ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

MVSc THESIS

EVALUATION OF THE IMMUNE RESPONSE OF NEWCASTLE DISEASE VIRUS VACCINES, IN LAYER CHICKENS

By

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JUNE 2018

BISHOFTU, ETHIOPIA

EVALUATION OF THE IMMUNE RESPONSE OF NEWCASTLE DISEASE VIRUS VACCINES, IN LAYER CHICKENS

A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

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As members of Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by **Abel Sorsa**entitled: **Evaluationof the Immune Responses of Newcastle Disease Virus Vaccines, In Layer Chickens**and recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Veterinary Science in Veterinary Microbiology.

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First, I declare that this thesis is my bonafide work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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ABBREVIATIONS

AMPV-1	Avian Paramyxovirus, Type 1
ANOVA	Analysis of variance
CFSPH	Center for food security and public health
CMI	Cell mediate immunity
DMRT	Duncan multiple ranges taste
DPC	Day post challenge
ELISA	Enzyme linked immune sorbent assay
F	Fusion
GMT	Geometric mean titer
HIS	Hyper immune serum
HIT	Haemagglutination inhibition test
HN	Hemagglutinin-neuraminidase
IBD	Infectious bursal disease
ICPI	Intracerebral pathogenicity index
ICTV	International committee on taxonomy of viruses
loNDV	Newcastle diseases virus of low virulence
MDA	Maternal derived antibody
MHC	Major Histocompatibility
ND	Newcastle disease
NDV	Newcastle disease virus
NVI	National veterinary institutes
OIE	World organization for animal health
Rt-PCR	Real-time reverse transcriptase polymerase chain
SD	Standard deviation
SsRNA	Single stranded ribonucleic acid
VNDV	Virulent Newcastle disease virus

ABSTRACT

This study was undertaken to evaluate the immune responses of NDV vaccines in layer chickens in the National Veterinary Institutes (NVI), Bishoftu, Ethiopia, in the period of February to May 2018.A total of 140 layer day old chicks (Lohmann Brown) were randomly divided into seven groups such as 1, 2, 3, 4, 5, 6, and 7 of which group 1, 2, and 3 were vaccinated primarily with HitchnerB1, LaSota, and Thermos-table I2 vaccine respectively at day 5 of age and secondarily vaccinated using LaSota, LaSota and Thermostable I2; vaccine, respectively at day of 26, again thirdly (second boost) vaccinated using Thermostable I2, Thermostable I2,Thermostable I2; vaccines,at the day of 54 of age, through single eyeinstillationof the same schedules and group 4,5 and 6 were vaccinated with the same vaccines respectively via double eye instillation following the same schedule.Group 7 was kept as unvaccinated control chickens.Sera samples were collected after 10 vaccination days and at day 5, 15, 21, 31 of age from unvaccinated control whereas at day of 15, 36, and 64 of age from vaccinated chickens.Serum sample subjected to Haemagglutination Inhibition (HI) test for the determination of antibody titres. It was observed that after secondary vaccination the geometric mean (GM) titres of vaccinated chickens were induced highest antibody titer than primary vaccinationand third vaccination GMtiter.From the present research it may be concluded that LaSota, LaSota, thermostable I2vaccination schedules was the best combination of which produced highest immune response in single eye instillation whereas HB1LaSota, LaSota, vaccination schedules was the best combination of which produced highest immune response in double eye instillation.The persistence of passive immunity remains in chickens until the age of day 31.

Key words: *Immune response,Layer chickens, Newcastle disease, ND vaccines*

1. INTRODUCTION

Poultry production plays a major role in the economy particularly of developing countries.The larger proportion of rural poultry in the national flock population of developing countries makes them worth paying attention to improved management and breeding. At national level in Ethiopia, 99% of the total, 56.5 million, estimated chickens are contributed by village poultry production while only 1% is from intensive exotic breed maintained under intensive management system (Dinka*et al*., 2010).Furthermore, the poultry sector in Ethiopia has long traditional practices and can be characterized into three major production systems (back yard, small scale and commercial scale) based on some selected parameters such as breed, flock size, housing, feed, health, technology and biosecurity. Globally, poultry are used as an animal protein source, generation of extra cash incomes and religious/cultural considerations are amongst the major reasons for keeping village chickens by resource-poor rural communities (Mazengia, 2012).

Constraints which restrict the potential of village chickens in Ethiopia include; low inputs of feeding, poor management, the presence of diseases of various natures and lack of appropriate selection and breeding practices. Among these threats, viral diseases like Newcastle disease (ND) is the major health constraints inflicting heavy losses (Zeleke*et al*., 2005). Thus, Newcastle disease (ND) is caused by avian paramyxovirus serotype 1 (APMV-1) belonging to the family Paramyxoviridae, genus Avulavirus. NDV is an enveloped non-segmented, single-stranded negative-sense RNA virus with a helical morphology. Its genome has six open reading frames (ORF), which encode for the following proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and RNA-dependent RNA polymerase (L). During P-gene transcription, two additional nonstructural proteins, the V and W proteins are also generated through RNA editing (Mayo, 2002).

