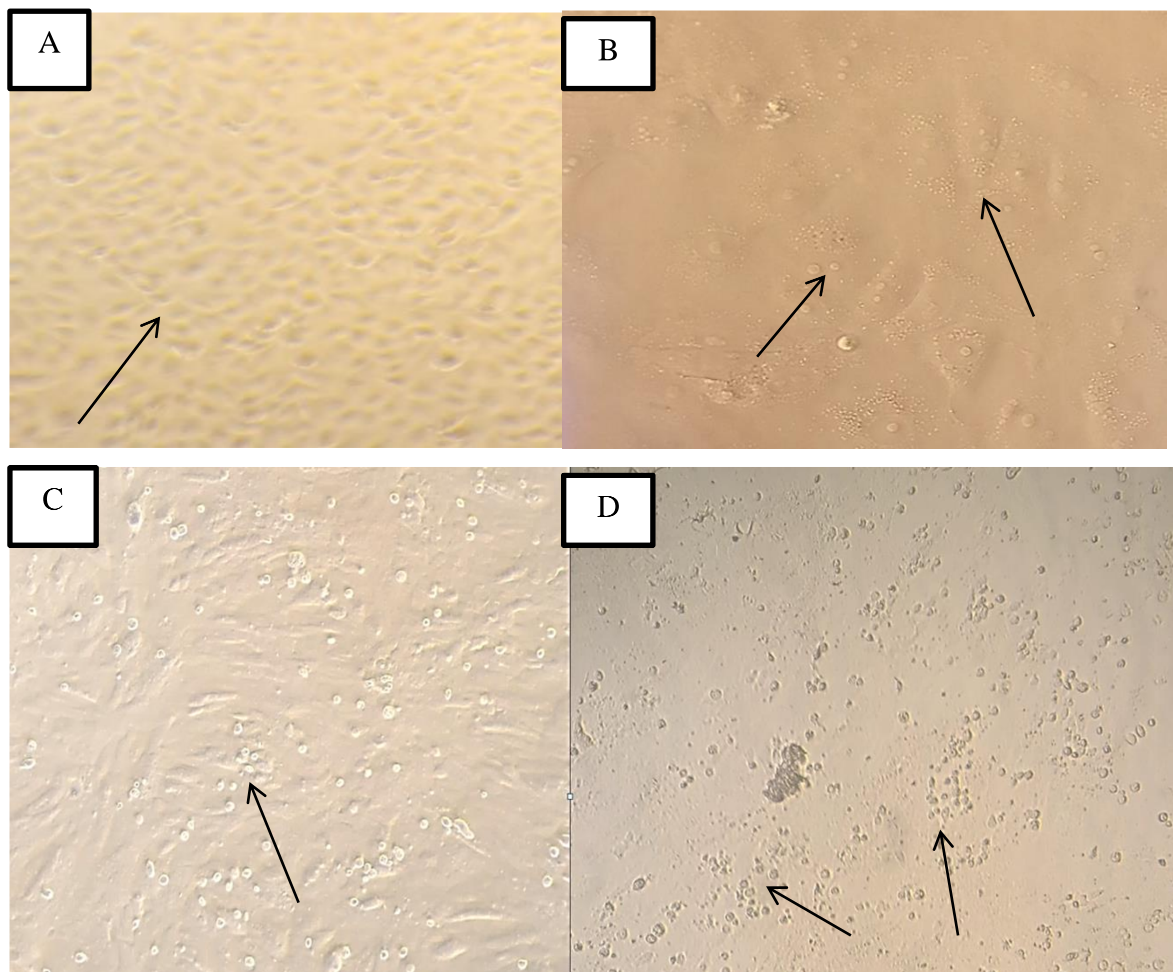


Figure 16: ND virus cytopathic effect on DF-1 cells. **(A)** Uninfected monolayer of DF-1 cell, and DF-1 cells infected by NDV showing: **(B)** Vacuolation**,** **(C)** cell swelling, rounding and **(D)** detachment & cells floating (arrows).

4.3.2. Reverse Transcriptase Real-Time Polymerase Chain Reaction result

From a total of 53 pooled (265 individual) samples RNA were extracted and tested by reverse transcriptase polymerase chain reaction (RT-PCR) for M gene-based NDV and 24 pooled samples (120 individual) of the samples were positive by RT-PCR. This pooled samples were from seven kebeles in Bishoftu Town, the positive results are in proportions of Dembi 6 (11.32%), Filtuu 2 (3.77%), Qurqura 3(5.66%), Kata 1(1.89%), Gorba 3 (5.66%), Calalaka 5 (9.43%) and from Dire Jitu 4 (9.45%) were positive for NDV. The samples and control RT-PCR amplification curve are indicated in Figure 17.

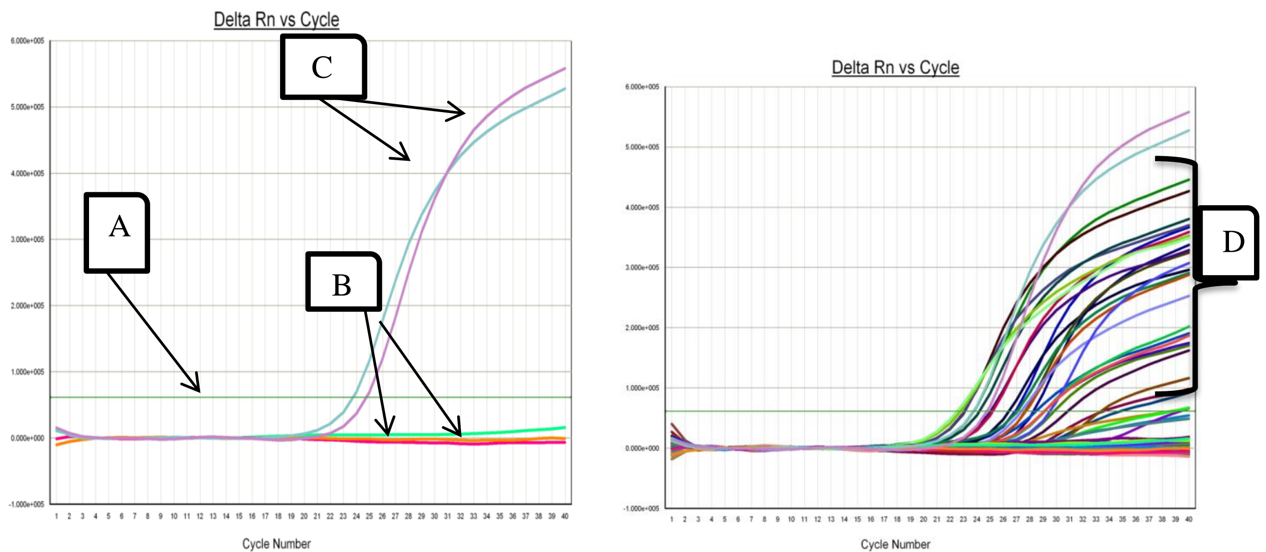


Figure 17: Amplification plot result of rRT-PCR.**(A)** Shows baseline**, (B)** shows negative controls, **(C)** shows positive controls and **(D)** shows positive samples result**.**

4.4. Questionnaire survey result

Fifty-three smallholder poultry farms were visited, 77% of them were layers farms. According to the present survey, 54.72% of farmers have experience in poultry production, and 37.74% get poultry production consultancy services. 35.9% poultry farms have footbath at the entry of the poultry house, 30.19 % have an isolation room for sick chicken and 37.73% of them have scheduled vaccination for their chickens regularly. Based on, the questioner survey 60.37% of households purchase chicken from growers/ brokers. 60.38% of the farms have contacts with other poultry farms and 56.60 % with wild birds and rodents while 45.28% of the farms throw waste in the compound.

