JIMMA UNIVERSITY

COLLEGE OF AGRICULTURE AND VETERINARY MEDICINE

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MOLECULAR AND SEROLOGICAL DETECTION OF NEWCASTLE DISEASEVIRUS IN BACKYARD CHICKEN PRODUCTION SYSTEM IN WOLISO DISTRICT, ETHIOPIA

MSC.THESIS

BY

ALEMAYEHU CHORAMO

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Molecular and Serological Detection of Newcastle Disease virus in Backyard Poultry Production System in Woliso District, Ethiopia

By

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MSc Thesis

Submitted to School of Research and Postgraduate Study, Jimma University College of Agriculture and Veterinary Medicine for the Partial Fulfillment of the Degree of Masters of Science /MSc/ in Veterinary Microbiology

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I have completed my thesis research work as per approved proposal and it has been evaluated and accepted by my advisors. Hence, I hereby kindly request the department to allow me to to present the findings of my work and submit the thesis.

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STATEMENT OF AUTHOR

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BIOGRAPHY

The author, Alemayehu Choramo, was born in Southern Nation Nationalities and Peoples Region (SNNPR),Dawro Zone, Tercha zuria woreda, Manta Guchili kebele in 1989 from his father Choramo Diko and from his mother Zerfnesh Dojamo. He joined primary school in 1997 and completed his primary education in Abba Bonga primary School. He attended his high school education at Tocha Secondary school in Tocha Wereda and attended preparatory school at Waka Senior Secondary School in Mareka Wereda ,Dawro Zone. He then joined Jimma University College of Agriculture and Veterinary Medicine in October 2009 and graduated with degree of doctor of veterinary medicine in June 2014 .After his graduation, he worked in Dawro zone, Tocha woreda livestock and fishery resource development office for about three years until he joined again Jimma University College of Agriculture and Veterinary Medicine in October 2017 for his post graduate study in veterinary microbiology.

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LIST OF ABBREVIATIONS AND ACRONOMYS

APMV	Avian Paramyxo Virus
cDNA	Complementary DNA
CSA	Central Statistics Agency
Ct	Threshold Cycle
CV	Coefficient of Variation
dNTPs	Deoxyribonucleotide Triphosphates
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
GDP	Gross Domestic Production
HA	Hemagglutination Test
HI	Hemagglutination Inhibition Test
HN	hem agglutinin-neuraminidase
NAHDIC	National Animal Health Diagnostic and Investigation Center
NCD	Newcastle Disease
NDV	Newcastle Disease Virus
OIE	Office International des Epizooties
OD	Optical Density
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipments
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
rRT-PCR	Real-Time Reverse Transcriptase Polymerase Chain Reaction
SPSS	Statistical Package for the Social Sciences
VNND	Velogenic Neurotropic Newcastle Disease
VVND	Velogenic Viscerotropic Newcastle Disease

ABSTRACT

Newcastle disease is one of the most important respiratory diseases. It is an infectious viral disease of domestic chicken and other species of birds regardless of variation in sex and age. It causes economic losses such as low growth rate and production, high expense on prevention and treatment, and high morbidity and mortality rate. Despite of these facts, no research report is available on Newcastle disease in the current study area. Therefore, A cross-sectional type of study was conducted with the objective of detecting Newcastle disease virus, using molecular and serological methods in Woliso district, South West Shewa zone, Oromia regional State from December 2018 to November 2019. Out 37 kebeles in the district, six kebeles were randomly selected. Convenience sampling method was used for swab and blood sample collection. For rRT-PCR detection, total of 76 pooled (380 individual) swabs and for serology, 348 serum samples, total of 728 sample collected .Real-time RT-PCR was done targeting matrix (M) gene, while indirect ELISA test was performed to detect anti-bodies against NDV and to determine its anti-body titer. Viral RNA extraction was conducted and rRT-PCR amplification was performed in SDS 7500 fast real time PCR machine (Applied Biosystems, USA), while ELISA test was performed using (ID.vet innovative version 2, Louis Pasteure-Grabels, France) procedures. In rRT-PCR test, 86.8% (66/76 pooled=330/380 individual) were positive for NDV, in indirect ELISA test 37.64 % (131/348) animals were positive and anti-body titer ranging from 998.01 to 11735.9 with mean value of (1761.9088) was scored. Standard deviation of 2592.42160 and percentage CV of 147% was scored. The mean antibody titer was significantly different (F = 1.993, P=0.0079) (Table 2) among the kebeles where the samples were collected. From the finding of this research, we conclude that both real-time PCR and indirect ELISA tests detected presence of the NDV, indicated circulation of the virus and heterogeneousity of anti-body titer in the study area. Therefore, further molecular characterization and epidemiological investigation should be carried out to distinguish circulating NDV genotype and associated risk factors respectively and also vaccine program should be scheduled and vaccination should be provided in the study area to prevent outbreak and economic loss that would occur.

Keywords: *Backyard-chicken, Newcastledisease, Molecular, Serology, Woliso*

1. INTRODUCTION

Poultry industry has been one of the most dynamic and ever expanding sectors, contributing much to the global economy. Poultry sector plays key role in poverty reduction at national and household levels in developing countries .Alarming poverty has been reported in Ethiopia with food and financial crisis (Habte *et al*., 2017). Poultry meat and egg are very important sources of nutrition and an important source of income for poor families, and therefore important for the rural development (Sonaiya and Swan, 2005).Poverty reduction and sustainable development of many developing countries rely on agriculture. Among agricultural sectors; livestock sector is important sector, contributing about 47% of the agricultural GDP, and 18.8% of the national GDP of Ethiopia (Habte *et al*., 2017).

Even though the commercial poultry production system is rapidly developening globally, it has been estimated that more than 80% of the global poultry population occurs in backyard family-based production systems and contribute up to 90% of the total poultry products in many countries (Sonaiya and Swan, 2005). In Africa, 80% of poultry production is in rural and peri-urban areas are based on traditional scavenging systems (Branckaert *et al*., 2000 and Habte *et al*., 2017).

In Ethiopian condition, Chicken production under backyard system has long been practiced in Ethiopia and almost every rural family owns poultry which has been widely used for egg, meat production and other purposes (Mogesse, 2007).Village chickens contribute more than 98% of the total poultry meat and egg production in the country (Udo *et al*., 2006). The total chicken population in Ethiopia is estimated to be 56.06 million out of which 97% is indigenous breed that are well adapted to the local environmental conditions (CSA ,2018).The majority (97%) of these chickens are maintained under this scavenging production system. However, in research, extension and development agenda the village indigenous chickens are poorly considered, focusing the commercial poultry sector which covers only approximately 3% (Reta, 2009).

Despite its role in raising incomes and reducing poverty in local communities of Ethiopia, backyard poultry production is hampered by wide arrays of constraints such as predators, poor management and nutrition (Selam and Kelay, 2013) and infectious diseases (such as Newcastle disease, infectious bursal disease, mycoplasmosis, pasteurellosis and salmonellosis, coccidiosis

and fowl pox) were also reported as the major causes of morbidity and mortality in poultry in Ethiopia (Chaka *et al*., 2012).

Among the poultry diseases, Newcastle disease (NCD) is one of the most important viral diseases .It is an acute infectious viral disease of domestic poultry and other species of birds regardless of difference in sex and age (Haque *et al,* 2010). The disease is characterized by respiratory problems, nervous system impairment, gastrointestinal and reproductive problems .The first outbreaks of Newcastle disease as a defined viral infection were reported in 1926 in Java, Indonesia and in Newcastle-upon-Tyne, England, from where it gained its name (Alexander, 2004).

There is no clear record about the introduction of the virus to the country (Ethiopia).however, NCD first occurred in and around seaports of the country and spread to the interior of the country along transport routs. The first documented outbreak of NCD in Ethiopia dates back to 1971 and reported from a small poultry farm in Asmara, Eritrea, located close to a seaport and the province of the country(former Ethiopia) (Sahlu *et al*. ,2015). The first NCD virus reported was a velogenic type, which caused about 80% mortality (Kebreab *et al.,* 2001). In the following years, the disease spreads fast to other parts of the country. In 1972, outbreaks had been reported in Addis Ababa , in 1974 in Haromaya (Alemaya) college of Agriculture poultry farm (Tadelle and Yilma, 2004) , in 1995, in the surrounding areas of Bishoftu (Debrezeit), Adama (Nazareth) and Addis Ababa that mortality was reported almost in 50% of the local birds. Since then it is occurring endemically in Ethiopiopia (Sahlu *et al*., 2015).

1.2. Background of the Problem

Intensification of agriculture is accelerating from time to time, in many countries of Africa due to improvement of novel technologies, financial initiatives, changing social infrastructure and private sector engagement (Pretty *et al*.,2011), and policies allowing increases in consumption and productivity for a variety of livestock species. Similarly, Ethiopian entrepreneurs are setting up large, intensively managed flocks of exotic breeds, particularly in areas close to Addis Ababa (Hutton *et al*., 2017).Government-owned poultry multiplication centers throughout the country,

non-governmental organizations and private individuals also distribute intensively reared chickens to smallholders. As a result, more urban and suburban households now keep flocks of 50 to 1000 birds under semi-intensive management (Godfray *et al*., 2010). The close links between intensive and smallholder farms could facilitate spread of diseases like Newcastle disease and other contagious diseases, exacerbated by low bio-security, and poor access to veterinary inputs and expertise among small holder producers (Sambo *et al.*, 2015).

The district is in close contact through live poultry marketing with Addis Ababa (114kms), which favors the flow of chicken to and from Addis Ababa .The movement of chicken marketing is from periphery to the center (rural to towns) and cross breed chicken multiplied in commercial farms around Addis Ababa, disseminated away from Addis Ababa(Hutton *et al*., 2017), which favors the spread of diseases either to Woliso or all over the country .Such movements of chicken gives high chance for spreading of diseases like Newcastle disease (Dessei and Ogle, 2001).

1.3. Justification of the Research

Newcastle disease (NCD) is one of the most devastating diseases of both domestic and wild birds that critically cripple the global poultry industry and it is a main constraint to poultry production system in Africa. The village chicken population in many parts of Ethiopia is endemically infected with NDV (Mazengia, 2012).Due attention is needed to control and prevent the disease as well as to combat economic loss occurrence due to the disease (Chaka *et al.*, 2013*).* Despite of these facts, no researcher or no attention has given to investigate the status of Newcastle disease in the current study area, only few research works were reported from northern parts of Ethiopia. Until this research work, there is no research work available on detection of presence of Newcastle disease in the current research area, which would help either as references for researchers or to design control and prevention method of the disease.

1.4. General Objective

To detect Newcastle disease virus in backyard poultry production system in Woliso district, South West Shewa, Ethiopia.

1.4.1. Specific objectives

- Molecular and serological detection of Newcastle disease virus in backyard poultry production system
- To determine the antibody titer against Newcastle disease virus in chicken sera

2. LITERATURE REVIEW

2.1. Definition

Newcastle disease is an acute, rapidly spreading, viral disease of domestic poultry and other birds in which the respiratory signs (coughing, sneezing, rales) are often accompanied or followed by nervous manifestations and infections with some strains result in diarrhea and swelling of the head (Sahlu *et al*. ,2015).

2.2. Synonym

As some other diseases, Newcastle disease also has many synonyms .These synonyms are: Avian Pneumoencephalitis, Ranikhet Disease, pseudo-fowl pest, pseudo poultry plagues, avian pest, avian distemper (Fikre, 2003 and Rahman *et al*., 2016).

2.3. Etiology

All avian paramyxoviruses(APMV) are part of the order Mononegavirales, family Paramyxoviridae subfamily Paramyxovirinae, genus Avula virus, (Lamb *et al*.,2005).There are 9 serotypes of APMV, but all isolates of Newcastle disease virus (NDV) belong to serotype 1 (APMV-1), therefore NDV is synonymous with APMV-1. The virus is single-strand, non-segmented, negative-sense and enveloped RNA virus, (wise *et al*., 2004).The APMV-1 viral genome of approximately 15 kb is composed of 6 genes encoding 6 structural proteins fusion (F), nucleoprotein (NP), matrix (M), phosphoprotein (P), RNA polymerase (L), and hem agglutinin-neuraminidase (HN)). Two additional proteins are encoded by RNA editing of the P- protein, namely proteins V and W. The cleavability of protein F is the main determinant for viral virulence, but other proteins such as HN and V are also believed to influence pathogenicity (Cattoli *et al*., 2011).

Although all NDV isolates belong to a single serotype (APMV-1), there is great genetic variability among different strains. Based upon phylogenetic reconstruction, NDV can be divided into 2 classes (I and II), each of those respectively subdivided into 9 and 14 genotypes (Miller *et al*., 2010 and Bello *et al*., 2018). Class I contains almost exclusively low virulence such as Strains recovered from wild waterfowl worldwide. Class II includes strains of low and high virulence isolated from poultry and wild birds (Chake *et al.*, 2013). Isolates belonging to class II

are all predicted to be virulent in chicken, except some isolates in genotypes I, II, and X (Bello *et al*., 2018).

In the last few years, there has been an increase of newly discovered geno-types, and some of the genotypes have been associated with increased virulence or expanded host range (Cattoli *et al.*, 2011). In addition, this genetic variability has raised concerns as to whether the commonly used commercial vaccines can provide protection against the very distant genotypes, not just in preventing clinical signs, but also in limiting shedding of the challenge viruses (Miller *et al*., 2010).