In addition, Newcastle disease virus (NDV) can be categorized into highly pathogenic (velogenic), intermediate (mesogenic), and less pathogenic (lentogenic) strains based on pathogenicity in chickens and are divided in two clades (class I and class II).Class I contains, almost exclusively, low virulence strains recovered from wild waterfowl worldwide. Class II

includes strains of low and high virulence isolated from poultry and wild birds. The disease is endemic in the village poultry population in Africa and is regarded as the most important constraint to the development, survival, and productivity of village chicken flocks (Alder, 2009).

Moreover, the virus is characterized by respiratory, nervous system impairment, gastrointestinal and reproductive problems. Sources of infection for NDV are exhaled air from infected birds and contaminated feed and water and transmission is mostly via aerosol. Feces, eggs lay during clinical diseases, and all parts of the carcass during acute infection and at death can also act as sources of infection. Chickens infected with virulent NDV may die without showing any clinical sign of illness though young chickens are more susceptible and show sign sooner than older ones. Much of the spread of ND in village is probably via human agents (Tiwari*et al.,* 2004).An outbreak of ND is unpredictable and discourage villager from paying proper attention to the husbandry and welfare of their chickens (Ashraf and Shah, 2014).

The generalized signs of ND include depression, loss of appetite, abnormal thirst, severe dehydration, emaciation and fever. Mortality can reach up to 100%. Mesogenic strains of NDV usually cause respiratory disease in adult chickens while lentogenic strains are not pathogenic. The first contact of the virus in permissive hosts occurs with respiratory epithelial cells (Miller *et al*., 2013).Newcastle disease (ND), called *"Fengle"* in Ethiopia, is the most important cause of economic loss in poultry production in this country. The disease was first reported in 1971 in Eritrea. In 1972, outbreaks occurred in Addis Ababa and in 1974 in the poultry farm of the Agricultural University at Harer and in the Shola and Bishoftu poultry farms (National Veterinary Institute (NVI), 1974). Vaccination against the disease was not practiced until 1974.The disease is enzootic in Africa and has a major impact on village poultry production as it can devastate flocks every year (Alexander, 2004).

Up to 90% of fowl die every year from this pestilence. Village poultry owners interviewed at various times between 2000 and 2009 indicated Newcastle disease as a sporadic problem with devastating losses once in four or five years. In spite of vigorous and regular vaccination, ND is still havoc to the poultry flocks and outbreaks have occurred even in vaccinated flocks.

According to the Ethiopian authorities reported only 14 outbreaks in 1998, 16 in 1999, 8 in 2000, 34 in 2001, 67 in 2002, 16 in 2003 and 40 in 2004 (OIE, 2006).

Vaccine failure results in the outbreak of ND. Vaccine failure results from, inadequate method of vaccination, vaccination during concurrent incubation period of ND, stress of extreme weather conditions (winter or summer), transportation of birds after vaccination, etc. Poor quality of vaccine is also one of the factors that can cause vaccine failure (Numan*et al*., 2005). Poor vaccine quality is due to poor manufacturing standards, low quantity of antigen (immunogen), faulty application in birds, lack of proper storage, exposure to direct sunlight, poor vaccine handling during transportation, non-maintenance of supply cold chain. The vaccine failure may also result due to the presence of high titers of maternal antibodies titer. Sometimes; the farmers are suspicious about the protective nature of those local NDV vaccines which manufactured in National Veterinary Institute, Bishoftu, Ethiopia. A number of relevant questions are faced by scientists and field Veterinarians of this country as to the immunogenicity, virus titer, stability and such other qualities of those vaccines (Muhammadamin and Qubih, 2010).

General objectives

This research was designed to determinethe persistence of maternally derived antibody titer of chicks they were came from vaccinated parent stocks.Thestudy evaluated the immune response of vaccinated layer chickensusing live Newcastle diseases virus vaccines produced in National veterinary institutes, Bishoftu and the protective level of all chickens against virus challenge.

Specific objectives

- To determine the persistence of maternally derived antibody in the layer chicks.
- To compare the antibody titres of chickens those vaccinated via single eye and double eyes Instillation with different vaccination schedule.
- To evaluate the protection level of chicken vaccinated through single eye and double eyes Instillation and unvaccinated control group against virus challenge.

2. LITRATURE REVIEW

2.1.History of Newcastle diseases Virus

Newcastle disease (ND) was first recognized ninety years ago and continues to be a problem for poultry producers. At least four defined panzootics have been recognized (Miller and Koch, 2013); negatively impacting not only economic livelihoods, but also human welfare by decreasing food supplies After the initial, almost simultaneous, identification of Newcastle disease in 1926 in Indonesia, England, and possibly Korea, ND was identified to the Philippines, India, Japan, Australia, and Kenya. By 1952 it was also reported in Palestine, Syria, French Congo (present day Gabon, Republic of Congo, and Central African Republic), the island of Sicily, Europe, and the United States. In the 1960's as part of the 2nd and 3rd panzootics, ND was reported in Hawaii, Canada, Mexico, Central and South America, China and throughout Europe. These panzootics were aided both by the trade and movement of exotic psittacine birds without strict quarantine guidelines, the ubiquitous and synanthropic nature of pigeons, and the industrialization of the poultry industry (Dimitrov*et al*., 2017).