Table 1: Biosecurity practices in the study households

Variable	Frequency	Percent
Farming experience		
Yes	24	54.72
No	29	45.28
Source of chickens purchase		
Broker\out grower	33	60.37
From hatchery	21	39.62
Waste management		
Burning	14	26.42
Fertilizer	5	9.43
Buried	10	18.87
Throw in the compound	24	45.28
Interaction of chicken with other chicken or wild birds		
Yes	30	56.60
No	23	43.40
Contact of farm with other farm		
Yes	32	60.38
No	21	39.62
Isolation pen		
Yes	16	30.19
No	37	69.81

Selling the diseased or recovered		
Yes	32	38.46
No	20	61.54
Advisory service on poultry production		
Yes	20	37.74
No	33	62.26
Protective vaccination for Newcastle		
Yes	20	37.73
No	16	31.9
Unknown	17	32.08
Presence of Footbath		
Yes	19	35.9
No	34	64.16

5. DISCUSSION

Newcastle disease is one of the most economically important diseases of chickens that cause serious losses in Ethiopia (Chaka *et al.,* 2012). This has been evidenced by the present study, where from 53 households, 265 chickens, (195 clinically sick, 70 apparently healthy), were examined and clinical signs, gross and microscopic lesions characterized. In addition NDV isolation and detection was performed during this study period. The current study showed that from 53 pooled specimens of tracheal and cloacal swabs and pooled tissue samples, NDV was molecularly detected from 24/53 (120/265) and isolated from 12 NDV positives using DF-1 chicken fibroblast cell line. It is therefore construed that high pool prevalence of NDV implies the maintenance of the virus and the spread of the disease within the study area.

The clinically sick chickens were physically examined and characterized by the clinical signs. Accordingly, clinical signs recorded in this study were: mild to severe depression with drooping wings in all clinically sick chickens, from which 32.35% showed greenish-white diarrhea and pasty vent, 26.47% were observed with torticollis, paralysis of legs and wings which was either unilateral or bilateral and 41.18% showed respiratory disorders which were labored breathing, increased rales, wheezing, and open-mouthed breathing with watery/tenacious mucus discharge from the nostrils in close agreement with Brown *et al*. (1999), Alexander, (2003), Kommers *et al.* (2003) and Wakamatsu *et al.*(2006) who reported as one of the clinical signs of ND.

Gross lesions in tracheas were hemorrhages and mucoid exudates; in the lungs the gross pathological changes observed were black spot pigmentation in some cases and severe hemorrhages. These gross pathological changes including haemorrhagic tracheitis and pulmonary congestion are in agreement with the earlier descriptions of ND (Balachandran *et al*. 2014 and Khorrajiya *et al.* 2015). The histological alterations observed in this study, namely loss of cilia in the trachea, congestion, severe cellular infiltration of lymphocytes and diffuse marked destruction of epithelium observed are in agreement with earlier reports (Alexander and Senne 2008).

The proventriculus showed petechial hemorrhage and hemorrhagic proventriculitis. Microscopic observation revealed proventriculitis, marked by desquamation of proventriculus epithelial

surface and epithelial gland, hyperemia of the muscularis layer and infiltration of inflammatory cells to sub-mucosal layer of proventriculus glands similar to results reported by Nakamura *et al*. (2008) and Bwala *et al*. (2012). In the present study, the intestine suffered enteritis, pinpoint hemorrhages and microscopically, segments of intestines were similarly characterized by presence of sloughed mucosal epithelium, moderate to severe infiltration of leucocytes, mostly mononuclear cells, congestion and hemorrhages. These gross and microscopic lesions in intestines in the present study corresponded with the findings of Alexander and Jones (2001) who stated that marked hemorrhages in the intestine and within intestinal lymphoid follicle are due to blood vessel injury caused by replication of virulent NDV.

In the current study, gross examination of livers found severe diffuse areas of paleness which indicates hepatic necrosis. There were also areas with widespread hemorrhages. Microscopically the hepatocytes were necrotized with infiltration of lymphocytes and macrophages. These changes are in line with Wakamatsu *et al*. (2006) and Susta *et al*. (2011) where VVND infection has been reported to cause multifocal areas of necrosis in the liver.

Gross lesions were also observed in cecal tonsils, with swelling and moderate to severe hemorrhages. Microscopic examination of cecal tonsil revealed that there was necrosis of epithelial mucosal cells, hemorrhage, and infiltration of inflammatory cells into the submucosal layer. The results of the present study agree with the results of Ecco *et al*. (2011) and Wakamatsu *et al. (*2006) which described especially prominent gut lymphoid aggregates in cecal tonsils located in the proximal portion of the ceca, and stated that these are often regarded as the "old faithful" lesion for VVND as they most consistently display hemorrhage and necrosis grossly.

The spleens examined were found to have gross lesions evidenced by the presence of congestion, hemorrhage and abnormally enlargement. Microscopic examination revealed hemorrhage, necrosis, and multifocal inflammatory cell infiltration. Similar lesions in the spleen of chickens with severe systemic ND were reported by Mohammadamin and Qubih (2011) and Kommers *et al.* (2003).

Atrophy and marked degeneration of lymphocytes in the bursa were observed, which was in line with the findings of Eze *et al*. (2014) that stated lymphocytic depletion in the lymphoid organs and ballooning degeneration in the bursa of Fabricius were the predominant lesions observed and can serve as useful features in the diagnosis of VVND in chickens.

Gross lesions observed in hearts were gray to white small nodules which were many around and over the apex of the heart. Microscopic observation found myocarditis marked by myocardium degeneration, and necrosis, with mononuclear cell infiltration. NDV distribution in the heart matches a previous report by Bwala *et al.* (2012) that stated chickens infected by velogenic viscerotropic NDV isolate showed macrophage cell accumulation at the myocardium. NDV distribution in heart tissue was caused by virus carried by the blood into the heart.

In the present study the kidney had nephritis with gross changes marked by swelling and fragility with multiple necrotic foci. Microscopic examination revealed hemorrhage, tubular epithelial cell necrosis, and inflammatory cell infiltration in the interstitium. These pathological findings agree with previous research by Nakamura *et al.* (2008) who stated that nephritis occurs as the virus invades through the respiratory tract and is then carried by blood circulation to the kidney. Virus replicates inside the mucosal epithelial cells of the upper respiratory and digestive tracts then spreads through blood circulation to the kidney and bone marrow, causing secondary viremia.

On gross examination the brain showed encephalitis; the microscopic examination showed that there were excess lymphocytes in the brain capillaries and multifocal hemorrhages. These lesions are identical to those reported by Ecco *et al.*(2011) who stated that encephalitis was found in brains of chickens infected with s velogenic viscerotropic isolate and clinical signs observable originating from the nervous system were caused by nerve cell destruction in the brain from infection and NDV replication. Additionally, Zachary, (2012) stated that in the presence of neurotropic velogenic strains within the brain can cause vascular and neuronal damage which will result in an inflammatory response. This then spreads to surrounding astrocytes. In addition, this pathotype also leads to neuronal degeneration and significant lesions are often observed in the caudal central nervous system (spinal cord, brain stem).

Chicken embryo fibroblast cells infected with viruses isolated from 12 positive samples showed cell swelling, rounding, detachment & cells floating and vacuolation which are related to the virulence of the virus. This finding was in close agreement with reports of Haque *et al*. (2010) and Li *et al*. (2009) which found the same CPE characteristics in NDV isolated from birds.