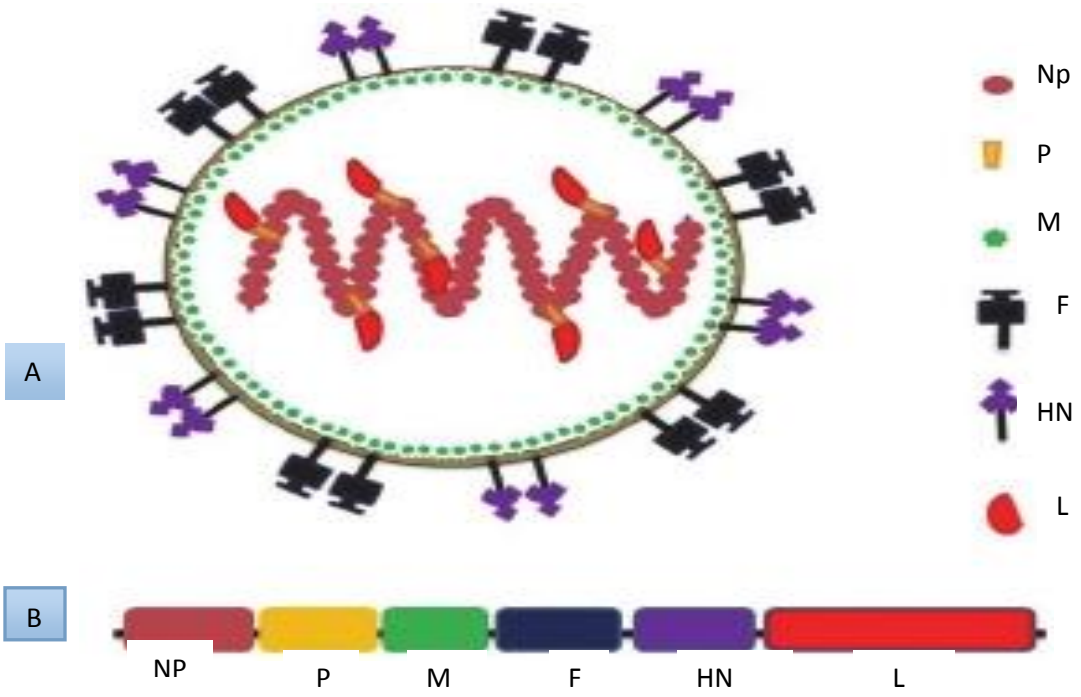

Figure 1: The structure of Newcastle disease virus: A, Morphology of the virion showing the locations of the viral proteins.NP, P, and L proteins associate with the RNA genome to form RNP(ribonucleic protein),while the M, F, and HN are membrane associated. B, Arrangementof the genes in the viral genome.

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

2.4. Virus Characterization

Newcastle disease affects a wide range of domestic and wild avian species; however, the severity of the disease varies greatly, spanning from per acute disease with almost 100% mortality to subclinical disease with no lesions (Cattoli *et al.,* 2011). Such variability makes it impossible to pinpoint NCD as a single clinic-pathologic entity. Based on severity of clinical disease, the strains of NDV were originally classified into 4 pathotypes, known as Doyle, Beach, Baudette, and Hitchner forms (Cattoli *et al.,*2011) .At present, patho-types are more commonly classified based on pathogenicity from least most pathogenic: "asymptomatic enteric," "lentogen" (formerly Hitchner), "mesogen" (formerly Beaudette), and "velogen." .The velogens have been further divided into "viscerotropic" (formerly Doyle; velogenic viscerotropic (VVNDV) or "neurotropic" (formerly Beach; velogenic neurotropic(VNNDV) according to their ability to cause primarily visceral or nervous signs(Alexander, 1998) .

Additionally, some laboratory classify the virus into 3 categories depending upon MDT (mean death time), IVPI (intravenous pathogenicity index), and ICPI (intra-cerebral pathogenicity index). All involve the use of numeric criteria. The MDT is the time to death, measured in hours, after inoculation of embryonated eggs (if the embryos die in less than 60 hr, it is classified as a velogen; if the embryos survive for more than 90 hr, it is classified as a lentogen; anything in between is a mesogen as shown in table 1 below) (OIE, 2008).

The IVPI test involves scoring illness (0 =normal; 1 = sick; 2 = paralyzed or nervous signs; 3 = death) after intravenous inoculation of 6-week-old chickens. The score of the IVPI test is calculated using the mean score per bird, per observation, over the 10-day period .The IVPI scores are computed similarly to ICPI and range from 0 to 3 (Cattoli *et al.,* 2011). According to some authors, velogenic NDV have IVPI scores between 2 and 3, mesogenic between zero (0) and 0.5, while lentogens have 0; however, there are no IVPI cut-off values to define notifiability to the international community. The IVPI test is not in widespread use today (Alexander, 1998). At present, according to international standards, (OIE,2008) the definitive in vivo assessment of virus virulence is based on the ICPI test, which is regarded as the most sensitive and widely used test for measuring virulence (Cattoli *et al*.,2011 and OIE,2008) .

The ICPI test is based on scoring sick or dead birds (0 = normal; 1 = sick; 2 = dead) every day for 8 -10 days after inoculation of virus intra-cerebrally into ten 1-day-old chicks(OIE, 2008).The score of the ICPI test is calculated using the mean score per bird, per observation, over the 8-day period. Scores range from 0 to 2, and any strain with an ICPI ≥0.7 is considered virulent or "notifiable" to the OIE (OIE, 2008).

Table 1: Pathotype designation of Newcastle disease virus strains based on standard pathogenicity tests.

Pathotype	MDT	IVPI	ICPI
Velogenic	<60	2-3	>1.5
Mesogenic	60–90	0.0-0.5	0.7–1.5
Lentogenic	>90	0	<0.7

*MDT = mean death time, measured in hours to death; IVPI =intravenous pathogenicity index, measures average scores; range from 0 to 3, ICPI =intra cerebral pathogenicity index, based on an average score of clinical signs over time (min. 0.0–max 2.0).

Source: (Cattoli *et al.*, 2011)

2.5. Persistence of Newcastle Disease Virus

New castle Disease can survive for several weeks in a warm and humid environment and indefinitely in frozen material. When using agents to inactivate virus, it is critical that the manufacturer's directions for the correct concentration of the solution, and the time needed for complete inactivation to occur, be followed. In addition, appropriate PPE (goggles, glove, and respirator) should be used. Newcastle Disease virus is inactivated by being heated at 56°C (132.8°F) for 3 hours, or 60°C (140°F) for 30 minutes (OIE, 2009). It inactivated by acidic pH (PH≤ 2) , multi-purpose disinfectants(such as ether or formalin), phenolics and oxidizing agents (e.g. chlorhexidine, sodium hypochlorite (6%).Survival of the aerosolized virus and long distance transmission are still subject to further study; aerosolized survival is likely dependent on humidity and a number of other environmental factors. Some the reports indicate that the virus is destroyed by dehydration and exposure to ultraviolet rays (CFSPH, 2008).

2.6. Pathogenesis

The pathogenesis of NCD Virus Strains varies greatly with and/or within the host. Chickens are highly susceptible but Turkeys, ducks and geese may be infected and show few or no clinical signs, even with strains lethal for chickens. In chickens, the pathogenicity of NCDV is determined chiefly by the Strain of the virus, although the dose, route of administration, age of the chicken and environmental conditions all has an effect. In general, the younger the chicken, the more acute the disease. With virulent viruses in the field, Younger chickens may experience sudden deaths without major clinical signs while in older birds the disease may be more protracted and with characteristic clinical signs. Breed or genetic stock appears to have very little effect on the susceptibility of chickens to the disease (Fikre, 2003).

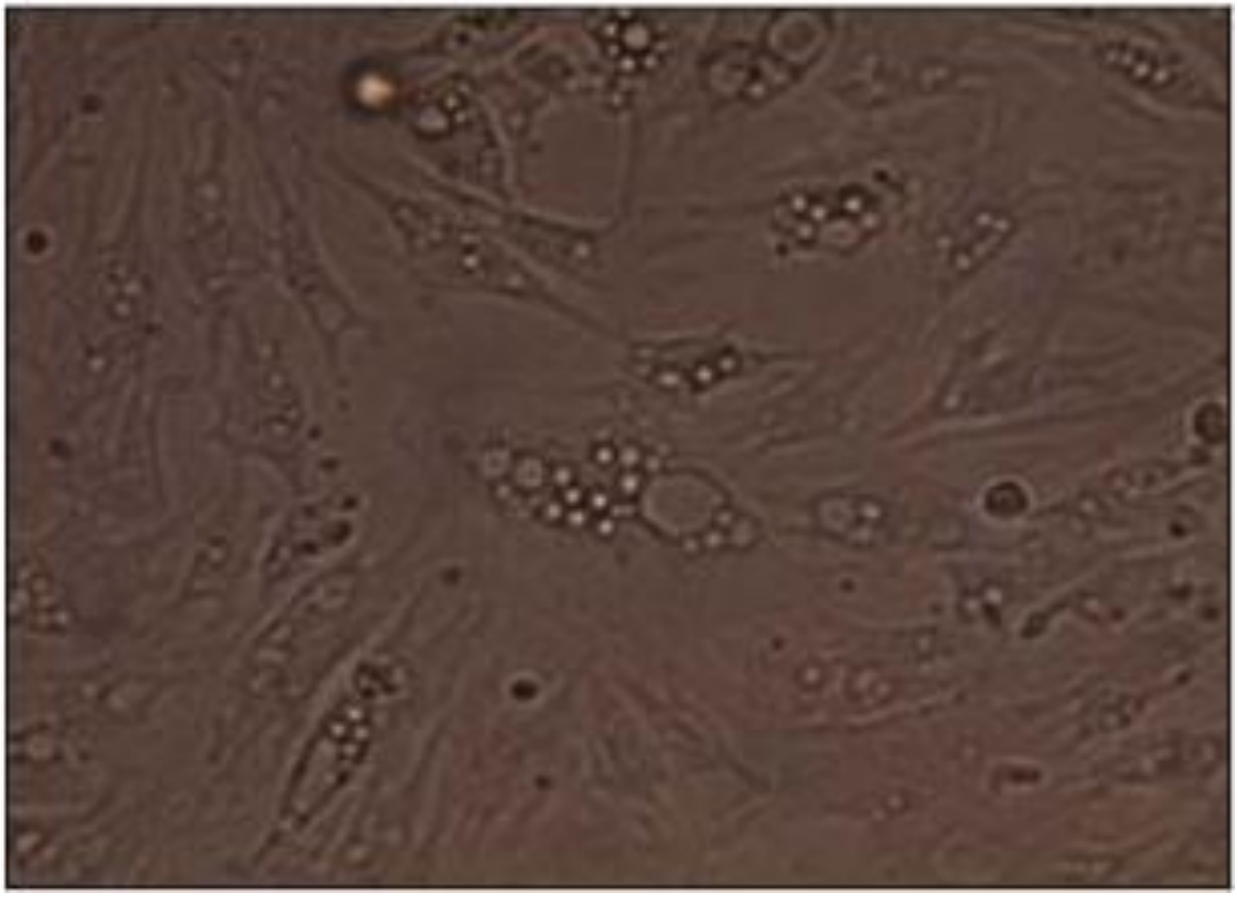

Figure 2: Chicken embryo fibroblast cells 72hrs Post-infection showing rounding, failure of cell adhesion, vacuolization, cell fusion and clustering and syncitium formation

Source: (Maqbool *et al.*, 2017)

2.7. Transmission

Newcastle disease is very contagious and is easily spread from one bird to another. The infection is usually transmitted by direct contact with sick birds or unaffected birds carrying the virus. Even vaccinated birds that are clinically healthy can excrete virulent virus after they have been exposed. Virus can also be transmitted indirectly by people, other animals, equipment, and vehicles. The infection takes place by inhalation or ingestion of the virus or by contact with mucous membranes, specially the conjunctiva. Infected birds shed virus in aerosol, respiratory discharge and feces. Infected birds start to excrete virus during the incubations period and continue to excrete virus for a varying but limited time during convalescence (Caupa, 2009). During the course of infection of most birds with NDV, large amounts of virus are excreted in the feces. Ingestion of feces results in infection; this is likely to be the main method of bird-to bird spread for a virulent enteric NDV and the pigeon variant virus, neither of which normally produces respiratory signs in infected birds (Caupa and Alexander, 2009).Vertical transmission (i.e., passing of virus from parent to progeny via the embryo) remains controversial. The true significance of such transmission in epizootics of NCD is not clear. Experimental assessment using virulent viruses is usually hampered by cessation of egg lying in infected birds. Infected embryos have been reported during naturally occurring infections of laying hens with virulent virus, but this generally results in the death of the infected embryo during incubation (Samrawit and Mulat, 2018).

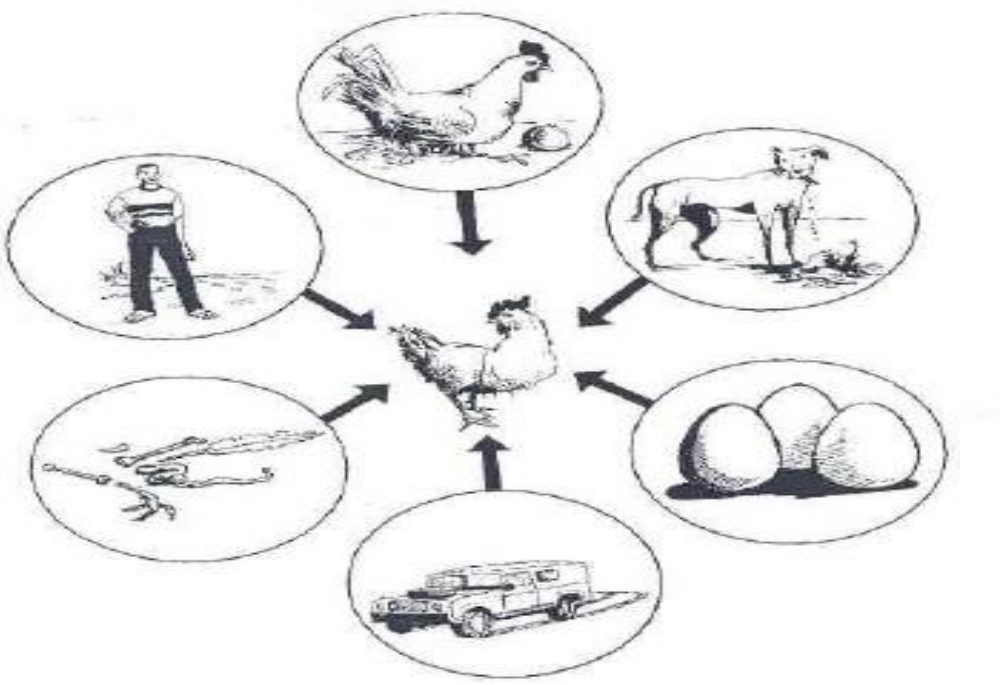

Figure 3: Source for transmission for NDV

Source :(Sahlu *et al*., 2015).