The widespread distribution of ND and the high number of annual outbreaks demonstrate that although globally used, current ND vaccines and vaccination practices alone cannot control the disease. Countries most affected during 2006 to 2009 in descending order were Iran, South Africa, Israel, China, Vietnam, Columbia, Romania, South Korea, Kuwait, and Sweden. With 56 countries reporting ND outbreaks on average per year from 2006 to 2009, ND ranked 2nd only behind rabies in the reported disease outbreaks. From 2008 to 2010, 77 countries confirmed ND outbreaks in domestic poultry with 68, 61 and 56 countries reporting ND outbreaks in 2013, 2014, and 2015, respectively. Underreporting of ND, especially in areas where virulent NDV is endemic in poultry may mean that these numbers are underestimating an already bad situation. Many of the countries affected by ND also lack good biosecurity practices. Thus, ND will likely persist in these areas of the world until both vaccines and biosecurity practices are improved (Anonymous, 2011).

During 2012, severe outbreak of ND occurred in Jallo Wildlife Park in Lahore, Pakistan, caused by APMV 1 serotype. Within a week, it took the lives of approximately 190 peacocks with a 100% mortality rate and 50% loss of the susceptible birds. Isolation of virus and serological diagnostics, such as HI Test, ELISA and molecular diagnostic tests like real time PCR confirmed the presence of velogenic Newcastle Disease Virus (Munir*et al*., 2012).According to the Ethiopian authorities reported only 14 outbreaks reported in 1998, 16 in 1999, 8 in 2000, 34 in 2001, 67 in 2002, 16 in 2003 and 40 in 2004 (OIE, 2006).

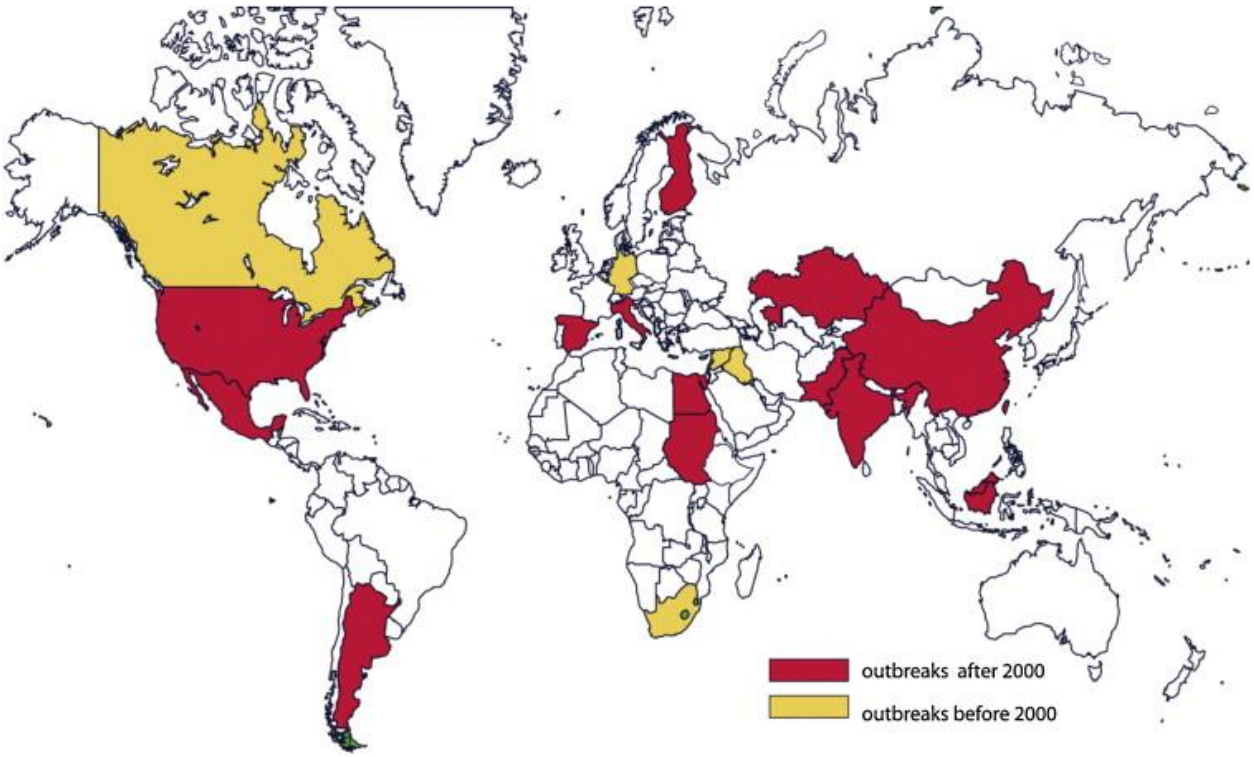

Figure 1: Newcastle disease virus (NDV) outbreaks in different parts of the world.
Source: www.science direct, 2014

2.2.Etiological Agent

The virus responsible for causing ND is "a member of the family: Paramyxoviridae, in the genus: Avulavirus."NDV is also known as avian paramyxovirus, of serotype 1 (APMV-1). Only infections with the virulent APMV-1 (vNDV) are responsible for ND. Strains of NDV that are of

low virulence (loNDV) are often used as vaccines to prevent disease and death from ND (OIE, 2009).According to the international committee on taxonomy of viruses (ICTV), this virus has the following characteristics: Family: Paramyxoviridae, Genus: Avula virus Serotypes: one, Classes: two, the virus exists in 10 serotypes; APMV-1 to APMV-10, but all NDV isolates belong to serotype 1 (APMV-1)(Waheed*et al*., 2013).