Because of its high sensitivity, specificity, efficiency, and, most importantly, its ability to detect the virus, reverse transcriptase real-time PCR was used. NDV was found in a pooled sample of 24/53 (45.3%) of the chickens tested. The current investigation found that ND was endemic in the kebeles investigated, with an overall pool prevalence of 45.3% using RT-PCR. Dembi (11.32%) had the largest proportion of this finding, followed by dire jitu (9.45%) and Calalaka (9.43%). The results were found to conflict with earlier reports of low real time PCR findings from Bishoftu 25% (2/8) by Worku *et al*. (2022) and East Shewa zone, Ethiopia (14.2%) by Chaka *et al*. (2012). However, Miressa *et al*. (2016) reported (26.7%) from East Shewa, Ethiopia and Mulisa *et al.* (2014) reported lower PCR findings, 30.1% (44/146) from live poultry market of Ethiopia.

The difference could be attributed to the sample size and seasonal variation in case of Worku *et al.* (2022) who tested only eight commercial farms. Chaka *et al*. (2012) used a fusion (F) gene detection assay but in the present study we used real-time PCR which is very sensitive for matrix (M) gene detection. Real time PCR is highly sensitive to M-gene of NDV which is more diverse than fusion (F) gene. Fusion (F) gene is specific and attributes to pathogenesis of the virus (Rahman *et al*., 2016). Lower PCR finding of Mulisa *et al.* (2014) could be attributed to the large sampling area covered by researchers as they collected samples originating from the entire country. In case of Miressa *et al.* (2016), the difference may be attributed to sampling method since they collected samples individually. Nevertheless the present finding is in line with the findings of Maqbool *et al.* (2017), who reported 90.0%, using real time PCR detection method. The overall variation of the kebeles might be due to variations in the management system and biosecurity practices that may serve as a stress factor and contact among households. In Dembi there are densely populated small-scale farms as compared to other kebeles.

During the current study, biosecurity practices that might contribute to the occurrence of ND were assessed in 53 small-scale farms. Several practices that potentially cause ND were observed. According to Okwor and Eze (2010), inadequate biosecurity is conducive to the transmission of ND, and ND may be controlled by strong biosecurity and limiting interaction with rodents and other animals. During the building of new poultry farms, proper distances should be maintained between chicken farms. Geresu *et al*. (2016) found that the interaction of healthy birds with migratory birds resulted in the outbreak and spread of ND. Additionally, previous reports of Joshi *et al*. (2021) and Sahoo *et al*. (2022) state that dead birds are source of spread of ND; mortality may be constructed within the boundary wall of the farm area thus, all the dead birds should be buried in the pit for proper disposal and for preventing the spread of ND. However, in the present study 45.28% of the farms throw waste in the compound, which is in line with Haftom *et al.* (2015) where 56% of the farms throw dead birds in the compound.

Regarding accessibility to wild birds and rodents, 56.60 % of farms have contact with wild birds and rodents which was lower than Haftom *et al.* (2015) where 84% of the farms were found to be free from wild birds and rodents. In the current study 35.9% of poultry farms have a footbath at the entry of the poultry house, which was in line with a report of Elelu *et al.* (2012) and contrary to the report of Haftom *et al.* (2015) who noted that about 80% of the small-scale commercial poultry farms in and around Mekelle had a foot bath at the entrance. In the current study, 30.19 % of the farms have an isolation room for sick chickens which was in agreement with Haftom *et al*. (2015) where 32% of the farms have an isolation pen for sick chickens. Regarding vaccination, the current result is in contrast to the findings of Birhanu *et al.* (2010) who noted 84% of small-scale poultry farms in Addis Ababa have regular scheduled vaccination for their chickens. In the present study the farms having contact with other farms is 60.38% in contrast to the findings of Haftom *et al*. (2015) with 88% of the farms preventing contact with other farm

6. CONCLUSION AND RECOMMENDATIONS

Based on the present finding, Newcastle disease is still prevalent and lethal poultry diseases causing death, loss of production and limiting chicken production in the study area. Despite the availability of vaccines for ND, poultry farmers continue to experience losses due to mortalities, drop in production, diagnostic and control costs.

The following recommendations are forwarded:

- Poultry producers should regularly vaccinate their chickens and, based on the chicken type, booster vaccines should be given.
- In some farms disease occurred even though chicken were vaccinated so the root cause of this should be carefully evaluated and be answered
- Day-old chick distributers should administer the first Newcastle disease vaccine before distribution (some already doing that) to growers
- Small-scale poultry farmers should get management and biosecurity training.
- Poultry producers should practice strict biosecurity. Footbaths should be inspected and the disinfectant regularly changed. The wire mesh used to prevent entry of wild birds should be inspected and should not allow access of wild birds. The crates and vehicles used to move chickens should be washed and disinfected. Material used in the poultry house should be kept clean and attendants should have limited access to another poultry houses and should change clothes when they exit and enter poultry house.

7. REFERENCES

Abah H. O., Abdu, P. A. & Assam A. (2016): Molecular detection and viral isolation of Newcastle disease viruses from chickens in Benue state, Nigeria. *International Journal of Animal and Veterinary Advances*, **8**(4), 40-45.

Abdisa T. &Tagesu T. (2017): Review on Newcastle disease of poultry and its public health importance. *J. Vet. Sci. Technol*, **8**(3), 441.

Abel S. (2018): Evaluation of the immune response of newcastle disease virus vaccines, in layer chickens, Msc thesis, Addis Ababa University, College of Veterinary Medicine and Agriculture, Bishoftu Ethiopia.

Alem T. (2014): Production and reproduction performance of rural poultry in lowland and midland agro-ecological zones of central Tigray, Northern Ethiopia. *African Journal of Agricultural Research*, **9**(49), 3531-3539.

Alemayehu A., Yilma T., Shibeshi Z. &Workneh T. (2015): Village chicken production systems in selected areas of Benishangul-gumuz, western Ethiopia. *Asian Journal of Poultry Science*, **9**(3), 123-132.

Alexander D, J. 2003: Newcastle disease, other avian paramyxoviruses, and pneumovirus infection. In: Disease of poultry, ed. Shaif YM, Barnes HJ, Glisson JR, et al., 12th ed., pp. 75–100. Blackwell, Oxford, UK.

Alexander D. and Jones R. (2001): Paramyxoviridae. Poultry Disease, 5th edition. London: W. B. Saunders., Pp: 257-267.

Alexander D. J. &Senne D. A. (2008): Newcastle disease and other paramyxoviruses. *Swayne DE GJR, Pearson JE, Reed WM, Jackwood WM, Woolcock PR and Dufour-Zavala, editor.A laboratory manual for the isolation identification and characterization of avian pathogens. 5th ed: OmniPress, Inc*, 135-41.

Alexander J. T. (2004): Coccidosis in poultry disease 5 th ed. *edited by Jordan FTW, pattison M., Alexander D, farager TWB sauaders London, Edinburgh. New YorkPhiladelphia. St Loius Sydney Toronto*, 405-420.

Al-Habeeb M. A., Mohamed M. H. A. &Sharawi S. (2013): Detection and characterization of Newcastle disease virus in clinical samples using real time RT-PCR and melting curve analysis based on matrix and fusion genes amplification. *World*, **6**(5), 239-243.

Asfaw Y. T., Ameni G., Medhin G., Gumi B., Hagos Y. & Wieland B. (2021): Poultry disease occurrences and their impacts in Ethiopia. *Tropical Animal Health and Production*, **53**, 1-10.