2.8. Incubation and Infectious Periods

Incubation periods for ND are variable depending on the host species. Newcastle disease incubation periods also vary depending on the strain of the virus; age, health, and other environmental factors. Most commonly, after natural exposure the period can be from two to twelve days with the average being five to six days (Alexander *et al*., 2008). However, the OIE Terrestrial Animal Health Code (2013) gives the incubation period for NCD as 21 days (OIE, 2013). It is possible for a bird to shed the virus before and after the appearance of clinical signs. Depending on the species of bird, virus shedding can last anywhere from one week to a year (Alexander *et al*., 2008).

2.9. Clinical Signs

The onset of the disease is often rapid and the first signs are usually seen throughout the flock. Spread is slower, if the fecal-oral route is the primary means of transmission, particularly for caged birds. Observed signs depend on whether the infecting virus has a predilection for respiratory, digestive, or nervous systems (Miller *et al.,* 2010). Young birds are usually more susceptible for infection but the disease causes heavy losses in birds of all ages (Kahn, 2005).Clinical signs vary considerably according to the virulence and tropism of the NCD virus involved, the species of bird, the age of host, the immune status of the host and environmental conditions. As a result, none may be regarded as a specific sign of NCD. Chickens infected with virulent NCD virus strains may die without showing any signs of illness. The chicken flutes its feathers ,appears to have its coat dragging on the ground, lethargy , in appetence, respiratory signs such as mild rales and snick can be detected by careful observation. Severe respiratory distress , gasping, Swelling of the head , neck, greenish diarrhea, marked decrease in egg production sometimes deformed eggs may be produced. Nervous signs of tremor, torticollis, convulsions and paralysis of wings and legs will not be seen until the disease is advanced. Mortality may be very high, often reaching 50 to 100%.Other domestic poultry such as turkeys and pigeons may also be affected. Normally ducks are resistant to the disease but on occasions, ducklings may be affected (Sahlu *et al*., 2015).

2.9.1. Signs among Young Chicks and Growing Chickens

In young chickens and growing chickens gasping, coughing, aphonia, depression, partial or complete inappetance, increased thirst is the common findings of respiratory system (Cattoli *et al.*, 2011). Nervous signs including partial or complete paralysis of extremities, muscular tremor and rhythmic, clonic spasms. Peculiar attitudes including “Torticollis”, opisthotonus, emprosthotonus and lateral deviation of head, partial or complete paralysis of one or two legs were the only sign observed in several outbreaks of NCD (Miller *et al.,* 2010).

2.9.2. Signs in Laying Flock

The disease usually appears suddenly and spreads quickly through susceptible flocks, drop in egg production, laying of soft or imperfectly shelled, profuse fluid diarrhea, rapid dehydration of body, also effects like Absence of air cells, watery albumen and a rough discolored and chalky shells egg quality. A high per cent of hens lay abnormal eggs up to 45 days after infection (Cattoli *et al.*, 2011).

Figure 4: Deformed eggs due to Newcastle disease

Souce :(Miller *et al.,* 2010)

2.9.3. Clinical Signs Based on Tissue Tropism and phatogenecity

2.9.3.1.Velogenic Viscerotropic Newcastle Disease

In case of velogenic viscerotropic Newcastle disease (VVND), mortality can easily reach 100%, and in experimental conditions, the course of disease is rapid, usually 2–4 days. Clinical signs are first recognizable starting at 2 days post infection (dpi)(Brown *et al.,* 1999).The main signs are conjunctival swelling and reddening centered over the lymphoid patch located in the lower eyelid, anorexia, ruffled plumage, prostration, weakness, tremors, and diarrhea; labored breathing is variably reported (Cattoli *et al.,* 2011).some experimental work reports show that in numerous animal experiments conducted with the same techniques used in the same laboratory

(infection via eye-drop instillation in 4-week-old chickens), respiratory signs were observed very rarely and were limited to open-mouth breathing in a few animals. In the absence of respiratory lesions, the open-mouth breathing was interpreted as polypnea and a consequence of a generalized febrile state (Kommers *et al*.,2003).

2.9.3. 2.Velogenic Neurotropic Newcastle Disease

Morbidity with velogenic neurotropic Newcastle disease (VNND) often reaches 100%, and mortality is usually 50% (but can rise to 100% in young chickens). The most prominent clinical signs are neurologic and consist of head twitch, tremors, opisthotonus, and paralysis (Kommers *et al*., 2003). Despite the fact that the neurologic involvement can be dramatic, the animals are characteristically bright and alert, and if able to reach food, will eat. The course of the disease is longer than with VVND, and the neurological signs are most prominent between 5 and 10 days post infection(dpi), which is beyond the point of survival with most VVND strains, where animals often die at 4 or 5 days post infection(dpi) (Cattoli *et al.*,2011).

Figure 5: Torticollis (twisting of head) due to Newcastle disease (VNND).
Source :(Habte *et al*.,2017)

While, according to some reviews (Alexander *et al*., 1998) respiratory signs are considered a prominent feature of infection with velogenic neurotropic strains, there is an absence of original reports (at least in the recent literature) that describe respiratory clinical signs or respiratory lesions in animals experimentally infected with VNNDV. According to Brown *et al* report, when 4-week-old chickens were infected via eye-drop instillation with 4 neurotropic strains respiratory distress was not observed, and the neurologic signs predominated (Cattoli *et al.,* 2011). Experimental infection with VNNDV, described severe respiratory signs (i.e., mouth breathing and gasping by 4 dpi) followed by nervous signs at 11–12 dpi (Brown *et al.,* 1999).

Figure 6: Sick chicken with acute respiratory distress due to NCD

Source :(Caupa, 2009).

2.9.3. 3.Mesogenic Newcastle Disease

Mesogenic viruses in field conditions cause mild clinical signs, mainly respiratory signs. Field

outbreaks with mesogenic strains also have been associated with a drop in egg production and misshapen eggs (figure 4) (Cattoli *et al.,* 2011). Concurrent viral and secondary bacterial infections are thought to be common complications of mesogenic NDV that result in more sever morbidity (Brown *et al.,* 1999).

In contrast to what is observed in the field with mesogenic strains, experimental inoculation of specific pathogen free (SPF) chickens with most mesogenic strains causes very minimal clinical signs (mostly slight depression), but not any signs specifically related to the respiratory system. In numerous animal experiments conducted with similar methodology, mesogenic strain infection will in rare cases result in neurologic signs, similar to those observed with VNND, but much milder, and with lower mortality rates (Kommers *et al*., 2003).

2.9.3.4. Lentogenic New Castle Disease

It is generally accepted that lentogenic viruses do not cause disease in adult chickens. Although some textbooks refer to La Sota as causing severe respiratory disease in very young animals, no references could be found in the scientific literature (Alexander *et al*., 1998). When the lentogens B1 and QV4 were experimentally inoculated into 4-week-old chickens, or when QV4 was inoculated into 7-week-old chickens, in both cases via eye-drop instillation, no clinical signs were observed. Some lentogenic isolates in Australia have been associated with respiratory disease in commercial broilers in the field ("late respiratory syndrome") with very low mortality, detectable gross lesions (reddening of the trachea), and chronic non-suppurative tracheitis histologically. However, *Escherichia coli* were consistently isolated from the tracheas of the diseased birds, indicating that the clinical disease may well have been multi-factorial in nature. In another report, there were mild clinical signs consisting of rales, coughing, anorexia, and depression observed between 2–12 dpi when a lentogenic strain was aerosolized at high concentration into 40-day-old SPF chickens. However, the high dose delivered directly to the respiratory system may have affected the clinic-pathologic syndrome (Cattoli *et al.,* 2011).

2.10. Pathology

Pathological conditions observed, either the gross lesions or the organs affected in birds infected with NDV are dependent on the strain and patho-type of the infecting virus, in addition to the host and all the other factors that may affect the severity of the disease (Piacenti *et al*., 2006).Accordingly, various patho-histological conditions of Newcastle disease are discussed as follows.

2.10. 1.Velogenic Viscerotropic Newcastle Disease.

Common gross lesions are: the presence of multifocal hemorrhages seen through the serosal surface of the intestines, multifocal areas of necrosis and ulceration of the gut-associated lymphoid tissue, and disseminated foci of necrosis in the spleen are highly suggestive of VVNDV infection (Susta *et al*., 2011). The ceacal tonsils, which are especially prominent gut lymphoid aggregates located in the proximal portion of the ceca, are often regarded as the"old faithful" lesion for VVND, as they most consistently display hemorrhage and necrosis grossly. Peri-thymic hemorrhages are occasionally observed, and as the disease progresses, there is severe atrophy of thymus and bursa. Comb and wattle edema are variably present. Eyelid edema and hemorrhage are consistent findings in animals inoculated via the conjunctival route. They are markedly hemorrhagic and appear to result from necrosis of the intestinal wall or lymphoid tissues such as cecal tonsils and Payer's patches (Miller *et al*., 2010).The most unifying microscopic lesion histological feature is severe necrosis of the lymphoid tissues scattered throughout the body, most especially prominent in spleen and gut-associated lymphoid tissue, which corresponds to the foci hemorrhage and ulceration noted grossly (Alexander, 2008). In the less severe, or initial stages, there is lymphoid depletion and hyperplasia of macrophages with large vacuolated cytoplasm (commonly referred to as the "starry-sky" effect). In later stages, there is accumulation of cellular and karyorrhectic debris, pyknosis, and numerous macrophages with vacuolated cytoplasm that contain nuclear debris (Miller *et al*., 2010).

2.10.2. Velogenic Neurotropic Newcastle Disease

Gross lesions are often absent, and the involvement of the visceral organs appears to be minimal, although animals euthanized in the early stages of disease may have splenic or proventricular

congestion. Despite the neurotropism of these strains, gross lesions in the central nervous tissue are not present. In comparison to VVND, there are no characteristic gross lesions for VNND (Miller *et al*., 2010). Microscopic histo-pathologic changes in chickens infected with VNND strains are largely restricted to the central nervous system. There is multifocal mononuclear peri-vascular cuffing associated with hypertrophy/hyperplasia of vascular endothelium, moderate gliosis, and multifocal necrosis of the Purkinje cells. Other reported histologic lesions with VNNDV are lymphoid depletion, and myocarditis. No reports of documented pneumonia with VNNDV were found in the literature (Brown *et al.,* 1999).

2.10.3. Mesogenic Newcastle Disease

Gross lesions with mesogenic strains are minimal. 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, which have their own set of morphologic correlates (Alexander, 2004).Microscopic histological lesions, there is a range of changes 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 cases caused by the VNND strains (i.e., peri-vascular cuffing and gliosis) (Susta *et al*., 2011).

2.10.4. Lentogenic Newcastle Disease

Grossly, lentogenic strains produce mild pulmonary hemorrhages and splenomegaly was described. Lentogenic strains of NDV had 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 (Hooper *et al*., 1999). Microscopic lesion of these strain are hyperplasia of the lymphoid follicles in spleen and air sacs were present, lymphoid follicle proliferation mainly in the lamina propria of the trachea (Susta *et al*., 2010). Some lentogenic isolates in Australia caused non-suppurative tracheitis in association with E. coli in field outbreaks, or, when experimentally inoculated in SPF chickens, induced mild changes, including lymphocytic infiltration, loss of cilia, and squamous metaplasia in the proximal trachea. Aerosol delivery of the virus causes congestion, goblet cells hyperplasia,

edema, and multifocal submucosal infiltration of scattered heterophils, lymphocytes, and plasma cells) (Hooper *et al.*, 1999).

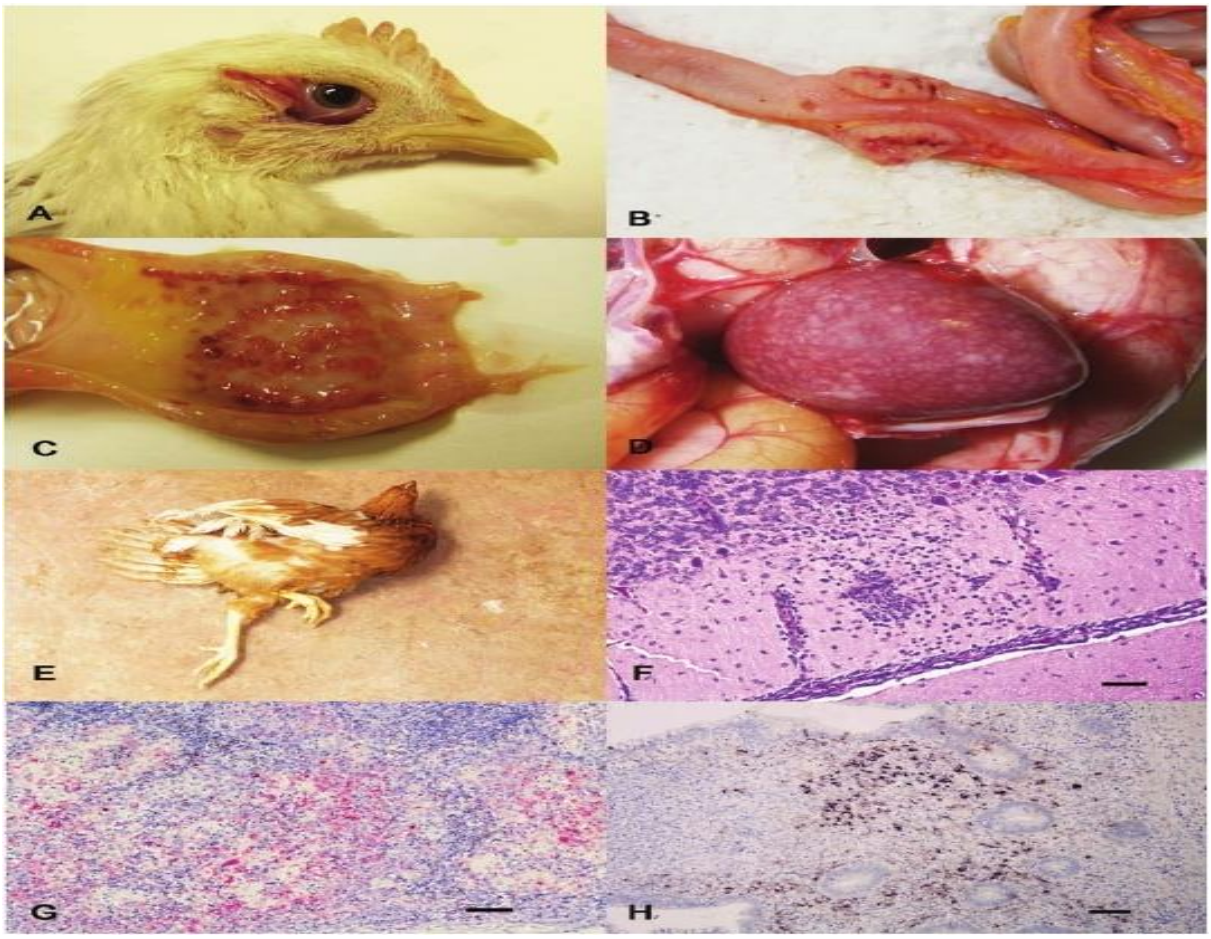

Figure 7: Clinical and pathologic features of Newcastle disease virus (NDV).