2.2.1. The Virion.

The virions are pleomorphic in shape, and consist of single stranded, non-segmented, negative sense RNA genomes (Miller*et al*., 2010). NDV virions are 100 nm or more in diameter, pleomorphic, but mostly spherical in shape. The virion is enveloped with a lipid membrane from the host cell plasma membrane. The envelope contains two transmembraneglycoproteinsthe HN and the fusion (F) protein. These proteins are present as homooligomers and form spikelike projections of 8 nm length on the outer surface of the envelope (Samal, 2011).

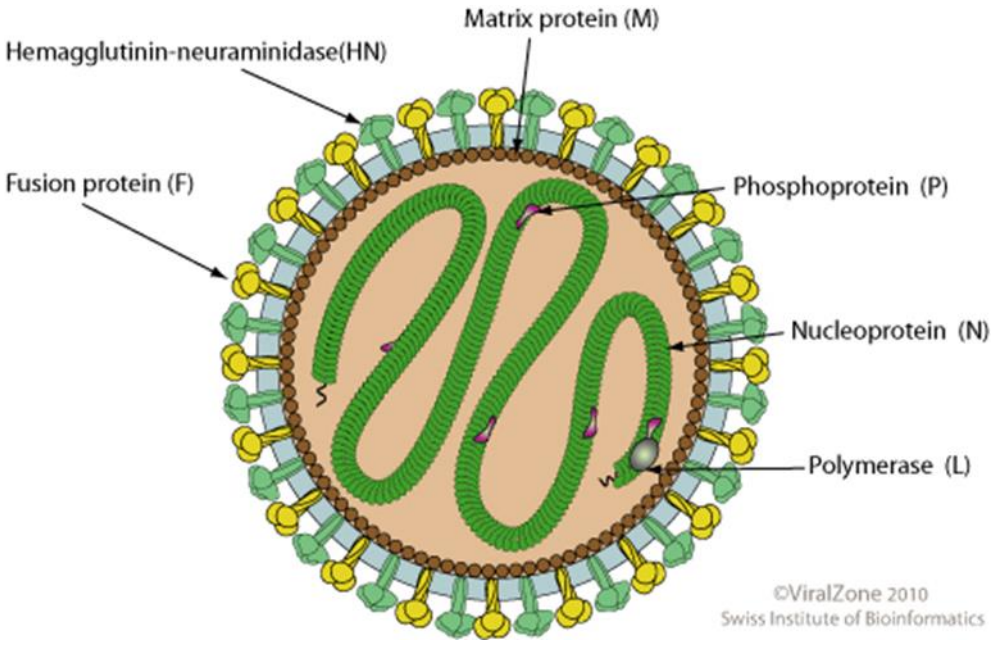

Figure 2: Schematic diagram of a paramyxovirus showing major components.

Source: Viral zone, 2015.

The viral matrix protein (M) underlies the lipid bilayer. Inserted through the viral membrane are the hemagglutinin neuraminidase (HN) attachment glycoprotein and the fusion (F) glycoprotein. Only some paramyxoviruses contain the SH protein. Inside the virus is the negative strand virion RNA, which is encased in the nucleocapsid protein (N). Associated with the nucleocapsid are the L and P proteins, and together this complex has RNA-dependent RNA transcriptase activity. The V protein is only found in rubulavirusvirions.There are at least three different genome lengths(15,186, 15,192 or 15,198), with six genes that produce six structural proteins in a 30to 50order: nucleocapsid (N), phosphoprotein(P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) andthe RNA dependent RNA (large) polymerase (L). Editing of P produces at least one other protein, the V protein, which has anti interferon properties (Czegledi*et al*., 2006).