Ashraf A. & Shah M. S. (2014): Newcastle disease: present status and future challenges for developing countries. *African Journal of Microbiology Research*, **8**(5), 411-416.

Assefa H. (2019): The role of poultry for poor livelihoods in Ethiopia. *Int. J. Vet. Sci. Anim. Husb*, **4**(3), 1-4.

Assefa, H., 2019. The role of poultry for poor livelihoods in Ethiopia. *Int. J. Vet. Sci. Anim. Husb*, *4*(3), pp.1-4.

Balachandran P., Srinivasan, P., Sivaseelan S., Balasubramaniam G. A. & Murthy T. G. (2014): Isolation and characterization of Newcastle disease virus from vaccinated commercial layer chicken. *Veterinary World*, **7**(7).

Belayneh G., Moses N., Melese B. and Fufa D. (2014): Seroprevalence of Newcastle Disease Virus Antibodies in Village Chickens in Kersana- kondalaity District, Ethiopia. Glob. Vet. 12 (3): 426-430.

Bello M. B., Yusoff K., Ideris A., Hair-Bejo M., Peeters B. P. & Omar A. R. (2018): Diagnostic and vaccination approaches for Newcastle disease virus in poultry: the current and emerging perspectives. *BioMed research international*, *2018*.

Birhanu G.,(2010): Husbandry and health care practice in small scale poultry production in Addis Ababa, DVM thesis, Addis Ababa University., DebreZeit, Ethiopia.

Brown C. O., King D. J. & Seal B. S. (1999): Pathogenesis of Newcastle disease in chickens experimentally infected with viruses of different virulence. *Veterinary pathology*, **36**(2), 125-132.

Bush J. (2006): The threat of avian flu predicted impacts on rural livelihoods in Southern Nation, Nationalities and Peoples Region (SNNPR), Ethiopia. *The Food Economy Group*.

Bwala D. G., Clift S., Duncan N. M., Bisschop S. P., & Oludayo F. F. (2012): Determination of the distribution of lentogenic vaccine and virulent Newcastle disease virus antigen in the

commercial hen using immunohistochemistry. *Research in Veterinary Science*, **93**(1), 520-528.

Cappelle J., Caron A., De Almeida R. S., Gil P., Pedrono M., Mundava J. & Gaidet N. (2015): Empirical analysis suggests continuous and homogeneous circulation of Newcastle disease virus in a wide range of wild bird species in Africa. *Epidemiology & Infection*, **143**(6), 1292-1303.

Carvallo F. R., Moore J. D., Nyaoke, A. C., Huang L., Crossley B. M. &Uzal F. A. (2018): Diagnosis of virulent Newcastle disease in southern California, May 2018. *Journal of Veterinary Diagnostic Investigation*, **30**(4), 493-494.

Cattoli G., Susta L., Terregino C. & Brown C. (2011): Newcastle disease: a review of field recognition and current methods of laboratory detection. *Journal of veterinary diagnostic investigation*, **23**(4), 637-656.

Chaka H., Goutard F., Bisschop S. P. & Thompson P. N. (2012): Seroprevalence of Newcastle disease and other infectious diseases in backyard chickens at markets in Eastern Shewa zone, Ethiopia. *Poultry Science*, **91**(4), 862-869.

Chaka, H., Goutard, F., Gil, P., Abolnik, C., Almeida-de, S., Shahn, P. and Peter, N. (2013): Serological and molecular investigation of Newcastle disease in household chicken flocks and associated markets in Eastern Shewa zone, Ethiopia. *Trop. Anim. Health. Prod.*, **45**:705–714.

Corrie B., Raquel R., Daniel R. and Taiana C. (2008): Poultry Necropsy Manual (The Basics). Department of Pathology, University of Georgia, Athens, Georgia., Pp:1-14

CSA. (2009): Agricultural sample survey 2003/2004. Volume 2: Report on livestock and livestock characteristics, 2. Statistical Bulletin No. 302. Federal Republic Ethiopia, Addis Ababa, Ethiopia

Dawit A., Tamrat D., Stotaw F., Nzietcheung S. & Roy D. (2011): Overview and background paper on Ethiopia's poultry sector. *Relevance of Reseasrch in Ethiopia.www. hpai-reserach net. Accessed*, june *6*

Dessie T. &Jobre Y. (2004): A review of the importance and control of Newcastle disease in Ethiopia. International Livestock Research Institute. *Eth. Vet. J.*, **1**:71-81.

Desvaux S., Grosbois, V., Pham T. H., Fenwick S., Tollis S., Pham N. H., & Roger F. (2011): Risk factors of highly pathogenic avian influenza H5N1 occurrence at the village and

farm levels in the Red River Delta Region in Vietnam. *Transboundary and Emerging Diseases*, **58**(6), 492-502.

Dzogbema K. F. X., Talaki E., Batawui K. B. & Dao B. B. (2021): Review on Newcastle disease in poultry. *International Journal of Biological and Chemical Sciences*, **15**(2), 773-789.

Ecco R., Susta L., Afonso C.L., Miller P.J. & Brown C. (2011) Neurological lesins in chickens experimentally infected with virulent Newcastle disease virus isolates. *Avian.Pathol.*,**40**(2): 145-152.

Elelu N., Jaji Z., Badiru A., Olowoleni F., &Ambali A. G. (2012): Assessment of Management and Health Practices in Some Selected Poultry Establishments in Ilorin, Kwara State, Nigeria. *International Journal of Poultry Science*, **11**(8), 524.

Etriwati R., Handharyani E. &Setiyaningsih S. (2017): Pathology and immunohistochemistry study of Newcastle disease field case in chicken in Indonesia. *Vet. Wor.*,**10**(9): 1066-1071

Eze C. P., Okoye J. O. A., Ogbonna, I. O.,Ezema W. S., Eze D. C., Okwor E. C. &Salihu E. A. (2014): Comparative study of the pathology and pathogenesis of a local velogenic Newcastle disease virus infection in ducks and chickens. *International Journal of Poultry Science*, **13**(1), 52.

Fentie T., Dadi K., Kassa T., Sahle M. &Cattoli G. (2014). Effect of vaccination on transmission characteristics of highly virulent Newcastle disease virus in experimentally infected chickens. *Av. Pathology,* **43** (5), 420-426.

Fikre M. (2003): Poultry Disease Lecture Notes, Proceedings of a Postgraduate Refresher Course Conducted at Mekelle, in May 2003. By the Tigray Agriculture and Natural Resources Development pp 2-3.

Gelana H. (2017): Conventional and molecular epidemiology of new castle disease in wild and domestic birds in selected rift valley areas, Ethiopia, Msc thesis, Addis Ababa University, College of Veterinary Medicine and Agriculture, Bishoftu Ethiopia.

Geresu M. A., Elemo K. K. &Kassa G. M. (2016): Newcastle disease: Seroprevalence and associated risk factors in backyard and small scale chicken producer farms in Agarfa and Sinana Districts of Bale Zone, Ethiopia. *Journal of Veterinary Medicine and Animal Health*, **8**(8), 99-106.

Getabalew M., Alemneh T., Akeberegn D., Getahun D., &Zewdie D. (2019): Epidemiology, Diagnosis & Prevention of Newcastle disease in poultry. *Am J Biomed Sci Res*, **16**, 50-9.

Girmay A. M., Alemu Z. A., Mengesha S. D., Dinssa D. A., Wagari B., Weldegebriel M. G. &Tollera G. (2022): Effect of demographic disparities for water, sanitation, and hygiene services in Bishoftu Town, Ethiopia. *Discover Water*, **2**(1), 8.