Velogenic viscerotropic Newcastle disease (VVNDV): **A**, hemorrhage within the crescent-shaped lymphoid patch in the lower eyelid is a characteristic early feature of NDV. **B**, Focal hemorrhage and necrosis of cecal tonsils occurs in infection. **C**, hemorrhagic foci in the proventriculus correspond to necrosis of underlying lymphoid tissue. **D**, mottled spleen indicating multifocal necrosis. Velogenic neurotropic Newcastle disease (VNNDV): **E**, birds are often bright and alert but have hemiparesis. **F**, histologically, brain lesions are prominent in velogenic neurotropic ND and consist of extensive gliosis and astrocytosis; cerebellum. **G,** Velogenic viscerotropic (VVNDV), immunohistochemical staining for viral nucleoprotein reveals numerous infected cells in spleen. **H**,riboprobe in situ hybridization for matrix gene reveals abundance of infected cells morphologically compatible with macrophages.

Source :(Cattoli *et al.*,2011)

2.11. Diagnosis

Diagnosis is based on history, signs and lesions may establish a strong index of suspicion but the Laboratory confirmation must be done. Hemagglutination test and hemagglutination inhibition test, virus neutralization test, Enzyme linked immune-sorbent assay, plaque neutralization test and reverse-transcriptase polymerase chain reaction (RT-PCR) can be used for confirmation of the NCD virus (Kim *et al.*, 2007). Now RT-PCR is the most exclusively used method to detect AIVs and NCD Virus. Reverse-transcriptase RT-PCR assay is more sensitive, specific and less labor intensives as compare to other conventional methods used for lab diagnoses such as virus isolation, Immuno Fluorescence Staining, Neuraminidase Inhibition and ELISA (Samrawit and Mulat , 2018). Using modern technologies, new diagnostic techniques are being developed for identification and differentiation of NDV strains. Other molecular diagnostic test like nucleotide sequence analysis is also important in viral disease diagnosis (Kim *et al*., 2007).

2.11.1. Culturing and isolation of the Virus

Suspension of homogenated organs, feces, or swabs prepared as for isolation in eggs may be used for attempted isolation in cell cultures. The APMV-1 strains can replicate in a variety of cell cultures of avian and non-avian origin, among which the most widely used are: chicken embryo liver cells, chicken embryo kidney cells, chicken embryo fibroblasts, African green monkey kidney cells, avian myogenic and chicken embryo–related cells (Cattoli *et al.*,201). Primary cell cultures of avian origin are the most receptive. Viral growth is usually accompanied by cytopathic effects typically represented by disruption of the monolayer and formation of syncytia .The virus also causes the formation of plaques, which according to the level of the cytopathic effect can appear clear, dull, or very dark and have a variable diameter from 0.5 to 4.0 mm. The majority of velogenic and mesogenic strains cause the formation of clear plaques. Effective replication and plaque formation in chick embryo cells for lentogenic viruses is conditioned on the presence in the culture of Mg^{2+} ions and diethylaminoethyl dextran or trypsin(0.01 mg/ml) in the culture medium(Terregino and Capua, 2009)

2.11.2. The ELISA

The ELISA test works on the principle of recognition of anti-NDV antibodies of a viral antigen-coated plate, the bound serum antibodies are consequently detected by anti-chicken antibodies produced in another species that is conjugated in a reporter molecule (Alexander *et al.*, 2004). An ELISA kit for the detection of antibodies against NDV, which is designed to be easily transportable and to give uniform results under widely varying ambient temperatures, has been developed by the Animal Production and Health Section of the Joint Food and Agricultural Organization the United Nations (FAO) and International Atomic Energy Agency (IAEA) division .This ELISA is considered as accurate, rapid and sensitive compared to the HI test (Tabidi *et al.*, 2004).

2.11.3. Haemagglutination (HA) Test

The allantoic fluid containing dead embryos, or those chilled at the end of the fourth through seventh day, are tested for hemagglutinating (HA) activity, as hemagglutination is a key feature of ND viruses. However, avian influenza (AI) viruses and other avian paramyxoviruses will also cause hemagglutination, so distinction is essential. If HA activity is detected, the hemagglutinating agents should be identified by means of the hemagglutination inhibition (HI) test, which uses specific sera, or by molecular tests, which may provide information on the pathotype and genotype. Some APMV-1 strains lose the hemagglutinating capacity when heated at 56°C for 5 minutes, but retain infectivity for chicken embryos even after 30 minutes at the same temperature. Influenza viruses, instead, always lose their infectivity before the loss of HA ability. On the basis of the response to heat treatment, it may be also possible to distinguish between 2 types of lentogenic viruses. In fact, classical vaccine viruses (e.g., La Sota or B1 strain) can be heat-inactivated while other lentogenic viruses as well as mesogenic and velogenic strains remain infectious after the treatment (Cattoli *et al.*,2011),

2.11.4. Hemagglutination Inhibition (HI) Test

The HI test is based on the principle that the haemagglutinin on the viral envelope can bring about the agglutination of chicken red blood cells and that this can be inhibited by specific anti-

bodies. The HN is a surface protein believed to be a key in determining the serological classification of viruses currently based on the serological HI assay .The HI test is simple to perform, but difficult to standardize amongst laboratories. HI is considered cheaper than ELISA as no microplate reader is required in addition to the cost of the ELISA kit (Tabidi *et al.*, 2004).

In the HI test, some level of cross-reactivity may be observed among the various avian paramyxovirus serotypes. Cross-reactivity can be observed between APMV-1 and APMV-3 viruses (particularly with the psittacine variant of APMV-3, commonly isolated from pet or exotic birds) or APMV-7(Cattoli *et al.*, 2011*).* The risk of mistyping an isolate can be greatly reduced by using a panel of reference sera or monoclonal antibodies (mAbs) specific for APMV-1, APMV-3, and APMV-7. The use of monoclonal antibodies also permits characterization of antigenic differences within different strains of APMV-1 or even between subpopulations of the same strain (Chaka *et al.,* 2013).

2.11.5. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Using PCR, specific sequences within a DNA or cDNA template can be copied, or "amplified", many thousand to a million-fold using sequence specific oligo-nucleotides, heat stable DNA polymerase, and thermal cycling. In traditional (end point) PCR, detection and quantification of the amplified sequence are performed at the end of the reaction after the last PCR cycle, and involve post-PCR analysis such as gel electrophoresis and image analysis (Pham *et al*., 2005).

The short comings of the conventional diagnostic techniques warrant the need for the development of more rapid, yet very accurate methods of NDV diagnosis in poultry. The most commonly used molecular test in NDV diagnosis especially in the developing countries is RT-PCR. The test can rapidly and accurately detect viral genome in clinical samples with high sensitivity especially if appropriate sample are taken (Bello *et al.,* 2018).

The first reverse-transcriptase polymerase chain reaction (RT-PCR) for the detection of NDV was introduced by Jestin and Jestin (1991) in infected allantoic fluids employing universal primers to amplify a 238 base pair (bp) section of the F gene. Later on a system that enabled

detection of the virus directly in tissue or feces from infected birds was introduced (Gohm *et al*., 2000). Since then several reverse transcription-based molecular techniques including real-time RT-PCR (rRT-PCR), targeting specific portions of the genome have been developed (Wise *et al*., 2004).

Real-time RT-PCR compared to conventional PCR, came with the advantage that a post-PCR processing step is avoided, which allows a savings in time and labor. In real-time quantitative PCR, PCR product is measured at each cycle. By monitoring reactions during the exponential amplification phase of the reaction, users can determine the initial quantity of target with great precision (Life Technologies, 2012).

The molecular techniques afford the possibility of differential diagnosis by multiplex rRT-PCRs of pathogens causing similar clinical signs, e.g. avian influenza virus and NDV. Although extensive variation among NDVs still poses technical problems, e.g. false negatives because of genetic variability of the nucleotide composition of the region targeted by probes (Cattoli *et al.*,2010), the real and potential advantages of a molecular biological approach to ND diagnosis appear to be overwhelming. In addition to RT-PCR methods, other molecular approaches, such as loop-mediated isothermal amplification (LAMP), have also been developed for detection of NDV (Pham *et al*., 2005).

2.11.6. Differential Diagnosis

The clinico-pathological picture of NCD gives important clues in making clinical diagnosis. However, a number of viral and bacterial diseases may manifest similar clinical features that could be confused with NCD. The commonest differentials of NCD include highly pathogenic avian influenza, avian infectious bronchitis, infectious laryngotracheitis, and diphtheritic form of fowl pox. Others include fowl cholera, mycoplasmosis, and psittacosis in psittacine avian species (Bello *etal.*, 2018). Distinguishing ND from all these diseases is a crucial task in arriving at tentative diagnosis. Some important thumb rules to differentiate the two in an ordinary laboratory are: - (1). The avian influenza virus can haemagglutinate rabbit erythrocytes, whereas NCD virus does not, and (2). Avian influenza virus does not produce disease in pigeons, whereas NCD virus cans (Sahlu *et al*., 2015).

2.12. Zoonotic Importance of Newcastle Disease

Newcastle disease is a zoonotic disease, though not one that poses a significant threat to public health (Bello *et al*., 2018). Human infection via exposure to infected birds can cause disease with symptoms of headache, flu, and mild conjunctivitis (4 to 7 days), rarely becoming severe or leading to lasting visual impairment in severe cases (Swayne and King, 2003).Individuals most likely to become infected are those working in the poultry industry or in laboratories; evidence has linked past human NCD infection with lack of correct eyewear while working with commercial poultry. Although there is no evidence to indicate that the virus is contagious from one human to another, responder groups and vaccination crews should comply with the appropriate biosecurity and safety measures, including the use of personal protective equipment (PPE). Transmission of NCD through the consumption of properly cooked poultry products has not been reported. Immuno-suppressed individuals are urged to take extra care to avoid exposure (Goebel *et al.,* 2007).

2.13. Economic Importance of Newcastle Disease

Newcastle disease poses major problem to the nation as the disease is a sporadic epizootics despite implementation of routine vaccination programs (Kryger *et al*., 2010). The consequences of the disease are low growth rate and production, high expense on prevention and treatment, and high mortality rate (Amanu and Rohi, 2005). The lentogenic form is responsible for erosive losses in broilers including lowered gain and feed conversion efficiency and elevated mortality and condemnation. Where chickens are raised commercially, either in developing or developed countries, outbreaks have occurred in many locations, causing massive economic damage through control efforts and trade losses. For instance, during the last major outbreak in the United States, in California in 2002–2003, more than 2,500 premises were depopulated (4 million birds) at a cost of US$162 million (Cattoli *et al.,* 2011).

The severity and financial impact depends on climatic and management stress and inter current exposure to pathogenic E. coli and other viral respiratory disease and immunosuppressive agents. The cost and consequences (respiratory stress) of vaccination are significant, especially during winter and following immune-suppression. Disruption of trade and the cost of eradication of VVND in non-endemic countries impose a significant burden on producers and the public sector

after outbreaks (Chang and Dutch, 2012).

The Office of International des Epizooties (OIE) classified ND as list "A" disease (Cattoli *et al.,* 2011). According to OIE (1996), list A diseases are defined as "transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders, that are of serious socio-economic or public health consequence and that are of major importance in the international trade of animals and animal products (Alexander, 2000).

2.14. Status of the Disease in Ethiopia

Newcastle disease is considered as endemic in the village chicken population in Ethiopia. A number of studies have been conducted to determine the prevalence of NCD in different agro-ecology and season of Ethiopia. It is mentioned as one of the most important disease problems in backyard chickens in most parts of Ethiopia. Mortality may be very high, often reaching 50 to 100 %. The prevalence of NCD varies among years in Ethiopia. Literature review shows that, starting from 2005 the prevalence of NCD is endemic as reported by different researchers. Some previous research work reported from some parts of the country include: Zeleke *et al*. (2005) reported 0%, 0%, 12.9%, 16.7%, 35.9% and 47.6% from Arbegona,Shebedino, Hawassa, Butajira, Alage, Hossana, respectively; Serkalem *et al*. (2005) reported 28.6%, 29.7% and 38.% at Debreberhan, Sebeta and Adama, respectively; Mazengia *et al*.(2010) reported 21.7% from Bahir Dar; Chaka *et al*. (2012) reported 5.14% and 7.12% from Adamitulu gido kombolcha (ATGK) and Adea, respectively; Nega *et al*. (2012) reported seroprevalence of 55.8% ; Chaka *et al.* (2013) reported 16.73% and 32.2% from Adamitulu gidokombolcha and Adea, respectively; Belayheh *et al*. (2014) reported 5.6% at Kersna Kondality; Desalegn *et al.*(2016) and Miressa *et al*.(2016) reported 28.6% and 26.7% from east Shewa respectively .In the same year Minda *et al*.(2016) reported prevalence of 27.86% from Bale zone.

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Woliso district, South West Shewa, Oromia Regional State,Central Ethiopia and 114km away from the capital city Addis Ababa. Woliso geographically lies between longitude of $37^0$58'16.3" E and latitude of $8^0$32'23.0" N and 2,063 meters above sea levels. The area is characterized by binomial rain fall, long season (from June-September) and short season (March-April) and minimum annual temperature is 13.6^0c and maximum annual temperature is 25^0c with average annual temperature19.3^0c .The livestock population for the district is:224334 cattle, 39543 sheep, 51042 goat, 7625 horse, 6164 mule,16320 donkey and 147,679 poultry.