2.2.2. The Genome Structures

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

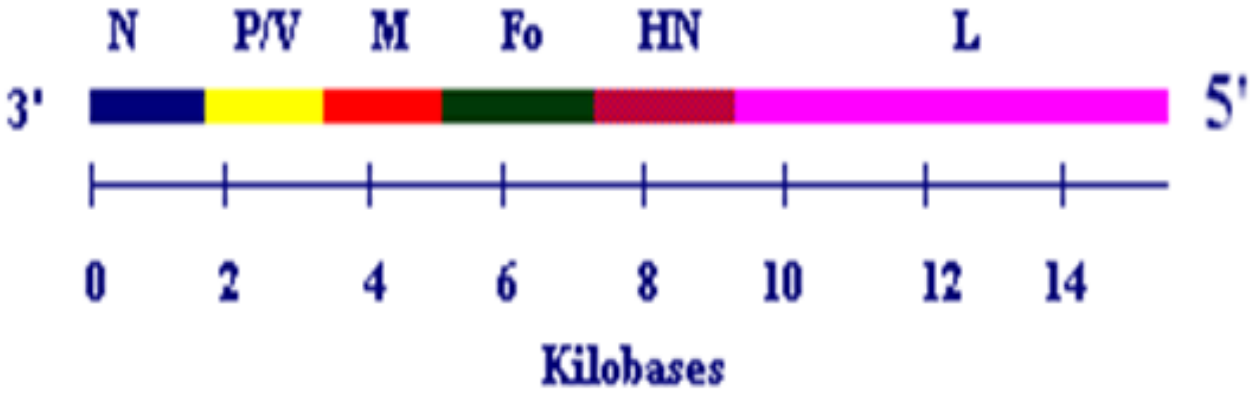

Figure 3: Schematic representation of NDV genomic structures**.**

Source: Virus, 2016

NEnvelope: lipoprotein bilayer contains projections of F and the larger HN (8 nm).**P/VP**polymeraseassociated or phosphoprotein which is part of the transcriptase complex**V** two non-structural virus encoded proteins produced in infected cells may have function in virus replication. **M**Matrix protein interacts with cytoplasmically located nucleocapsids and membrane associated glycoproteins in cell envelope to produce virus bud. **F** - Fusion protein enables virus and host cell membrane to fuse. **HN** - haemagglutinin or attachment protein enables virus to attach to host cell membrane. Also possesses neuraminidase activity (in some strains HN is derived from a larger precursor: HN0). **L** - Large protein, largest virus structural protein, acts as virus transcriptase and replicase in association with P.

2.2.3. Property of Virus Protein

In virus particles, NP is the most abundant protein which provides the NDVs core helical nucleocapsid structure. NP is the main regulator in replication of viral genome. The genomic RNA is associated with NP, P and L proteins to form RNP complex, which serve as template for RNA synthesis. NP is found to be highly immunogenic, as it induces antibody responses in chickens (AhmadRaus*et al*., 2009).

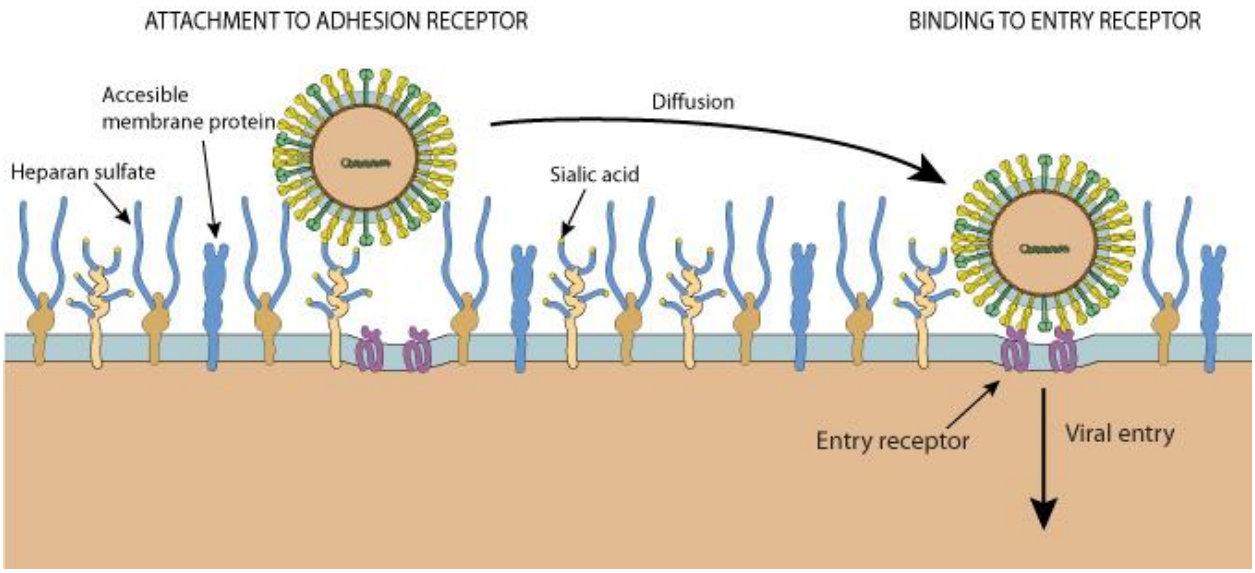

Figure 4: Diagrammatic representation of viral attachment

Sources: Viralzone. 2008

2.2.4. Virus replication

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

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

2.3.The Diseases

2.3.1. Signs and symptoms of Newcastle diseases

Neurotropic velogenic ND virus (nvNDV) affects the nervous system and causes paralysis of legs and wings, the neck twists to one side the birds may depict the picture of star gazing like torticulus, birds move in a circle and exhibit opisthonic posture. Viserotropicvelogenic ND virus (VVNDV) affects the digestive tract of birds, results in decrease feed and water intake, and copious greenish white diarrhea. Pneumotropicvelogenic ND virus (PVNDV) causes respiratory signs in chicks which result in difficult breathing (Oladele*et al*., 2005).