Hadipour M. M., Habibi G. H., Golchin P., Hadipourfard M. R. &Shayanpour N. (2011): The role of avian influenza, newcastle disease and infectious bronchitis viruses during the respiratory disease outbreak in commercial broiler farms of Iran. *Int J Anim Vet Adv*, **3**(2), 69-72.

Haftom B, Tehetna A, Yohannes H, Awot T (2015): Assessment of Bio-Security Condition in Small Scale Poultry Production System in and Around Mekelle, Ethiopia. *Eur. J. Biol. Sci.***7**(3):99-102.

Hailu M. (2012): Review on major viral diseases of chickens reported in Ethiopia. J. Infect. Dis. Immu., **4**(1): 1-9

Haque M. H., Hossain M. T., Islam M. T., Zinnah M. A., Khan M. S. R. & Islam M. A. (2010): Isolation and Detection of Newcastle disease virus from field outbreaks in Broiler and Layer chickens by Reverse transcription Polymerase chain reaction. *Bangladesh Journal of Veterinary Medicine*, **8**(2), 87-92.

Jarso D. (2015): *Epidemiology of village chicken diseases: a longitudinal study on the magnitude and determinants of morbidity and mortality—the case of Newcastle and infectious bursal disease* (Doctoral dissertation, Addis Ababa University).

Jordan F. T. W., Williams N. J., Wattret A. & Jones T. (2005): Observations on salpingitis, peritonitis and salpingoperitonitis in a layer breeder flock. *Veterinary Record*, **157**(19), 573-577.

Joshi V. G., Chaudhary D., Bansal N., Singh R., Maan S., Mahajan N. K. &Goyal S. M. (2021): Prevalence of Newcastle disease virus in commercial and backyard poultry in Haryana, India. *Frontiers in Veterinary Science*, **8**, 725232.

Khalifeh M. S., Amawi M. M., Abu-Basha E. A. & Yonis I. B. (2009): Assessment of humoral and cellular-mediated immune response in chickens treated with tilmicosin, florfenicol, or enrofloxacin at the time of Newcastle disease vaccination. *Poultry Science*, **88**(10), 2118-2124.

Khorajiya J. H., Pandey S., Ghodasara P. D., Joshi B. P., Prajapati K. S., Ghodasara D. J. &Mathakiya R. A. (2015): Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms. *Veterinary World*, **8**(3), 372.

Kommers G. D., King D. J., Seal B. S. & Brown C. C. (2003): Pathogenesis of chicken-passaged Newcastle disease viruses isolated from chickens and wild and exotic birds. *Avian diseases*, **47**(2), 319-329.

Kryger K. N., Thomsen K. A., Whyte M. A. & Dissing M. (2010): Smallholder poultry production–livelihoods, food security and sociocultural significance. *FAO Smallholder Poultry Production Paper*, *4*.

Li X., Qiu Y., Yu A., Chai T., Zhang X., Wangb J., Wang H., Wang Z. & Song C. (2009): Degenerate primers based RT-PCR for rapid detection and differentiation of airborne chicken Newcastle disease virus in chicken houses. *Journal of virological methods*, **158**(1-2), 1-5.

Lizin S., Rousseau S., Kessels R., Meulders M., Pepermans G., Speelman S., &Verbeke W. (2022): The state of the art of discrete choice experiments in food research. *Food Quality and Preference*, 104678.

Maqbool R., Wani S. A., Wali A., Kashoo Z., Dar P., Qureshi S. &Bukhari S. (2017): RT-PCR based assay for detection of Newcastle disease virus isolated from poultry in Kashmir. *Journal of Entomology and Zoology Studies*, **5,** 1835-1839.

Mazengia H. (2012): Review on major viral diseases of chickens reported in Ethiopia. *J Infect Dis Immun*, **4**(1), 1-9.

Miller P. J., Decanini E. L. &Afonso C. L. (2010): Newcastle disease: evolution of genotypes and the related diagnostic challenges. *Infection, genetics and evolution*, **10**(1), 26-35.

Miller P.J. and Koch G.H. (2013): Newcastle disease. In: Diseases of Poultry. 13th (ed.): Wiley-Blackwell; Hoboken, NJ, USA: Pp. 89–138.

Miressa Y., Cherinet Y., Bsrat A. &Tadesse F. (2016): Investigation of Newcastle disease virus using reverse transcription polymerase chain reaction in selected districts of Eastern Shewa, Ethiopia. *J Vet SciTechnol*, **7**(384), 2.

Mohammadamin O. G. &Qubih T. S. (2010): Effect of industrial product on immunosuppressed broilers vaccinated with Newcastle disease vaccine. *Iraqi Journal of Veterinary*

Sciences, **24**(1), 37-40.

Mohammadamin O. G. &Qubih T. S. (2011): Histopathology of virulent Newcastle disease virus in immune broiler chickens. *Iraqi Journal of Veterinary Sciences*, **25**(1), 9-13.

Mohammed A. (2018): Major constraints and health management of village poultry production in Ethiopia: review school of veterinary medicine, Jimma University, Jimma, Ethiopia. *Journal of Research Studies in Microbiology and Biotechnology*, **4**(1), 1-10.

Molia S., Samake, K., Diarra, A., Sidibe, M., Doumbia, L., Camara, S., Kante, S. and Kamissoko, B. (2011): Avian influenza and Newcastle disease in three risk areas for H5N1 highly pathogenic avian influenza in Mali, 2007–2008. *Avian diseases*, **55**(4), 650-658.

Mulisa D. D., Alemu R. B., Keno M. S., Furaso A., Heidari A., Chibsa T. R. &Chunde H. C. (2014): Characterization of Newcastle Disease Virus and poultry-handling practices in live poultry markets, Ethiopia. *SpringerPlus*, **3**(1), 1-6.

Mulualem A. (2017): Sero-prevalence of Newcastle disease in scavenging chicken in selected districts of Arsi Zone, Ethiopia. *Int Journal of Agriculture and Veterinary Sciences,* ***3****(1)*, 1-5.

Munir M., Abbas M., Khan M. T., Zohari S., & Berg M. (2012): Genomic and biological characterization of a velogenic Newcastle disease virus isolated from a healthy backyard poultry flock in 2010. *Virology journal*, **9**(1), 1-11.

Nakamura K., Ohtsu N., Nakamura T., Yamamoto Y., Yamada M., Mase, M. & Imai K. (2008): Pathologic and immunohistochemical studies of Newcastle disease (ND) in broiler chickens vaccinated with ND: severe nonpurulent encephalitis and necrotizing pancreatitis. *Veterinary Pathology*, **45**(6), 928-933.

Nyoni N. M. B., Grab S., & Archer E. R. (2019): Heat stress and chickens: climate risk effects on rural poultry farming in low-income countries. *Climate and Development*, **11**(1), 83-90.

Nyoni, N.M.B., Grab, S. and Archer, E.R., 2019. Heat stress and chickens: climate risk effects on rural poultry farming in low-income countries. *Climate and Development*, *11*(1), pp.83-90.

OIE (2004): *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: (Mammals, Birds and Bees)*. OIE.

OIE (2013): Newcastle disease Epidemiology Diagnosis Prevention and Control.

OIE (2020): OIE World Animal Health Information System. Weekly Animal Disease Sevice Global Report.