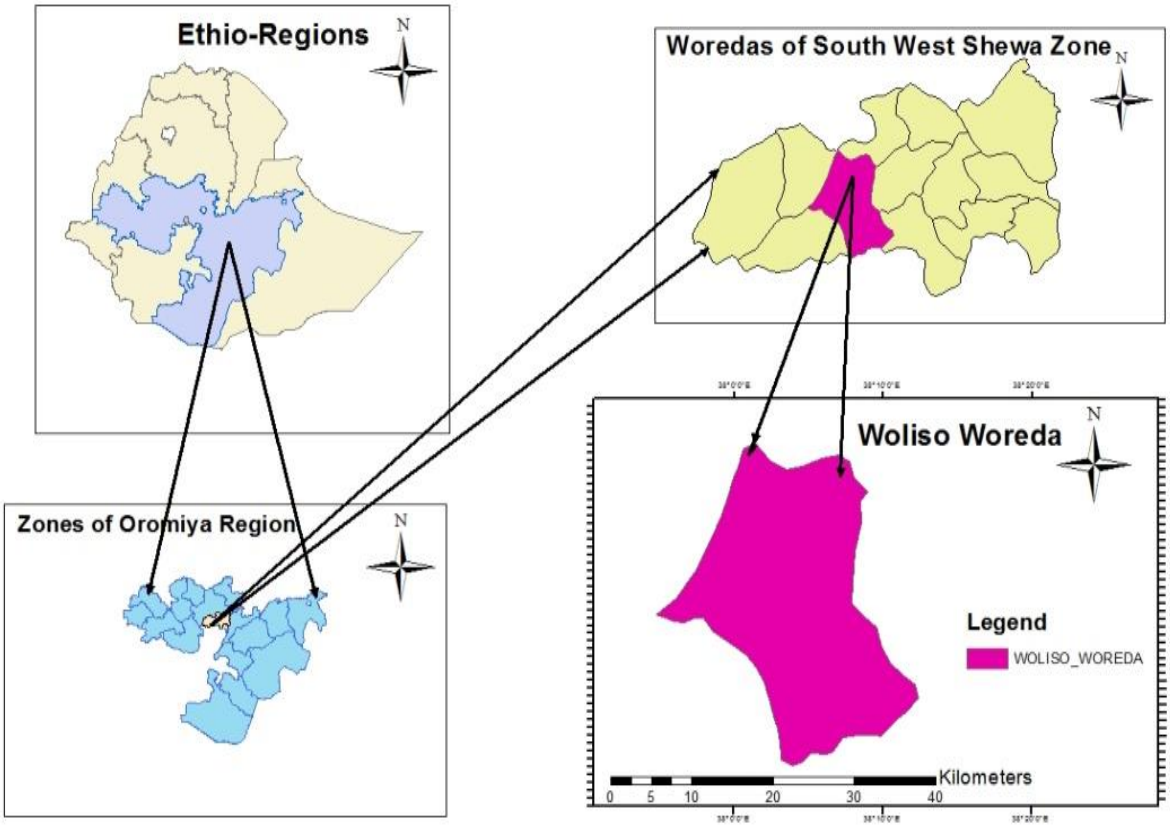

Figure 8: Map of the study area

3.2. Study Animals

The study population includes chicken of all ages, both sexes, both cross and local breeds which were managed under backyard production system and chicken that have no history of vaccination against Newcastle disease.

3.3. Study Design and Sample Size Determination

A cross-sectional type of study was carried out from December 2018 to November 2019 to detect gene of Newcastle disease virus and antibodies against Newcastle disease virus in backyard poultry production system. Accordingly, six kebeles (Badessa Koricha, Obbi Koji, DireDuleti, GururaBaka, Tombe Anchabi, and Fedu Gora) were randomly selected from 37 kebeles in Woliso district. Each household, who has chicken, was registered and number of chicken (samples) needed from each kebele determined. Convenience sampling method was used to collect sample data for this study. The number of animals that has be sampled determined by the formulae disease of detection given by (Stevenson and EpiCentre ,2008):

$$n = (1 - \alpha^{1/D}) \times (N - \frac{D - 1)}{2}$$

Where, N: the population size=147679

α: 1 - confidence level (α = 0.05)

D: the estimated minimum number of diseased animals in the group

(Population size × the minimum expected prevalence)=147679×16.7 %(NCD prevalence)

$$n = (1 - \alpha^{1/D}) \times (N - \frac{D - 1)}{2} = (1-0.05^{1/24662}) \times (147679-\frac{24662-1}{2}) = 553$$

Accordingly,for molecular detection a total of 380 swab samples from (190 chicken), of which 190 individual oropharengial samples in 38 pools each containing 5 samples and 190 individual cloacal samples in 38 pools each containing 5 samples were collected from the same chickens. For serology, 348 blood samples collected from those six peseantory associations (kebeles) listed above (Table2). Totally, 728 samples collected from 538 chickens.

3.4. Sample collection

3.4.1. Swab samples

Swab samples, including tracheal (oropharyngeal) and cloacal samples were collected from backyard chickens (Annex 4). Cotton swabs and viral transport medium in cryovial tubes were used to collect samples. Briefly, oropharyngeal and cloacal areas of the chicken was wiped using separate cotton swabs .Each swab was immersed in 2 ml cryovials containing 1.5 ml VTM (virus transport media) solution and squeezed to release the sample, then cotton swabs were removed and appropriately disposed. The cryovials were labeled and kept in icebox during samplingand transportation to National Animal Health Diagnostic and Investigation Center (NAHDIC) and stored at -80^{o}c until processing.

3.4.2. Serum samples

For serology test, 348 blood samples of 2 ml was collected from the brachial vein in 3ml disposable syringes, left horizontally for 3hours, and then vertically for the serum to ooze out. Serum was collected in labeled 2ml cryovial tubes and kept cool in icebox during collection and transportation to National Animal Heath Diagnostic and Investigation Center (NAHDIC), Sebata. The serum in the cryovial tubes was stored at −20°C until processing.

3.5. Laboratory Analysis

3.5.1. Real Time Reverse-Transcriptase Polymerase Chain reaction (rRT-PCR)

3.5.1.1. RNA extraction

Viral RNA extraction was conducted using QIAamp, USA viral RNA extraction kit according to manufacturers' 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 ml micro centrifuge tube and 140 µl of sample was added to the buffer AVL carrier RNA in the micro centrifuge tube. The solutions were then mixed by pulse-vortexing and incubated at room temperature (15-25°C) for 10 minutes. The tubes were then

briefly centrifuged to remove drops from inside the lid. Then equal amount of 560 µl of absolute ethanol was added to filtrate and mixed thoroughly and washed with 500 µl washing (AW 1 and AW 2), any unwanted protein and DNA were removed. Then, 60 µl (micro liter) of elute solution (AVE) was added to collect the RNA extract. Finally, the eluted RNA was kept at-20°C (Annex1 and Qiagen, Inc., Gaithersburg, MD, USA) for 1 day until it is amplified.

3.5.1.2. Preparation of master mix

All essential components for real time PCR were brought into safety cabinet in the master mix room. These were two (2) positive controls (master mix positive control and exposed positive control) and two (2) negative controls (master mix negative control and exposure free positive control), RNase free H_2O(6.45µl), five-x (5x) PCR buffer (5µl), $Mgcl_2$(1.25 µl), dNTPs (0.8µl), Primer :FP (APMV-1, M+4100,5'AGTGATGTGCTCGGACCTTC-3')(0.5µl), RP(APMV-1,M-4220, 5'CCTGAGGAGAGGCATTTGCTA 3') (0.5 µl), Probe (APM-1, M+4100, 5'-(FAM) TTCTCTAGCAGTGGGACAGCC (TAMRA)-3') (1 µl) and inhibitor(0.5 µl) and one-step RT-PCR enzyme mix(1 µl).All the reagents were added in to master mix tube and shaked in vortex. Then, 17 µl master mix was taken and added into applied biosystem plate and 8 µl RNA extract was added into each applied bio-system plate (96).The mixture was transferred into amplification room and sealed with optical caps to prevent evaporation loss .All the preparation was done in the PCR working station(Annex 2).

3.5.1.3. RNA amplification

The real time reverse-transcriptase Polymerase chain reaction(rRT-PCR) amplification was performed in SDS 7500 Fast real time PCR machine (Applied Biosystems, USA) using AgPath–IDTM One Step RT-PCR Kit (Life Technologies, USA) as recommended by the manufacturers'. The reaction was performed in 25 µl volumes in 0.1 mL MicroAmp Fast PCR 8-tube strips; 8 µl RNA templates were added to 17 µl reaction mix (Annex 2). The duration of the amplification lasted 2 hours and 23 minutes for 40 thermal cycles (Wise *et al.*, 2004).

3.5.1.4. Thermal cycler protocol (40cycles)

It has two stages, stage1:-At this stage cDNA synthesized at 50^{o}c for 30 minutes and initial denaturation at 95 ^{o}c for15 minutes, this stage is one cycle replication. Stage2:-Denaturation at 94^{o}c for 10 seconds, annealing at 52^{o}c for 30 seconds and extension at 72^{o}c for 25 seconds, this stage is forty (40) term long cycle replication.

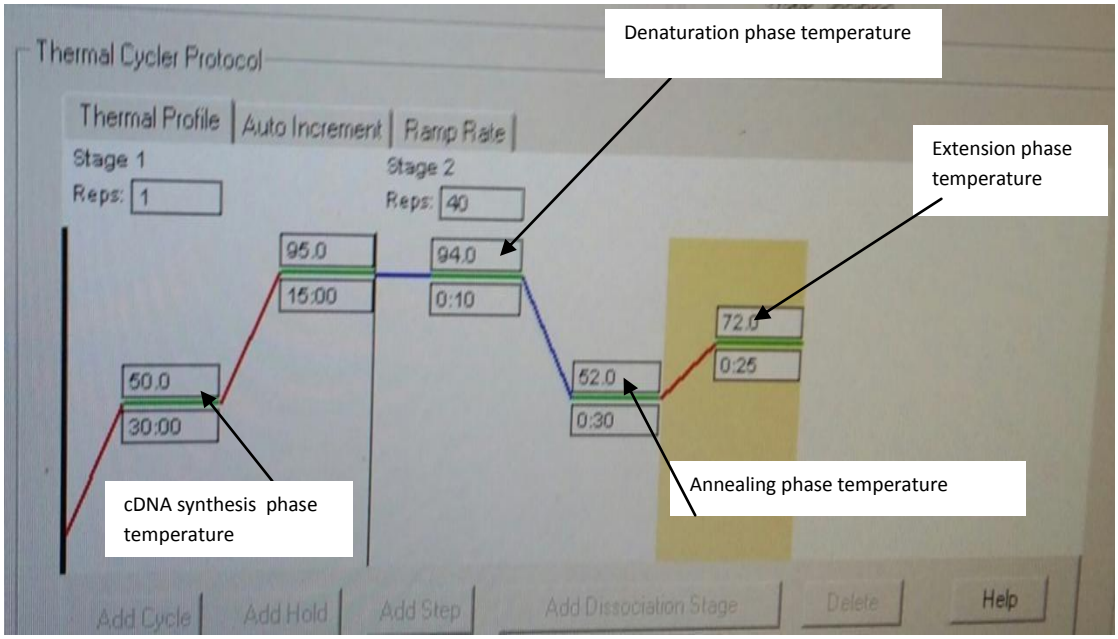

Figure 9: Thermal cycler protocol for rRT-PCR

3.5.1.5. Real Time PCR Test Validity

The test is valid, when positive control has expressed amplification approximately at 'ct ' value of 25 and no amplification or ct>35 in the negative control, when there is no amplification of positive control or amplification appear in the negative control is ct≤35,the test is rejected. Any amplification of ct>35 will be considered as negative or non-specific reaction to the test (wise *et al*., 2004).

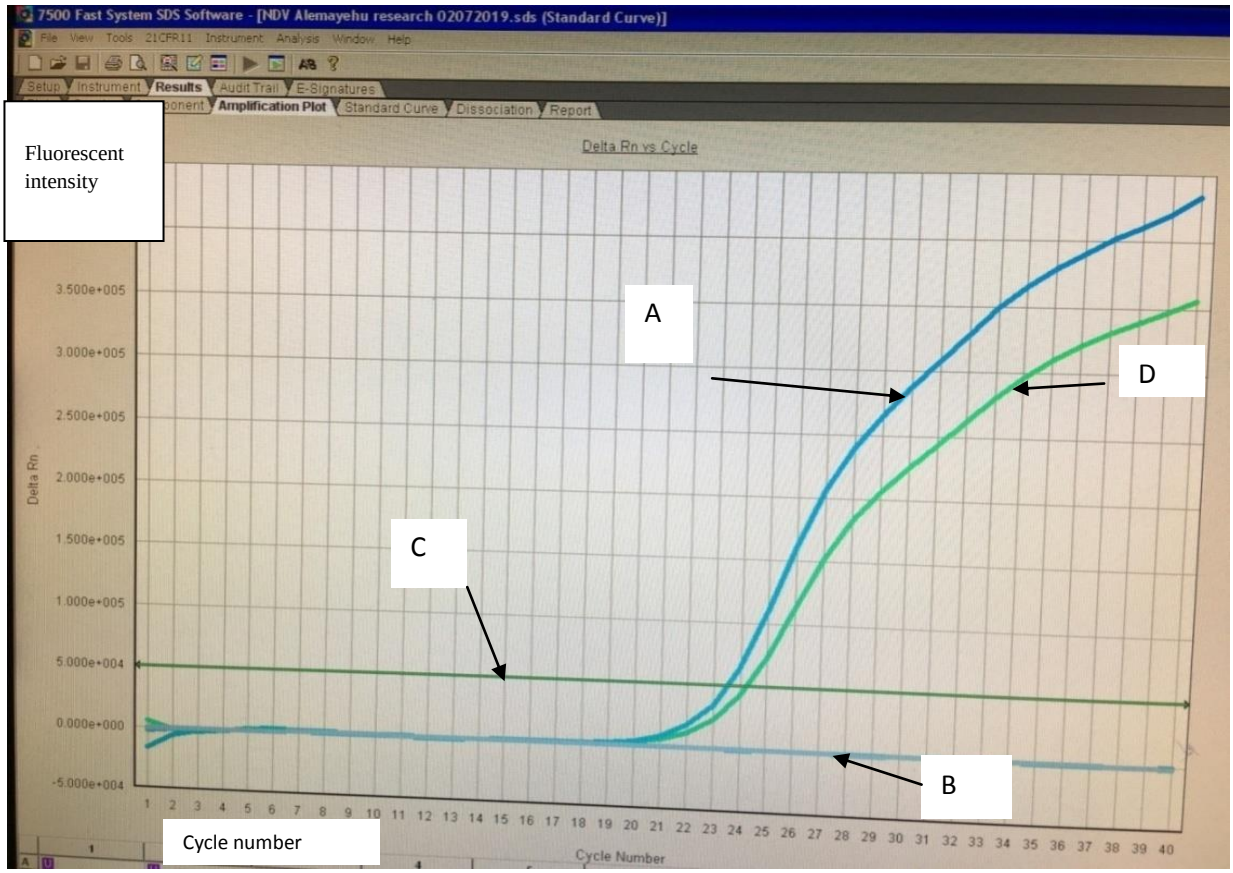

Fig. 10: Fluorescent intensity versus cycle number graph of rRT –PCR illustration: "A" shows exposed positive control, "B" shows negative control, "C" shows baseline and "D" shows master mix positive control.