The incubation period of ND virus ranges from 2 to 15 days. Cough flu sneezing, tracheal rales, fever,and depression, watery discharge from nose, decreased feed intake, decreased water intake, diarrhea, conjunctivitis, ruffled feathers, torticolis, and blindness was observed in birds. Diarrhea,

nervous signs, shivering and paralysis of legs and wings has been observed in pigeons (Shaheen*et al*., 2005). In addition, the clinical signs and symptoms of ND also include depression, weakness, loss of appetite, dehydration, inability to stand, cyanosis of comb and wattle, greenish watery diarrhea, nasal and eye discharges, decreased egg production, loss of weight followed by death (Pansota*et al*., 2013).

There are many factors which effect severity of clinical signs of ND in birds mainly; age, route of infection, immune status and concomitant environmental stress. Young birds have more severe and acute disease in comparison to older birds. The intravenous inoculation /infection elicit neurologic signs and aerosolization of high viral doses impact the upper respiratory infection (Alexander, 2003). There is also species variation regarding expression of clinical signs in birds. The clinical signs in chickens are recognizable at second day of the onset of the disease (Susta*et al*., 2011), birds become off feed and dull on third day and chicks become severely depressed and inactive with hard ruffled feathers on fourth day, prostrated position and open mouth breathing starts on fifth day of ND infection. Nervous signs i.e. blindness and torticollis and incoordination on seventh day of ND virus infection (Sa'idu*et al*., 2006).

The pigeons develop ND after fifth day of infection and became dull, anorexic and have droopy and paralyzed wings. Loss of appetite, greenish diarrhea, swelling of head, respiratory distress, sneezing and coughing was observed in turkeys and guinea fowls, quails. The redness of eyes, and swelling around neck and ear was also seen in infected birds. In case of acute outbreak of ND there may be sudden death of birds in the flock in a period of overnight incubation with virulent strain of ND virus. Sometimes only death is the symptomatic feature of ND in poultry flocks. Rise in temperature, dullness, thirst, anorexia, staggering gait, shaking of head and paralysis of legs and wings in last stages is also observed. Birds also become lame, have drooping wings, unable to fly, walk in circles and older birds may also moult(Oladele*et al*., 2008).

2.3.2. Transmission

NDV can infect more than 240 species of birds and it spreads primarily through direct contact between healthy and infected birds. The disease transmits through droppings and secretions from

the nose, mouth and eyes of infected birds. The disease spreads by contaminated water, feed and transport. Airborne transmission of the virus is also an important route of transmission for ND (Li *et al*., 2009).Mechanical transfer of infected faeces occurs by rodents, insects, dogs, fleas, or scavenging animals. Infection takes place by virus inhalation, ingestion or by contact with conjunctiva. The disease may vary from subclinical with no mortality to severe infection, with 100% mortality(Ullah*et al*., 2004)

The primary route of transmission is either by ingestion of fecal contaminated material or inhalation of droplets containing the organism. Viral replication in the respiratory tract of infected birds allows for dissemination of the virus during nasal discharge. When the virus reaches the mucous membranes of susceptible birds, the virus is likely to reach the upper respiratory tract. Replication in the respiratory tract of newly infected birds allows for the potential to expose more susceptible birds and the virus easily spreads through the flock. The success of this mode of transmission hinges on the environment temperature and humidity and the viral load contained in the aerosolized droplets. The virus is also able to replicate in the intestinal tract which can then be excreted in the feces. It has been shown that large amounts of virus are commonly excreted in the feces of NDV infected birds (Alexander *et al.,* 2004).

Several methods of virus transmission have been linked to the introduction of NDV to new premises. Direct ingestion of feed or water contaminated with feces delivers a high virus load to susceptible birds. This was demonstrated by the PPMV-1 transmission to chickens that occurred in Great Britain in 1984. Importation of sick pet or exotic birds, movement of commercial poultry and game birds or the sport of racing pigeons allows for dissemination of the virus across vast distances. A broad range of animals including reptiles and humans can be infected with NDV and are able to distribute the virus to other vulnerable animals. The virus particles have been shown to enter the eggshell after it has been laid which gives rise to the potential for virus spread during transport of table or hatching eggs. Live or attenuated vaccines may also be a source of infection if the virus used to prepare the vaccine is not properly killed or the vaccine is contaminated. Vaccination and insemination crews as well as veterinarians have been shown to transmit the disease from farm to farm due to improper cleaning and disinfecting of equipment (Li and Qiu, 2009).

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

Biosecurity of commercial poultry facilities is an important step in preventing transmission of NDV and large economic loss. It is recommended that poultry farms and hatcheries should not be in close proximity to each other to protect highly susceptible young birds. Poultry farms and flock houses should also be spread apart from each other to avoid transfer of contaminated material between premises. Movement of equipment and materials between farms should be restricted and subject to thorough cleaning and disinfecting. Humans may also harbor the virus in the conjunctival sac resulting in conjunctivitis and possible dissemination of the virus. It is not advised for people to move between premises unless appropriate biosecurity procedures are followed. Separation of farms based on species is important to prevent introduction of exotic diseases to new avian species. The water supply should be clean and should not come from surface water where migratory birds have the potential to contaminate the water source (Yu *et al.*, 2009).