OIE (2021): Newcastle Disease (Infection with Newcastle Disease Virus). Chapter 3.3. 14. *Manual of diagnostic tests and vaccines for terrestrial animals. Available online: http://www. oie. int/standard-setting/terrestrial-manual/access-online*.

Okwor EC, Eze DC (2010): The Annual prevalence of Newcastle disease in commercial chickens reared in south eastern savannah zone of Nigeria. *Res. J. Poul. Sci.* **3**:23-26.

Oladele S. B., Nok A. J., Esievo K. A. N., Abdu P. &Useh N. M. (2005): Haemagglutination inhibition antibodies, rectal temperature and total protein of chickens infected with a local Nigerian isolate of velogenic Newcastle disease virus. *Veterinary research communications*, **29**, 171-179.

Pansota F. M., Rizvi F., Sharif A., Javed M. T., Muhammad G., Khan A. & Khan M. Z. (2013): Use of hyperimmune serum for passive immunization of chicks experimentally infected with Newcastle disease virus. *Pak. J. Agri. Sci*, **50**(2), 279-288.

Prosser D. J., Cui P., Takekawa J. Y., Tang M., Hou Y., Collins B. M. & Newman S. H. (2011): Wild bird migration across the Qinghai-Tibetan plateau: a transmission route for highly pathogenic H5N1. *PloS one*, **6**(3), e17622.

Rahman M. M., Barman L. R., Chowdhury E. H. & Islam M. R. (2016): Detection of Newcastle disease virus of poultry by real time reverse transcription-polymerase chain reaction. *Bangladesh Veterinarian*, **33**(1), 16-22.

Ramos-Vara J. A. & Miller M. A. (2014): When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry—the red, brown, and blue technique. *Veterinary pathology*, **51**(1), 42-87.

Rasamoelina A., Lancelot R., Maminiaina O., Rakotondrafara T. &Jourdan M. (2012): Risk factors for avian influenza and Newcastle disease in smallholder farming systems, Madagascar highlands. *Prev. Vet. Med.,* **104**(1–2): 114–124.

Rauw F., Gardin Y., Palya V., Van Borm S., Gonze M., Lemaire S., Van Den Berg T. &Lambrecht B. (2009): Humoral, cell-mediated and mucosal immunity induced by oculo-nasal vaccination of one-day-old and conventional layer chicks with two different live Newcastle disease vaccines. *Vaccine*, **27**(27), 3631-3642.

Sahlu B. W., Gugssa T. &Gebrekidan B. (2015): Newcaste disease In Ethiopia: a review. *Advances in Life Science and Technology*, **31**, 51-58.

Sahoo N., Bhuyan K., Panda B., Behura N. C., Biswal S., Samal L. &Goyal S. M. (2022): Prevalence of Newcastle disease and associated risk factors in domestic chickens in the Indian state of Odisha. *PloS one*, **17**(2), e0264028.

Saif Y. M., Fadly A. M., Glisson J. R. &McDougald L. R. (2008): "Newcastle disease, other avian paramyxoviruses, and pneumovirus infections," in Diseases of Poultry, pp. 75–93, Ames, Iowa, USA.

Samal S. K. (2011): Newcastle disease and related avian paramyxoviruses. *The biology of paramyxoviruses*, **1**, 69-114.

Samour J. (2014): Newcastle disease in captive falcons in the Middle East: a review of clinical and pathologic findings. *Journal of Avian Medicine and Surgery*, **28**(1), 1-5.

Shabbir M., Goraya M., Abbas M., Yaqub T., Shabbir M., Ahmad A., Anees M. &Munir M. (2012): Complete Genome Sequencing of a VelogenicViscerotropic Avian Paramyxovirus 1 Isolated from Pheasants (Pucrasiamacrolopha) in Lahore, Pak. *J. Virol.,* **86**(24): 13828-13829.

Shaheen S., Anjum A. D. &Rizvi F. (2005): Clinico-pathological observations of pigeons (Columba livia) suffering from Newcastle disease. *Liver*, **2**(7.14).

Sime, A.G., 2022. Review on poultry production, processing, and Utilization in Ethiopia. *Int J Agric Sc Food Technol*, *8*(2), pp.147-152

Sohail M. U., Ijaz A., Yousaf M. S., Ashraf K., Zaneb H., Aleem M. &Rehman H. (2010): Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: Dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poultry science*, **89**(9), 1934-1938.

Spickler A. (2016): *Newcastle Disease*. Retrieved from http://www.cfsph.iastate.edu/DiseaseInfo/factsheets.php.

Susta L., Miller P. J., Afonso C. L. & Brown C. C. (2011): Clinicopathological characterization in poultry of three strains of Newcastle disease virus isolated from recent outbreaks. *Veterinary pathology*, **48**(2), 349-360.

Talukder S. (2007): Histopathology techniques: Tissue processing and staining. *Mymensingh Medical College, Department of Pathology, Mymensingh*, *11*.

Tang Q., Wang J., Bao J., Sun H., Sun Y., Liu J. &Pu J. (2012): A multiplex RT-PCR assay for detection and differentiation of avian H3, H5, and H9 subtype influenza viruses and Newcastle disease viruses. *Journal of virological methods*, **181**(2), 164-169.

Terregino C. & Capua I. (2013): Méthodeconventionnelle de diagnostic de l'infection par le virus de la maladie de Newcastle. In *Influenza aviaireetmaladie de Newcastle* (pp. 129-131). Springer, Paris.

Waheed U., Siddique M., Arshad M., Ali M. &Saeed A. (2013): Preparation of Newcastle Disease Vaccine from VG/GA Strain and its Evaluation in Commercial Broiler Chicks. *Pakistan Journal of Zoology*, **45**(2): 339-344.

Wakamatsu N. O., King D. J., Kapczynski D. R., Seal B. S. & Brown C. C. (2006): Experimental pathogenesis for chickens, turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002-2003. *Veterinary pathology*, **43**(6), 925-933.

Wong JT, de Bruyn J., Bagnol B., Grieve H., Li, M, Pym R. & Alders, R.G. (2017). Small-scale poultry and food security in resource-poor settings: A review. Global Food Security 5: 43-52.

Worku T., Dandecha M., Shegu D., Aliy A. &Negessu D. (2022): Isolation and Molecular Detection of Newcastle Disease Virus from Field Outbreaks in Chickens in Central Ethiopia. *Veterinary Medicine: Research and Reports*, 65-73.

Zachary J.F. (2012): Nervous system. In: McGavin, M.D. and Zachary, J.F., editors. Pathologic Basis of Veterinary Disease.5th ed. Mosby Elsivier, St. Louis. 771-870

Zhang S., Wang X., Zhao C., Liu D., Hu Y., Zhao J. & Zhang G. (2011): Phylogenetic and pathotypical analysis of two virulent Newcastle disease viruses isolated from domestic ducks in China. *PLoS one*, **6**(9):1-9

8. APPENDICES

Annex I: Ethical Approval Sheet

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/03/01/15/2023

Name and affiliation of applicant: **Hana Dejene (DVM, MSc Student)**
Department of Pathology and Parasitology, Addis Ababa University

Title of the project: *Characterization of Newcastle disease associated pathological lesions, viral isolation and molecular detection of the virus in small scale poultry farms in Bishoftu town, Central Ethiopia*

Date of application:	**December, 2022**
Nature of the project:	**Farm investigation**
Target animal species:	**Domestic chicken**
Number of animals involved:	**240**
Study area:	**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/01/15/022, 16/12/2022**

The Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University has reviewed the above research project and unanimously approved the application of Hana Dejene.