3.5.2. Serological Analysis

An Indirect Enzyme Linked Immuno Sorbent Assay (ELISA) technique (ID.vet innovative version2, Louis Pasteure-Grabels, France) kit was used to detect the presence of anti-NDV antibodies and to determine anti-body titer level in the chicken serum following the kit manufacturers' recommended protocol. Briefly, the test sera were pre-diluted by dilution buffer 14 in a pre-dilution plate according to the established protocol or kit instructions, and each was dispensed into micro wells. In the ELISA plate pre-diluted samples and dilution buffer 14 were added and incubated for 30min $\pm$ 3min at 21^0C. After incubation, the sera were discarded from the plates, and each well was washed 3 times by 300µl of washing solution. 100µl anti-chicken immunoglobulin's peroxidase conjugate was dispensed into the wells and the plates were incubated for 30min $\pm$ 3min at 21^0C. After incubation, again the content were discarded from the

plates, and each well was washed 3 times by 300µl of washing solution. Then, 100µl substrate solutions were dispensed into each test well and again incubated for 15 min ± 2min at 21^0C in the dark place. After a final incubation, the substrate chromogen reaction was stopped by adding 100µl stop solution and the color reactions were quantified by measuring the optical density of each well at 450 nm using ELISA reader. The test is valid when the mean Optical Density (OD) value of positive control serum is greater than 0.250, and the ratio of the mean value of the positive and negative control (OD_{PC} and OD_{NC}) is greater than 3.

Serum sample to positive (SP) control ratio was calculated using the formula below

$$S/P = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}}$$

If SP value was ≥0.3, the NCD antibody status was considered to be positive and if SP value <0.3, it was taken as a negative.

The titer for the antibody in the sera sample of the chicken was determined using

$$Log_{10}(titer)=1.00*log_{10}(S/P)+3.520$$

$$Titer=10^{log_{10}}(tire)$$

Antibody titer results are interpreted as: If titer ≤993, it was considered to be negative and if titer ≥ 993 it was considered to be positive (ID.vet innovative version 2, Louis Pasteure-Grabels, France).

3.6. Data Analysis

The data collected for serology was entered into Microsoft Ex-Cell spread sheet, edited, coded, transferred to SPSS software (version 21) and analyzed by using descriptive statistics. Mean of anti-body titer between villages (kebele), standard deviation, percentage coefficient of variance and statistical significance (p-value) was computed. For mean comparison, one-way analysis method was used. The differences were considered statistically significant ($P<0.05$) at 95% confidence interval. The data of real time PCR was analyzed based on Newcastle disease test protocol of NAHDIC (National Animal Health Diagnostics and Investigation Center).

4. RESULTS

4.1 .Result for Real-Time Polymerase Chain Reaction (rRT-PCR)

Out of 76 pooled (380 individual) samples, 66 pooled (330 individual) samples (86.7%) were found positive for Newcastle disease virus gene. The positive "Ct" values range between 26.3 and 34.5, while the negative "Ct" values lay between 35.1 and 38.9 were scored. Newcastle disease virus gene was detected in 97% (37/38) and 76.3% (29/38) pooled samples of orophrengeal and cloacae samples respectively.

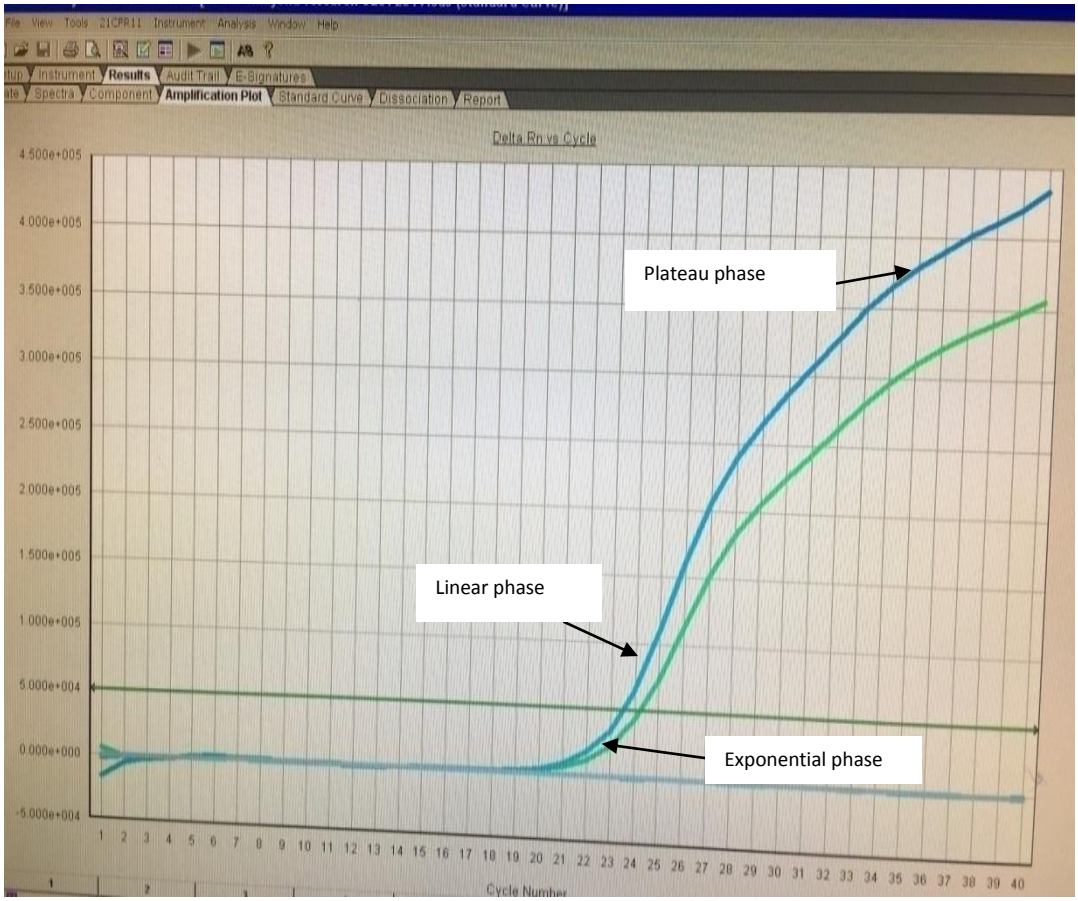

Figure 10: Flourescent intensity vs cycle number graph of RT-PCR, showing three phases of RNA amplification.

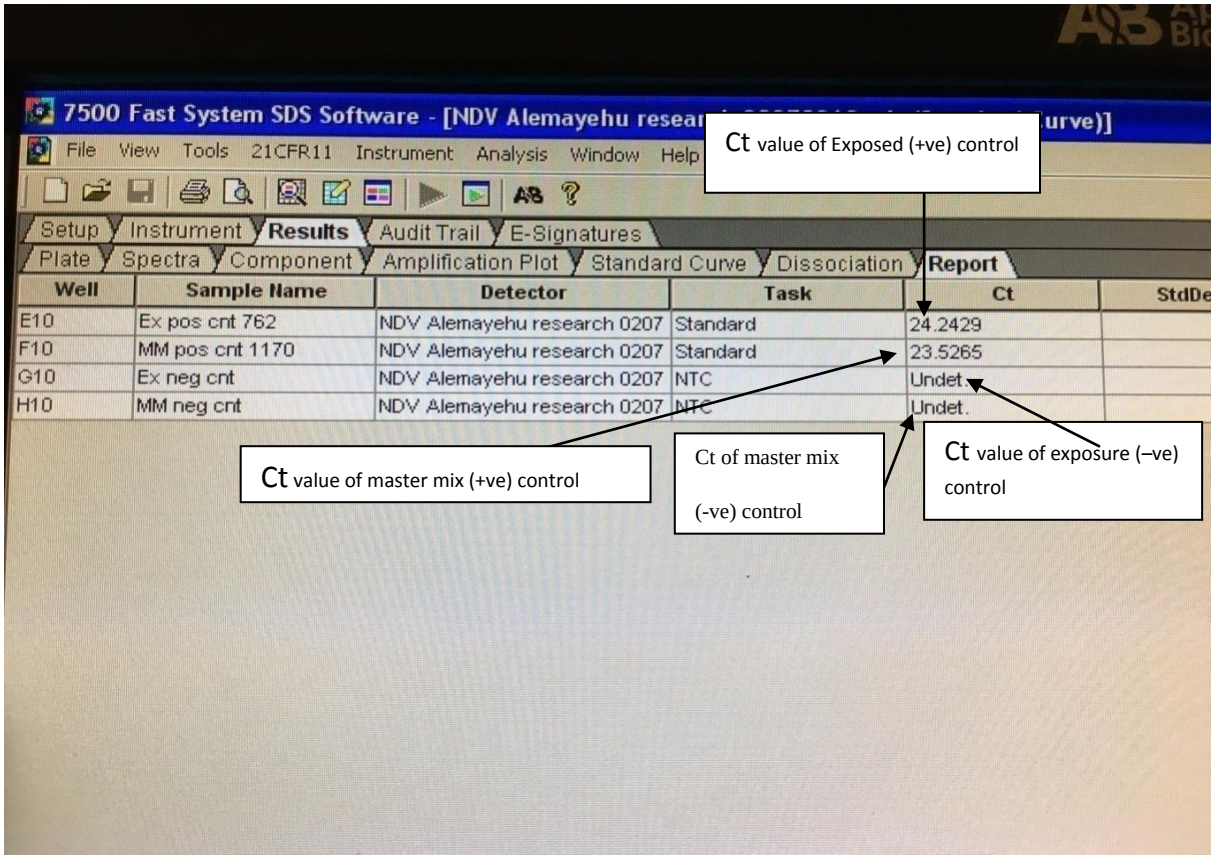

Figure 11: Tresholdcycle (Ct) Values of Positive Controls and Negative Controls:

In the above figure "F10" shows the $\mathbf{c}_t$ value of master mix positive control, "E10" shows the $\mathbf{c}_t$ value of exposed positive control, "G10" shows the $\mathbf{c}_t$ value of expose free negative control and "H10" shows the $\mathbf{c}_t$ value of master mix negative control. For both negative controls, the "ct" value was reported as undetected.

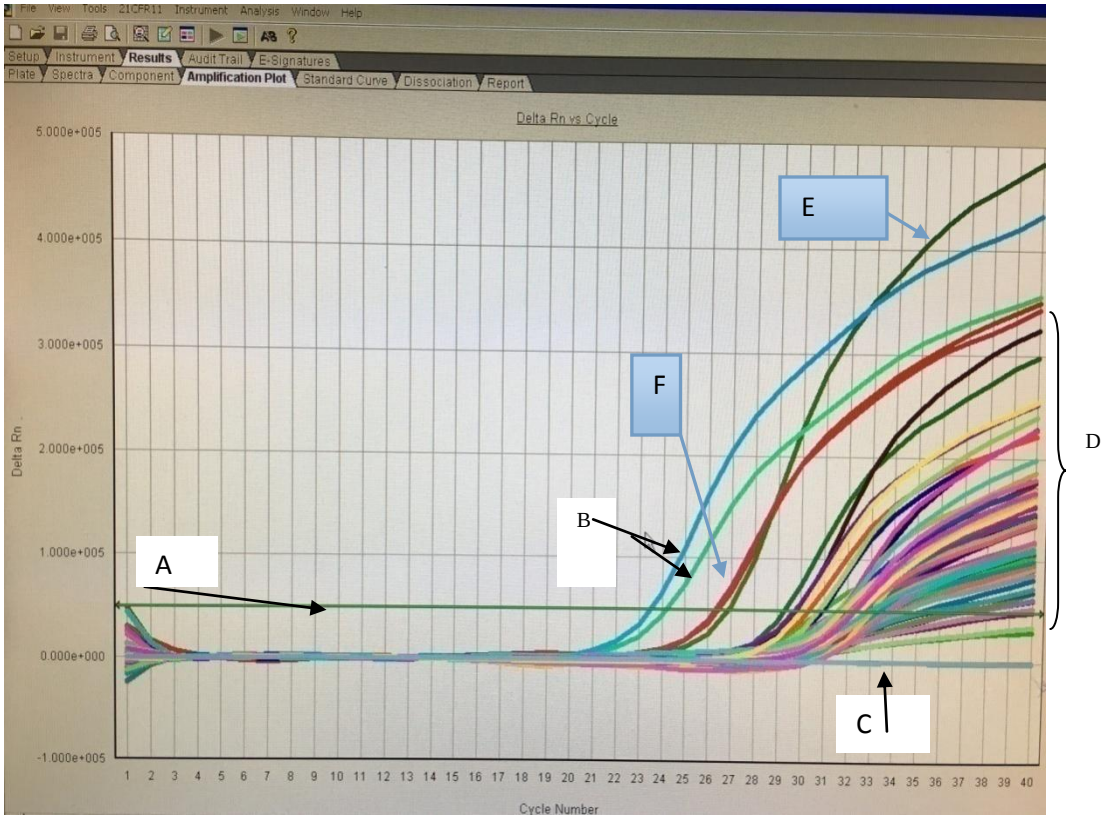

Figure 12: Graph showing Real Time RT-PCR result illustrations.