2.3.3. Pathotype and pathogenecity

For chickens, different strains of NDV have great variation in pathogenicity. On the basis of clinical signs in infected chickens, strains of NDV are grouped in to five pathotypes:1) Asymptomatic enteric: a form that has subclinical enteric infection without clear symptoms;2) Lentogenic: virus present with the mild respiratory infections;3) Mesogenic: virus presents with rare nervous and respiratory signs while mortality rate is related with the age of susceptible birds (young birds are more susceptible as compare to adults);4) Viscerotropicvelogenic: virus cause haemorrhagic intestinal lesions it is highly pathogenic;5) Neurotropic velogenic: virus cause high mortalities followed by respiratory and nervous signs (OIE, 2012).

The NDV isolates are differentiated on the basis of in*vivo* estimation of pathogenicity. These in *vivo* tests are mean death time (MDT) in SPF embryonated eggs of chicken, intracerebral pathogenicity index (ICPI) in 1 day old SPF chicks, and Intravenous pathogenicity index (IVPI) in six weeks old SPF chicks (Mohamed *et al*., 2011).

The MDT classifies ND virus strains into the groups: velogenic (takes less than 60 h to kill); mesogenic (takes from 60 to 90 hto kill); and lentogenic (takes more than 90 h to kill), The ICPI classifies ND virus strains by giving indices scores from 2.0 to 0.0. The maximum score of 2.0 is given to most virulent ND virus strain while lentogenic strains are given score close to 0.0; The IVPI classifies the ND virus strains from lentogenic to velogenic. Lentogenic strains and some mesogenic strains have IVPI values of 0.0, whereas the maximum IVPI indices for a virulent strain are 3.0 (OIE, 2004).

2.3.4. Molecular basis of pathogenicity

The genome of NDV encodes for six major structural proteins. Viral replication, transcription and translation occur in the cytoplasm of the host cell, while virus particles are assembled in plasma membrane by budding (Zanetti*et al*., 2003).Important pathogenic marker of NDV exists in F protein. Disulphide linkage is present between F1 and F2. These proteins enable the virus to attach to the host cell membrane. At cleavage site, F0 protein has two pair of basic amino acids that can be cleaved by the host proteases (Pham *et al*., 2005). Highly virulent NDV has three or more basic amino acids, which are lysine (K) or arginine (R) present at 113 - 116 residues and phenylalanine (F) at position 117. Cleavage of F0 protein is due to the presence of these basic amino acids in virulent NDV. It has been found that avirulent virus have 112G/E-K/RQ-G/E-R-L117 and virulent viruses have 112R/K-R-Q-K/RR-F117 amino acid sequence at cleavage site (Boostani*et al*., 2013)

Most of the pathogenic APMV-1 viruses for chicken have sequence 112R/K-R-Q/K/R-K/R-R116 Office of International Epizootics (OIE) accepts F cleavage sequence as determinant of primary virulence. However, if this cleavage sequence is not found, then an Intra Cerebral Pathogenicity Index (ICPI) is required for determination of the virulence (Wise *et al*., 2004).

2.3.5. Genetic diversity of Newcastle diseases virus

Genetic resistance to ND has been observed with variouslines within a breed for chickens and turkey and among breeds of chickens and ducks (Shi *et al.,* 2011). Concerning this it is important to note that each Newcastle disease virus may be better adapted to grow in one species versus another, like what is seen with PPMV1 (pigeon NDV) strains in chickens. Another example of this can be seen with the variability in the bird infectious dose 50 of one NDV forchickens, turkeys and ducks (Aldous *et al.,* 2010).While, improving genetic resistance to ND through breeding more resistant bird strains appears to be feasible, logistically it is very difficult due to the involvement of multifactorial components. Perhaps when the efficiency of producing transgenic birds is improved, more disease resistance breeds can be used for this purpose.While humoral immunity from vaccination is critical to ND control, another important aspect that is not a new concept, but is often neglected, is the differences in resistance to ND due to genetic variation. In addition, it is known that there is a negative correlation between a primary antibody response to NDV and favorable production traits(Zhang *et al.,* 2012).

2.3.6. Chicken immunity to Newcastle diseases virus

i. The innate immune response.

The innate immune response comprises factors that exist prior to the advent of infection, and are capable of exclusion or rapid response to microbes. The primary components of innate immunity of poultry are (1) physical and chemical barriers, such as feathers and skin, epithelia and production of mucus; (2) phagocytic cells, including macrophages and natural killer cells; (3) complement proteins and mediators of inflammation; and (4) cytokines. Overall, the innate immune response to virus infection is an immediate reaction designed to control and inhibits virus growth and spread and aid in developing pathogen-specific protection through the adaptive immune response. The early reactions of the innate immune system use germ-line encoded receptors, known as pattern recognition receptors (PRR's), which recognize evolutionarily conserved molecular markers of infectious microbes, known as PAMP's (Pathogen-associated molecular patterns). The virus is first recognized by host sentinel proteins, including TLR and NOD proteins, producing rapid signaling and transcription factor activation that lead to

production of soluble factors, including interferon and cytokines, designed to limit and contain viral replication (Kapczynski and Afonso, 2013; Miller, 2013).Adjuvant to improve the immune response of NDV vaccines were initially focused on inactivated vaccines, but now includes substances to favorably modulate the immune response from live NDV vaccines. Dietary supplements are commonly tested because the compounds may be locally available and/or because the compound maybe easily added to the diet to improve the immunity after vaccination. Lactobacillus-based probiotics have been shown to improve humoral immunity to live NDV vaccines in birds under heat stress (Sohail*et al.,* 2010). Antibiotics may be added to water at the time of vaccination to provide an undefined benefit to the birds (Khalifeh*et al.,* 2009). However, when antibiotics are evaluated for their ability to positively potentiate the Humoral immune response to NDV vaccines, typically they are found to decrease the response or not significantly improve the response (Munir*et al.,* 2007).