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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ፋክስ Fax 251-11-4339933 | ስልክ Tel. +251 114338450 | ፖ.ሣ.ቁ P.o.x. Box)34 | ቢሾፍቱ፣ ኢትዮጵያ Bishoftu, Ethiopia

Annex II: Chicken Necropsy Protocols (Corrie *et al*., (2008) and Chauhan and Chandra (2007)

A good necropsy involves paying attention to ALL the clues that can be provided, so the routine has to be followed, with attention to detail at every step. The consistent routine entails 6 steps:

Step 1: Obtain the History this history should include:

Bird's age

Sex

Breed

Clinical signs

History of trauma or disease

History of any treatments administered

Any other information that may be relevant to the case such as type of feed and water

If the bird is a member of a flock the following is also required:

- ✓ Number of birds in the flock
- ✓ Number of birds in the affected group
- ✓ Number of affected birds
- ✓ Clinical signs of the flock should also be noted

Step 2: Examine the Bird Externally

- ✓ Examine the bird for any signs of trauma and evaluate the bird's general body condition.
- ✓ If possible the bird should be weighed.
- ✓ Prior to opening the body:
- ✓ If it is planned for additional investigation, you might want to swab the trachea or cloaca. When swabbing the trachea, insert the swab up in the choana
- ✓ Dislocate cervical joints of the bird and dip the whole bird/carcass into a bucket of
- ✓ Soapy water to thoroughly wet all the feathers.

Step 3: Open the Body

- ✓ The body should be placed on its back with its feet facing you and reflect the wings back.
- ✓ Cut through the skin between the legs and the breast so the legs can be fully abducted and lie flat against the table.
- ✓ Remove the skin from the ventral surface of the bird by cutting across at the caudal edge of the keel and then pulling skin cranially and caudally - peel away from the muscle to expose the muscular body wall.

- ✓ Make a small cut into the body cavity using scissors or a scalpel blade just behind the breast bone, and then pull the abdominal muscle caudally to expose some abdominal viscera.
- ✓ Extend the cut up through the cervical area and cut open the beak at the angle of the jaw.
- ✓ Now oral cavity, esophagus, trachea and crop are all visible
- ✓ The keel bone and breast muscle is then removed by incising the pectoral muscles oneach side of the keel and cutting through the ribs. Use the heavy poultry shears. Remove the keel and breast muscles entirely - you should now be able to see the internal organs from oral cavity to rectum.
- ✓ At this point, all internal viscera should be examined in situ for any abnormalitiesbefore removing any organs.
- ✓ Note the color, position and size of all organs and look for any adhesions.
- ✓ Examine the air sacs for increased thickness or cloudiness (caudal thoracic air sac is at the end of the forceps).

Step 4: Remove the Organs and Set Aside For Examination

- It is probably easiest to remove the abdominal viscera first and then go back and remove the thoracic organs.
- Remove the liver - in birds the liver takes up a big portion of the abdomen.
- The spleen can be a difficult organ to find once everything is removed so it is a good idea to locate it now and set it aside.
- Pull the proventriculus aside and it should pop into view. Take it out now and set it in a clean dry spot.
- Find the junction of the esophagus and stomach, cut here and pull the digestive tract out, all the way to the cloaca.
- Cut at the end of the large intestine, but leave the bursa in the bird.
- Kidneys are nestled up against the body wall - there are three portions - cranial, middle and caudal poles.
- In females only the left side of the tract persists, the right is vestigial and too small to be identifiable.

- Pull the heart and lungs away - the lungs are closely adhered to the dorsal body wall, and therefore, careful teasing of the lungs away from the ribs may be necessary to remove them.
- Open up the nasal cavity to take a close look at the sinuses.
- Observe the sciatic nerve which is an important location where Marek's disease can be seen.
- Open some joints to observe the fluid and synovial membranes.
- The brain can now be removed. Using the smaller scissors (not poultry shears), chip away at the skull, beginning from the foramen magnum, and remove the calvarium covering the cerebral hemispheres and the cerebellum. Take the brain out - use care this organ is quite soft.

Step 5: Examine and Sample the Organs

A) Examining of the organs

- ✓ It is a good idea to go from the "cleanest" organs to the "dirtiest. Usually this order is:lymphoid tissue, brain, lungs, heart, kidneys, reproductive tract, liver, intestinal tract.
- ✓ Note any abnormalities for each (color, size, shape, consistency, exudates). Be sure to examine both capsular and cut surface.
- ✓ Make several cuts in each organ and collect specimens for further diagnostic work.

B) Sampling of the Organs

- Tissues should be collected in duplicate, with half going into 10% formalin for histopathology and half as unfixed tissues that will be used for bacterial culture etc. if necessary.
- The following tissues should be collected in all cases: lung, liver, spleen, bursa, brain, kidney, intestines.
- Additionally, any other tissue that might have lesions (skin, adrenal, ovary, heart etc.) should also be collected.
- Label the tissues that are collected fresh so that the technician performing thetests can determine what each tissue is.

- Crushing should be avoided by using a sharp blade and a hard surface when taking samples since this may cause artifact histologically.
- When examining any organ with a mucosal surface (trachea, esophagus, intestine, etc.) care should be taken not to damage or destroy the mucosal surface by rubbing the surface with fingers or instruments.
- In order to improve fixation of tissues, samples should not exceed 5mm in thickness and volume of fixative should be at least 10 times the volume of tissue.
- Fresh samples should be packaged so that they remain cool and to minimize possibilities of leaking.

Step 6: Write the Report

- No necropsy is complete until all findings have been recorded in written form.
- The report should include at least the following information:
- Species, breed, age, sex and history
- Dead or euthanized?
- Nutritional, hydration status
- Findings from external examination
- Findings by organ system: Lymphoreticular (spleen, bursa), Respiratory, Digestive, Urogenital, Musculoskeletal, Nervous.

Notes: When describing tissues, keep these four qualities in mind - consistency, color, size, and symmetry.

Annex III: Histopathological procedures (Takulder, 2007)

1. Fixation of tissue by 10% neutral buffered formaldehyde

2. Trimming part of the tissue in a way that the lesion we require be included or not missed and to fit standard histological processing tissue cassettes (5mm thickness).

3. Tissue Specimen Processing:

   - ✓ This process fixation of tissue by formalin, dehydrating tissue by increasing alcohols concentration, clearing of tissue by xylene, and impregnation of tissue by paraffin wax.

Formalin-I 2hr Formalin-II 2hr 70% Alcohol 1hr 95% Alcohol 1hr 100% Alcohol-I 1hr 100% Alcohol-II 2hrs 100% Alcohol-III 2hrs Xylene I 1:30hrs Xylene-II 1:30hrs Xylene-III 1:30hrs Paraffin-I 2hrs Paraffin-II 3hrs.

4. Embedding of processed tissue

   - ✓ Impregnated tissue is placed in a mould with their labels and then fresh melted wax (54-600C) is poured and allowed to settle and solidify.

5. Sectioning

   - ✓ Sectioning of tissue in 3-5 micron thickness and put on water bath to straighten the ribbon, and then adhere on the surface of frost ended and clear slide.
   - ✓ Later label and put an incubator overnight.

Annex IV: Staining Procedures (Talukder, 2007)

Hematoxyline and Eosine Stain Procedures

1. Deparaffinize slides in 2 changes of xylene for 5 minutes each.

2. Rehydrate slides in 100% alcohol (3times) and 95% alcohol and 70% alcohol respectively for 3 minutes each, and rinse in distilled water until ripples disappear from slides.