"A" shows baseline, "B" shows positive controls, "C" shows negative control ,"D" shows samples result and "E and F'' shows strong positives.

4.2. Result for Serology

Out of 348 serum samples, 131serum (37.64%) were positive for antibody against Newcastle disease virus. The range of the antibody titer was from 0 to 11735.9 in micro-liter of serum sample. The highest (2308.3) and lowest (1225.7) antibody titer was recorded in Obbi-Koji and Gurura-Baka kebeles respectively. The mean and standard deviation for the antibody titer was accounted, 1761.9 and 2592.4 respectively in microliter of serum samples (Table 2). The mean antibody titer was significantly different (F = 1.993, P=0.0079) (Table 2) among the kebeles where the samples were collected. The percentage coefficient of variation (%CV) from the analysis was 147% and this shows that the variation of antibody is very high, indicating the heterogeneity of antibody titer level (Table **2**).

Table 2: The level of antibody titer and comparison of mean antibody titer using ANOVA

Study areas	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	F test	P-value	%CV
					Lower Bound	Upper Bound					
1.B/ Koricha	56	1320.7	2335.3	312.1	695.2	1946.1	0	8311.52			
2.O/Koji	60	2308.3	2674.6	345.3	1617.3	2999.3	0	10033.67	1.993	0.0079	147
3.D/Duleti	64	1478.5	2611.6	326.5	826.2	2130.9	0	11735.9			
4. G/Baka.	58	1225.7	1998.5	262.4	700.3	1751.2	0	7472.78			
5.T/Anchabi	51	2131.4	2762.1	386.8	1354.6	2908.3	0	11341.63			
6.F/Gora	59	2140	2951.3	384.2	1370.9	2909.2	0	10358.96			
Total	**348**	**1761.9**	**2592.4**	**138.9**	**1488.6**	**2035.2**	**0**	**11735.9**			

Note:B/ Koricha= Badessa Koricha, O/Koji=Obbi Koji, .D/Duleti=.DireDuleti, G/Baka= GururaBaka, T/Anchabi= Tombe Anchabi, .F/Gora=.Fodu Gora

5. DISCUSSION

In this study, real time reverse transcriptase polymerase chain reaction (rRT-PCR) and indirect enzyme linked immune-sorbent assay (indirect ELISA) tests were conducted for molecular and serological detection of Newcastle disease virus as well as to determine anti-body titer . Higher proportion of test positive result was detected in real time polymerase chain reaction (rPCR) than in indirect ELISA test. This might be due to higher sensitivity of real time polymerase chain reaction (rPCR) (Bello *et al.*, 2018 and Rahman *et al*., 2016) and because of the samples were collected in pool. It could also indicate that virus circulation increases when the level of antibody decreases or the number of resistant chickens in a flock decrease (Chaka *et al*., 2013). Researchers in Morocco (Bell and Mouloudi, 1988) obtained more viruses from areas with lower antibody levels.

In previous PCR studies, low values were recorded by Emilia*et al. (*2016) who reported zero percent (0%) molecular detection from Philippines using real time PCR detection method. This showed that all samples (100%) were negative for NDV in both the primary RT-PCR and nested RT-PCR assays they used. The absence of Newcastle disease virus RNA from the samples collected in both tests in their research may indicate that there was no active NDV infections or no viral shedding were currently ongoing and that the Newcastle disease virus RNAs may have already been cleared from the tested birds (Alexander, 2000). Other low real time PCR findings were reported from East Shewa zone, Ethiopia (14.2%) by Chaka *et al*. (2012) and from Benue State in Nigeria by Abah *et al*. (2016), who reported (12%). Again Miressa *et al*. (2016) reported (26.7%) from East Shewa ,Ethiopia and Delesa *et al*. (2014) reported lower PCR findings,30.1% (44/146) from live poultry market of Ethiopia. The difference could be attributed to assays used, in case of Chaka *et al*. (2012), they used a fusion (F) gene detection assay and in case of Abah *et al*. (2016) finding, they used conventional PCR method to detect matrix (M) gene, 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 Newcastle disease virus which is very 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 Delesa *et al*. (2014) could attributes to large sampling area covered by researchers as they collected samples originated from country wide (Sodo, Hosaina, Dessie, Shashemane,Jimma, and Ambo) (Delesa *et al*. ,2014). In case of Miressa *et al*. (2016)

finding, the difference may be attributed to sampling method (they collected samples individually).However, the finding of this study is in agreement with the finding by Maqbool *et al*. (2017), who reported 90.0% , using real time PCRdetection method.

The serological finding obtained in this study is higher than the previous finding of Terefe *et al.* (2015), who reported (11.61%) from three rift valley districts namely Bishoftu, Tikur wuha and Ziway. This could be related to the sampling duration as they collected sample only within one month, in this study it was collected from December to November .Collecting samples for long duration gives chance to get pathogen at different infection stages and thus increases precision of the finding. Other low serological findings reported from Bale Zone (27.86%) and East Shewa zone (28.6%) by Minda *et al.* (2016) and Desalegn (2016) respectively. This could be attributed to different serological assays used .They used hemagglutination(HA) test and hemagglutination Inhibition (HI) test respectively, but in this research indirect ELISA test, which is considered as accurate, rapid and sensitive compared to the hemagglutination(HA) test and hemagglutination Inhibition (HI) test (Tabidi *et al.* ,2004) was used and in the HI tests, only the antibodies directed against the HN protein are detected, ELISA platforms utilizing whole virus as antigens can potentially detect antibodies directed against all the proteins in the NDV particle (Bello *et al.,* 2018).

However, the current serological finding is in line with the previous findings of Zeleke *et al*.(2005), who reported (35.9%) from Alage and Tadesse *et al*.(2005), who reported(38%) from Adama, Emilia *et al.(*2016) reported (38.11%) and Parvin *et al.(*2015) ,reported(40%) from Bangladesh. Finally, this serological finding is lower than the findings of Biswas *et al*. (2009), who reported 89% again from Bangladesh and Chaka *et al*., (2013), who reported 82.6% and 78.6% from East Shewa zone, Ethiopia. This difference might be attributed to different criteria of ELISA result interpretation methods used. They calculated percentage inhibition (PI) and classified as positive if one or more chickens in the flock tested positive (PI > 40), but we calculated serum sample to positive (SP) control ratio and used different criteria of interpretation as presented in section (3.5.2) above.

For indirect ELISA test positives, antibody titer ranges from 998.01 to 11735.9 with standard deviation of (2592.42160), which is very high**.** According to Chaka *et al*. (2012) reports, the

wide range of anti-body titer level shows presence of both class I (responsible for lower level anti-body titer) and class II (responsible for higher level anti-body titer) Newcastle disease virus. The mean anti-body titer of the whole kebeles' was 1761.9088 ranging from 1225.7to 2035.2352 (Table2) at 95% confidence interval (CI) and 5% precision. Our mean value of antibody titer finding is lower than Parvin *et al. (*20015) reports, who reported antibody titer mean value of 6291 from Bangladesh ,this variation of mean titer is may be due to different test kits used (they used rapid NDV antigen test kit, but we used innovative diagnostic indirect ELISA kit) and also higher antibody titer may be attributed to presence of velogenic strains of the virus dominating in their research area, which are known to produce higher antibody titers than lentogenic and mesogenic strains (Alexander *et al.,* 2004) . Lower mean antibody titer was reported (0 to 969) from Mozambique by (Frechaut *et al.,* 2015) .The lower mean values might be due to the reason that age groups of the participant chicken (in their study, only chickens of 30-40 day old are participated) or virus circulation increased and immunity of the flock decreased as antibody titer and virus circulation inversely proportionate (the chickens did not developed sufficiently protective immunity) as stated by Chaka *et al*. (2013).

As indicated in table 2, the mean antibody titer of each kebeles shows large values. The standard deviation of antibody of chickens both between groups (kebeles) and within groups (kebeles) shows great variations at 95% confidence interval (CI), which shows the natural exposure of the chickens to the virus, because in case of vaccination (experimental case) the standard deviation could not be such a large value (Frechaut *et al.*,2015 and Tesfaye *et al.*,2018). Also, the percentage coefficient of variation (%CV) computed was 147%. The CV is used mostly to evaluate effectiveness of vaccine programs and development poultry humoral immune response. Good CVs, between 30-50%, show that flock immunization was succeeded by generating uniform antibody titers (Frechaut *et al.* ,2015) .The higher score of percentage coefficient of variation (%CV) in this study shows that the variation of antibody is very high, indicating the heterogeneity of antibody titer level and natural infection. In case of vaccination it would be homogenous with percentage coefficient of variation (%CV) less than 30(strong) and between 30 to50 (good) (Tesfaye *et al*., 2018). The highest CV in this finding might be due to the natural infection acquired by chickens that provoke immunity.

6. CONCLUSION AND RECOMMANDATIONS

The study was conducted to detect Newcastle disease virus and to determine antibody titer using real time reverse transcriptase polymerase chain reaction (rRT-PCR) and indirect enzyme linked immune-sorbent assay (indirect ELISA) test methods. In real-time RT-PCR test 86.7% of the samples were positive for Newcastle disease virus, and indirect ELISA test revealed that 37.64 % of the chickens were positive for anti-bodies against Newcastle disease virus. This indicated that the virus was circulating in the study area. The mean anti-body titration was found 1761.9088 per micro litters of sera samples. The higher standard deviation (2592.42160) and percentage coefficient variation (147%) showed the existence of natural exposure to Newcastle disease virus.

Based on the above conclusions, the following recommendations forwarded:

- Further molecular characterization should be done to identify genotypes circulating in the study area.
- Epidemiological investigation should be carried out to distinguish associated risk factors for NCD in the study area.
- Vaccine program should be encouraged to prevent Newcastle disease outbreak and economic loss due to high morbidity and mortality.

7. REFERENCE

Abah, H.O. Abdu, P.A.and 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.

Ahlers, C., Alders, R., Bagnol, B., Cambaza, A.B., Harun, M., Mgomezulu, R., Msami, H., Pym, B., Wegener, P., Wethli, E. and Young, M., 2009. Improving village chicken production: a manual for field workers and trainers. *Australian Centre for International Agricultural Research (ACIAR).*

Alexander, D.J., 2000. Newcastle disease and other avian paramyxoviruses. *Revue Scientifique et Technique-Office International des Epizooties*, ***19***(2):443-455.

Alexander, D.J., Senne, D.A., Gough, R.A. and Jones, R.C., 2008. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. Diseases of Poultry.

Alexander, D.J. and Senne, D.A., 1998. Newcastle disease virus and other avian paramyxoviruses. *A laboratory manual for the isolation and identification of avian pathogens*, **4**:156-63.

Alexander, D.J., Bell, J.G. and Alders, R.G., 2004. A technology review: *Newcastle disease, with special emphasis on its effect on village chickens* (No. 161). Food & Agriculture Org..

Alexander, D.J., Calnek, B., Barnes, H., Beard, C. and McDougald, L., 2003. Diseases of poultry. *Diseases of poultry*, *11*.

Amanu, S. and Rohi, O.K., 2013. Studi Serologis Dengan Uji Hambatan Hemaglutinasi Terhadap Angsa. *Jurnal Sain Veteriner*, ***23*(**1):8-12.

Atunbi, O.A. and Sonaiya, E.B., 1994. An assessment of backyard poultry housing inOsogbo. *Osun State, Nigeria, African Network for Rural Development Newsletter*, *4*(7).

Belayheh, G., Kyule, M.N., Melese, B. and Fufa, D., 2014. Seroprevalence of Newcastle disease virus antibodies in village chickens in Kersana-kondalaity district, Ethiopia. *Global Veterinaria*, ***12*(**3):426-430.

Bell, J.G. and Mouloudi, S., 1988. A ireservoir of virulent Newcastle disease virus in village chicken flocks. Preventive Veterinary Medicine, **6**(1):37-42.

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

Biswas, P.K., Barua, H., Uddin, G.M.N., Biswas, D., Ahad, A. and Debnath, N.C., 2009. Serosurvey of five viruses in chickens on smallholdings in Bangladesh. *Preventive veterinary medicine*, ***88*(**1):67-71.

Branckaert, R.D.S., Gaviria, L., Jallade, J. and Seiders, R.W., 2000, August. Transfer of technology in poultry production for developing countries. In *Proceedings of the XXI World's Poultry Congress* (pp. 20-24).

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

Capua, I. and Alexander, D.J. eds., 2009. Avian influenza and Newcastle disease*: a field and laboratory manual*. Springer Science & Business Media.

Cattoli, G., Susta, L., Terregino, C. and 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.

Center for Food Security and Public Health (CFSPH),2008. Iowa State University and Institute for International Cooperation in Animal Biologics. 2008. "Newcastle Disease Fact Sheet.http://www.cfsph.iastate.edu/DiseaseInfo/disease.php?name=newcastle-disease&lang=en

Chaka, H., Goutard, F., Bisschop, S.P. and 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., De Almeida, R.S., Bisschop, S. and Thompson, P.N., 2013. Serological and molecular investigation of Newcastle disease in household

chicken flocks and associated markets in Eastern Shewa zone, Ethiopia. *Tropical animal health and production*, ***45***(3):705-714.

Chang, A. and Dutch, R.E., 2012. Paramyxovirus fusion and entry: multiple paths to a common end. *Viruses*, ***4***(4):613-636.

CSA , 2018.Report on livestock and livestockcharacteristics (private peasant holdings). AddisAbaba: Central Statistical Agency.

CSA, 2015.Report on livestock and livestockcharacteristics (private peasant holdings). AddisAbaba: Central Statistical Agency.

Delesa, D. M., Menbere, K. W., Redeat, B.A., Melaku, S. K., Alice, F..Alireza, H., Tesfaye, R.C.and Hassen, C. C., 2014.Characterization of Newcastle Disease Virus and poultry-handling practices in live poultry markets,Ethiopia . *SpringerPlus a Springer Open Journal*, **3**(1):459.