3. Place in Hematoxylin for 8 - 15 minutes.

4. Rinse in tap water until water runs clear.

5. Decolorize in 1% acid alcohol, 3 - 6 quick dips. Check differentiation microscopically: Nuclei Should be distinct; Cytoplasm should be uncolored.

6. Rinse in tap water until ripples disappear from slides.

7. Stain in Eosin (Counter stain) (3dips).

8. Wash in tap water

9. Dehydrate in 70%, 95%, 100% (3times) alcohol respectively for 3 minutes each.

11. Clear in xylene (3times) 5 minutes each.

12. Mount with cover glass (with canada balsam)

13. Finally examined under microscope starting from the lower to higher magnification (4x, 10x, 40x and 100x) for the presence and identification of the microscopic lesions.

Annex V: A questionnaire survey prepared with aim to collect information about Biosecurity Measures Virus in small scale farms. Location and biosecurity assessment checklist

1. How long have you been working with chicken keeping?
2. .Flock size at and age at present?
3. Sources/foundation of poultry breeds:
    A. Purchased from Govt./Pvt. Hatchery/ research center___ B brokers
4. How is waste dealt with? A. Burning, B. fertilizer C. buried D. throw in the compound
5. Interaction of chicken with other chicken or wild birds. Yes ____no_____
6. Presence of rodents in your backyard? Yes _____no_____
7. Separate house for sick chicken? yes_____ No____
8. Disposal of dead chicken in the compound? yes_____, No____
9. Is there chicken mortality in your flock? Yes_____ no
10. Did you sell the diseased / recovered chicken at market? Yes____ no_____
11. Did your chicken get treatment at time of morbidity? Yes ______no_____
12. Is there foot bath in the get of poultry houses ? yes______ no_______
13. Do you have advisory service on poultry production? Yes______ No_____
14. How do you get poultry health care services?
    A. A By employed animal health professional______
    B. B By part-time animal health professional______
    C. C From private or public vet clinic___________
    D. D NONE
15. Do your poultry get protective vaccination for Newcastle disease?
    A. Yes_____ B. No_____ C. not known________

Annex VI: Preparation of Clinical Specimens and Inoculation of Cell Lines

PROCEDURE

Sample preparation

Swab

An identical procedure is used for the preparation of nasal, throatand rectal.Theswab is macerated well in the transport medium itself used for collection. The resultingsuspensions transferred to a centrifuge tube and centrifuge at 3000 – 5000 rpm for 20 minutes.The supernatant is collected and ready to be inoculated in to the suitable cell lines.

Lesion scrapings

Scrapings such as vesicle crusts, pock lesions, and tongue epithelium are removed from thebuffered glycerin or VTM (virus transport media), then Put in a mortar, and washed severaltimes with PBS containing antibiotics and antimycotic. Then by using coarse sterile sand, it is triturated thoroughly and a 10 % suspension made in PBS with antibiotics and antimycotic. It iscentrifuged at 3000 rpm for 20 minutes and the supernatant is collected for inoculation.

NB. Postmortem materials are processed as described for lesion scraping.

- ✓ Inoculation of DF 1 monolayer cell with the collected supernatants
- ✓ Wash the culture twice with PBS
- ✓ Inoculate the specimen suspension on the test flask (24-wells) and PBS with antibiotics and antimycotic(VTM) on the negative control flask and incubate at 37 o C for 60 minutes to allow the virusto adsorb on to the cell culture.(1ml for 25cm 2 and 3ml for75cm 2 tissue culture flask)
- ✓ Add maintenance medium (DMEM with 2% FCS) and incubate the flask (24-wells) at 37 o C for appropriate time, 3-7 days. Keep also control flasks without any specimen inoculums.
- ✓ Observe for the effect of virus action.

- ✓ It is essential that each material be passed in cell culture at least three times before declaring any specimen negative. The presence of viruses can be detected by observing cytopathic effect for some virus.

Annex VII: RNA Extraction Procedure

1. Prepared buffer AVL 560 µl containing carrier RNA was added in to 1.5 micro centrifuge tubes.
2. Sample of140 µl to the buffer AVL carrier RNA added in to the micro centrifuge tube and Mixed by pulse vortexing.
3. Incubated at room temperature (15-250C) for 10 minutes
4. The tube was briefly centrifuged to remove drops from the inside of the lid.
5. Ethanol (100%) 560 µl was added to the sample, and Mixed by pulse-vortexing for 15 seconds. After mixing, the tube was briefly centrifuge to remove drops from the inside of the lid.
6. Solution of 630 µl from step 5 was carefully applied to the QIAamp mini spin column (in a 2 ml collection tube) without wetting the rim.
7. The cap was closed, and centrifuged at (8,000 rpm) for 1 minute.
8. The QIAamp mini spin column was placed in to a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
9. The QIAamp mini spin column was carefully open and step 8 repeated
10. The QIAamp mini spin column was carefully opened, and 500 µl buffer AW1 was added.
11. The cap was closed, and centrifuged at (8,000 rpm) for 1 minute. Place the QIAamp mini spin column in to a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
12. The QIAamp mini spin column was carefully was opened, and 500 µl of buffer AW2 was added. The cap was closed, and centrifuged at full speed at (14,000 rpm) for 3min.
13. The QIAamp mini spin column was Placed in to a new 2 ml collection tube (not provided), and the old collection tube with the filtrate discarded and centrifuged at full speed for 1 minute.
14. Then, the QIAamp mini spin column was placed in to a clean 1.5 ml micro centrifuge tube (not provided) and the old collection tube containing the filtrate was discarded. The QIAampmini spin column was carefully opened and 60 µl of buffer AVE added to equilibrate to room temperature.
15. The cap was closed, and incubated at room temperature for 1 minute and Centrifuged (8,000 rpm) for 1 minute. Finally, Viral RNA stored at -200C until master mixing (Qiagen, 2014).

Annex VIII: Mixing Master Mixes

QIAGEN quantitative RT PCR kit

1. Preparation of master mix

1.1. Two positive controls (master mix positive control and exposed positive control) and 2 negative controls (master mix negative control and exposure free positive control)

1.2. Rnase free H2O=6.45µl*80=516 µl

1.3. five-x (5x) PCR buffer= 5 µl *80=400 µl

1.4. Mgcl2=1.25 µl *80=100 µl

1.5. dNTPs=0.8 µl *80=64 µl

1.6. Primer: FP (APMV-1, M+4100, 5‟‟AGTGATGTGCTCGGACCTTC-3‟‟) =0.5 µl *80=40 µl

RP (APMV-1, M-4220, 5‟‟CCTGAGGAGAGGCATTTGCTA 3‟‟)=0.5 µl *80=40 µl

1.7. Probe (APM-1, M+4100, 5‟‟-(FAM) TTCTCTAGCAGTGGGACAGCC (TAMRA)-3‟‟) =1 µl *80=80 µl.

NB. Lyophilized primers and probes was centrifuged briefly to ensure that the DNA pellet is at the bottom of the tube before they opened and reconstituted.

1.8. Inhibitor=0.5 µl *80=40 µl (inhibitors inhibit the binding primer with probe which will result in false positive)

1.9. One-step RT-PCR enzyme mix=1 µl *80=80 µl

2. All the reagents were added in to master mix tube and shake in vertex.

3.17 µl master mix was pipette and added into applied biosystem plate

4.8 µl RNA extractions was added into each applied bio-system plate (96)

5. The mixture was Transferred into amplification room and sealed to prevent evaporation loss

6. Finally, the mixture was inserted into the r PCR amplification machine and adjusted according to the manfacturer‟‟s instructions.

All the preparation was done in the PCR working station.