Desalegn Jarso Modjo, 2016.Incidence of Village Chicken Diseases in Eastern Shewa Zone, Ethiopia: The Case of Newcastle and Infectious Bursal disease. *Open Access Journal of Veterinary Science and Research*, **1** (4):1-14.

Dessie, T. and Ogle, B., 2001. Village poultry production systems in the central highlands of Ethiopia. *Tropical Animal health and production*, *33*(6):521-537.

Emilia, A. L.T., Sofiea, M.C. T. and Dennis, V.U., 2016.Serological and Molecular Detection of Newcastle Disease Virus from CaptiveRaptors in a Wildlife Rescue Center in thePhilippines.In: *Philippine Journal of Veterinary Medicine,***53** (2): 96-102.

Fikre, 2003 .Poultry Disease Lecture Notes. In: A Postgraduate Refresher Course Conducted at Mekelle, in May 2003. By the Tigray Agriculture and Natural Resources Development pp 2-3

Frechaut, E.R.C.. Muchanga, E.F. Taunde, P,. Nhambirre, O. Pondja, A.J. and. Bila ,C.G.,2015. Evaluation of Serum Antibody Titers Against Newcastle Disease in Broiler Poultry in

Maputo and Matola Regions, Mozambique. *International Journal of Poultry Science* ,**14** (11): 622-624.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M. and Toulmin, C., 2010. Food security: the challenge of feeding 9 billion people. *Science*, ***327*(**5967):812-818.

Goebel, S.J., Taylor, J., Barr, B.C., Kiehn, T.E., Castro-Malaspina, H.R., Hedvat, C.V., Rush-Wilson, K.A., Kelly, C.D., Davis, S.W., Samsonoff, W.A. and Hurst, K.R., 2007. Isolation of avian paramyxovirus 1 from a patient with a lethal case of pneumonia. *Journal of virology*, ***81***(22):12709-12714.

Habte, T., Amare, A., Bettridge, J., Collins, M., Christley, R. and Wigley, P., 2017. Guide to chicken health and management in Ethiopia. *ILRI Manual*, *25*.

Haque, M.H., Hossain, M.T., Islam, M.T., Zinnah, M.A., Khan, M.S.R. and 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.

Hooper, P.T., Russell, G.M., Morrow, C.J. and Segal, Y., 1999.Lentogenic Newcastle disease virus and respiratory disease in Australian broiler chickens.*Australian veterinary journal*,**77**(1) :53-54.

Hutton, S., Bettridge, J., Christley, R., Habte, T. and Ganapathy, K., 2017.Detection of infectious bronchitis virus 793B, avian metapneumovirus, Mycoplasma gallisepticum and Mycoplasma synoviae in poultry in Ethiopia. *Tropical animal health and production*, **49**(2):317-322.

Kahn, C.M., Scott, L. and Aiello, S.E., 2005. The Merck veterinary manual 9th ed. Copyright (C) by Merck Co., Inc printed in the USA by National publishing. Inc. Philadelphia, Pensylvania, pp.146-148.

Kebreab, G., Mohamed, N. and Berhanu, B., 2001. Vaccination of layers against newcastle disease using inactivated oil adjuvant and live vaccines. Journal of the Ethiopian Veterinary Association, **5**(1):1-8.

Kim, L.M., King, D.J., Curry, P.E., Suarez, D.L., Swayne, D.E., Stallknecht, D.E., Slemons, R.D., Pedersen, J.C., Senne, D.A., Winker, K. and Afonso, C.L., 2007. Phylogenetic diversity among low-virulence Newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. *Journal of virology*, **81**(22):12641-12653.

Kommers, G.D., King, D.J., Seal, B.S. and 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., Whyte, M. and Dissing, M., 2010. Smallholder poultry production: Livelihoods, food security and sociocultural significance.

Lamb, R., Kolakofsky, C.P.L.D., Melero, J.A., Nagai, Y., Oldstone, M.B.A., Pringle, C.R. and Rima, B.K., 2005. The negative sense single stranded RNA viruses, p 607–738. In Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (ed), Virus taxonomy. *Eighth report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA.*

Life Technologies Corporation, 2012. Real-time PCR handbook: Basics of real-time PCR. Life technologies.com.

Maqbool, R., Wani,S.A., Wali,A., Kashoo,Z., Dar, P., Qureshi,S., Hussain,I.,Shah,A., Sikander,P., Ganaie,M.,Y., and Bukhari,S. ,2017. RT-PCR based assay for detection of Newcastle disease virus isolated frompoultry in Kashmir. *Journal of Entomology and Zoology Studies*, **5**(4): 1835-1839.

Mazengia, H., 2012. Review on major viral diseases of chickens reported in Ethiopia. Journal of Infectious Diseases and Immunity, 4(1):1-9.

Mazengia, H., Tilahun, S.B. and Negash, T., 2010. Newcastle Disease and Infectious Bursal Diseases are Threat to Village Chicken Production in Two Districts of Amhara National Regional State, Northwest Ethiopia. *IUP Journal of Life Sciences*, **4**(2):62-72.

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

Minda, A. G., Kemal, K.E. and Gezahegne, M. K., 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 andAnimal Health* , **8**(8):99-106.

Miressa, Y., Cherinet, Y., Bsrat, A. and Tadesse, F., 2016. Investigation of Newcastle Disease Virus Using Reverse Transcription Polymerase Chain Reaction in Selected Districts of Eastern Shewa, Ethiopia. *Jornal of Veterinary Science and Technolog*y, **7**(384):2.

Mogesse, H.H., 2007. Phenotypic and genetic characterization of indigenous chicken populations in Northwest Ethiopia (Doctoral dissertation, University of the Free State).

Nega, M., Moges, F., Mazengia, H., Zeleke, G. and Tamir, S., 2012. Evaluation of I2 thermostable Newcastle disease vaccine on local chickens in selected districts of western Amhara.*Online Journal of Animal and Feed Research (OJAFR),***2**(3):244-248.

Gohm, D.S., Thür, B. and Hofmann, M.A., 2000. Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR.*Avian Pathology*, **29**(2) :143-152.

Office International des Epizooties (OIE). 1996.Manual of Standards for Diagnostic Tests andVaccines. 3rd Edition., *OIE Manual*, 161-165.

Parvin, M. A., Rahman, M. M. , Akter, M. R. , Rumi, N. A. , Hossain M. K.and Rahman, M. S.,2015. Detection of Newcastle Disease Virus by rapid NDV antigen test kit and antibody titer level in pre and post vaccination.*International Journal of BioResearch*, **2** (4):01-12

Perttula,L.,2009. Epidemiology and Characterization of Newcastle Disease in Smallholder Poultry in Mozambique

Pham, H.M., Nakajima, C., Ohashi, K. and Onuma, M., 2005.Loop-mediated isothermal amplification for rapid detection of Newcastle disease virus.*Journal of clinical microbiology*, **43** (4):1646-1650.

Piacenti, A.M., King, D.J., Seal, B.S., Zhang, J. and Brown, C.C., 2006. Pathogenesis of Newcastle disease in commercial and specific pathogen-free turkeys experimentally infected with isolates of different virulence. *Veterinary Pathology*, **43**(2) :168-178.

Pretty, J., Toulmin, C. and Williams, S., 2011.Sustainable intensification in African agriculture.*International journal of agricultural sustainability*, **9**(1):5-24.

QIAGEN, 2014. SAMPLE AND ASSAY TECHNOLOGIES: QIAMP Viral RNA Mini Handbook (4th Ed). Www.qiagen.com. Date accessed, 20/5/2019

Rahman, M.M., Barman, L.R., Chowdhury, E.H. and 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.

Reta, D., 2009.Understanding the role of indigenous chickens during the long walk to food security in Ethiopia. *Livestock Research for Rural Development*, ***21***(8):116.

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

Sambo, E., Bettridge, J., Dessie, T., Amare, A., Habte, T., Wigley, P. and Christley, R.M., 2015.Participatory evaluation of chicken health and production constraints in Ethiopia. *Preventive veterinary medicine*, **118**(1):117-127.

Samrawit Melkamu and Mulat Asrat, 2018. Review on economic importance and current diagnostic techniques of Newcastle disease in poultry.*International Journal of Advanced Resarch and Biological Science*.,**5**(3): 117-125.

Selam, M. and Kelay, B., 2013.Causes of village chicken mortality and interventions by farmers in Ada'a District, Ethiopia. *International Journal of Livestock Production*, **4**(6):88-94.

Sonaiya, E. and S. Swan, 2005.Small-scale poultry production, technical guide manual.FAO Animal Production and Health.No.1. Food and Agriculture Organization (FAO), Rome, 2004, Http://Www.Fao.Org/ Docrep/008/Y5169e/Y5169e00.HTM.

Stevenson, M. and EpiCentre, I.V.A.B.S., 2008. An introduction to veterinary epidemiology. Palmerston North: Massey University.

Susta, L., Miller, P.J., Afonso, C.L. and 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.

Swayne, D.E. and King, D.J., 2003. Avian influenza and Newcastle disease.*Journal of the American Veterinary Medical Association*, **222**(11):1534-1540.

Sylvester, S.A., Dhama, K., Kataria, J.M., Rahul, S. and Mahendran, M., 2005. Avian infectious bronchitis: A review. *Indian Journal of Comparative Microbiology Immunology and Infectious Diseases*, ***26***(1):1-15.

Tabidi, M.H., Makkawi, A., Mahasin, E. and Ali, A.S., 2004. Comparative evaluation of haemagglutination inhibition test and Enzyme-Linked Immunosorbent Assay for detection of antibodies against Newcastle disease vaccine in broiler chicks.*International Journal of Poultry Science*, **3**(10):668-70.

Tadelle Dessie and Yilma Jobre ,2004.A Review of The Importance and Control of New castle Disease in Ethiopia. *Ethiopian Veterinary Journal*, **8**(1):71-79

Tadesse, S., Ashenafi, H. and Aschalew, Z., 2005.Seroprevalence study of Newcastle disease in local chickens in central Ethiopia. *International Journal* of *Applied Research and Veterinary Medicine*, **3**(1):25-29.

Terefe D, Belaineh R, Chaka H, Sombo M, Mekuria A, et al. (2015) Serological and Molecular Study of Newcastle Disease Virus inVillage Chickens in Selected Rift-Valley Areas, Ethiopia. *Journal of Veterinar Science and Technology*,**6**: 264.

Terregino ,C.and Capua, I., 2009. Conventional diagnosis of Newcastle disease virus infection. *In*: Avian influenza and Newcastle disease. Capua, I., and Alexander, D.J.,(Eds.) pp. 123–125. SpringerMilan, Milan, Italy.

Udo, H.M.J., Asgedom, A.H. and Viets, T.C., 2006.Modelling the impact of interventions on the dynamics in village poultry systems. *Agricultural Systems*, ***88***(2-3):255-269.

Wise, M.G., Suarez, D.L., Seal, B.S., Pedersen, J.C., Senne, D.A., King, D.J., Kapczynski, D.R. and Spackman, E., 2004. Development of a real-time reverse-transcription PCR for

detection of Newcastle disease virus RNA in clinical samples. *Journal of Clinical Microbiology*, **42**(1):329-338.

World Organization for Animal Health (OIE), 2009.OIE Technical Disease Card. Newcastle Disease. http://www.oie.int.

World Organization for Animal Health (OIE), 2013. Terrestrial Animal Health Code. Chapter 10.9.Newcastle Disease. http://www.oie.int.

World Organization for Animal Health (OIE),2008. Chapter 2.3.14 *In*: Manual of diagnostic tests and vaccines for terrestrial animals, 6th ed., pp. 576–589. OIE, Paris, France.

Yusoff, K. and Tan, W.S., 2001. Newcastle disease virus: macromolecules and opportunities. *Avian Pathology*, **30**(5):439-455.

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

8. ANNEXS

Annex 1: 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 (15-25^{0}C) 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 QIAamp mini 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 -20^{0}C until master mixing (Qiagen, 2014).

Annex 2: 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 H_2O=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 .TE buffer was used for intial reconstitution of lyophilized primer and probe.

1.8. Inhibitor=0.5 µl *80=40 µl (inhibitors inhibit the binding primer with probe wich 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.

Annex 3: Test Procedure for Indirect ELISA

Allow all reagents to come to room temperature ($21^0c \pm 5^0c$) before use. Homogenize all reagents by inversion or vortex.

The negative and positive controls are supplied ready to use. **DO NOT** add dilution buffer to the control wells A1, B1, C1, and D1-the controls tested to undiluted.

Sample, however, are tested at a final dilution of 1:100 in dilution buffer14 (1:50 pre-dilution, followed by1:2 in the micro plate).

1. In a pre-dilution plate, aside well A1, B1, C1, and D1 for controls, and add:

    -5µl each sample to be tested

    -245µl **Dilution Buffer14** to all well EXCEPT control wells A1, B1, C1, and D1.

Note: it is recommended to respect the indicated order of deposit to able to visually control addition of sample each well.

2. In the ELISA microplate, add:

    -100 µl of the negative control to wells A1 and B1.

    -100 µl of the positive control to wells C1 and D1.

    -50 µl of Dilution **Buffer14** to as many wells as there are samples to be tested (NOT to controls A1, B1, C1, and D1).

    -50 µl of the pre-diluted samples as prepared above.

3. Cover the plate and incubate for **30 min ± 3min** at 21^0c ($\pm 5^0c$).

4. Prepare the **conjugate 1x** by diluting the **concentrated conjugate 10x** to1:10 in **dilution buffer3.**

5. Empty the wells. Wash each well 3 times with at least 300 µl of the **wash solution 1x**.Avoid drying of the wells between washings.

6. Add 100 µl of the **conjugate 1X** to each well.

7. Cover the plate and incubate **30 min ± 3min** at 21^0c ($\pm 5^0c$).

8. Empty the wells. Wash each well 3 times with at least 300 µl of the **wash solution 1x**.Avoid drying of the wells between washings.

9. Add 100 µl of the substrate solution to each well.

10. Cover the plate and incubate **15 min ± 2 min** at 21^0c ($\pm 5^0c$).

11. Add 100 µl of the **stop solution** to each well in the same order as in step No.9, to stop the reaction.

12. Read and record the OD at 450nm (ID.vet innovative Indirect Version 2, louis Pasteur-Grabels-France)

Annex 4: Pictures captured during sample collection