Astragalus polysaccharides commonly used in Chinese medicines to enhance the immune response demonstrated slight improvements in the humoral immune response to NDV vaccination with or without sulfation (Huang *et al.,* 2008).Glycyrrhetinic acid liposomes demonstrated a significantly improved humoral response to NDV vaccination 21–42 days after vaccination (Zhao *et al.,* 2011).

ii. Cellular immunity induced by Newcastle diseases virus.

Cell-mediated immunity (CMI) is specific adaptive immunity mediated by T lymphocytes and has been suggested to be an important factor to the development of protection in chickens vaccinatedagainst NDV and contribute to viral clearance. Cell-mediated stimulation following NDV infection is detected as early as 2–3 days post infection. More recent studies also confirmed CMI responses to NDV may be detected shortly after vaccination with a live NDV vaccine). In those studies, chickens with CMI specific for NDV, determined by blastogenesismicroassay with inactivated NDV, were not protected from lethal challenge in the absence of HI antibodies. However birds with NDV specific antibodies were shown to be protected. The results indicate that antibodies are the key modulators of protection, but that CMI likely contributes to decrease viral shedding through target killing of NDV infected cells (Lwelamira*et al.,* 2009).

Subsequent studies have compared CMI responses between birds receiving live versus inactivated NDV vaccines. In one study, measurement of IFN-c by ELISA and proliferation to NDV from splenocytes obtained from chickens receiving live or inactivated NDV vaccines were compared. Results indicate increased CMI with the live NDV vaccination.Whereas, live NDV stimulates both major histocompatibility complex (MHC) class I (CD8+) and II (CD4+) presentation in the host, CMI derived from inactivated NDV vaccines take longer to develop and are not as robust. Additional studies examined the role of vaccine virulence in CMI. Not surprisingly, the virulence of the virus appears to play a role in CMI stimulation. Rauw*et al*. demonstrated an earlier and shorter CMI induced by a less virulent NDV vaccine strain, compared to a stronger and longer CMI mediated by a more virulent vaccines strain. Thus, the more virulent strain persisted longer in the bird and therefore was able to increase magnitude and duration of CMI (Rauw*et al.,* 2009).

In the chicken, IgM, IgY (avian IgG equivalent) and IgA antibodies are produced as part of the immune response. Antibodies are detected at the site of infection and in the blood starting at six days after infection or live virus vaccination and peaks 21–28 days after infection. Antibodies neutralize the ND virus particles by binding and preventing attachment of the virus to host cells (Al Garib*et al.,* 2003).Approximately 30% of the IgY and 1% of the IgM and IgA antibodies present in the hen's plasma will passively transfer to the offspring and if the NDV antibody levels are high enough can provide protection until the levels fall below a protective level. This maternal antibody can interfere with live vaccination by neutralizing the vaccine virus (Hamal*et al.,* 2006).

2.3.7. Newcastle diseases status in Ethiopia

Newcastle disease (ND) 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 variation in sex and age. The disease is characterized by respiratory, nervous system impairment, gastrointestinal and reproductive problems Sources of infection for NDV are exhaled air from infected birds and contaminated feed and water and transmission is mostly via aerosol. Feces, eggs lay during clinical diseases, and all parts of the carcass during acute infection and at death can also act as sources of infection. Chickens infected with virulent NDV may die without showing any clinical

sign of illness though young chickens are more susceptible and show sign sooner than older ones. Much of the spread of ND in village is probably via human agents (Ashraf and Shah, 2014).

An outbreak of ND is unpredictable and discourage villager from paying proper attention to the husbandry and welfare of their chickens. Various studies have been conducted to determine the epidemiology of ND in various countries in Africa.In study conducted in Ethiopia the seroprevalence rates of 28.57 29.69, 38.33 and 43.68% were found in DebreBerhan, Sebeta, Adamaand Central Ethiopia (among local scavenging chickens kept under a traditional management system), respectively (Tadesse*et al.*, 2005).

Another study conducted in two districts of Eastern Shewa Zone, Ethiopia, the seroprevalence of ND (and other poultry diseases being not considered in this study) in the wet and dry seasons and they reported the overall seroprevalence of ND was 5.9% during the dry season and 6.0% during the wet season In general, the epidemiology of ND in village poultry in Ethiopia is poorly understood and there is no appropriate investigation and control strategy designed against the disease. This is due to lack of disease monitoring capacity in the Veterinary Services Department of the Ministry of Agriculture and Rural Development. Farmers start to consider, therefore, losses due to diseases as normal and natural. And they fail to report outbreaks to the veterinary authorities.Though all the above study reveals that as ND seriously devastating poultry industry in Ethiopia, This paucity of information on the presence and seroprevalence of ND in backyard and small scale poultry producer farms may reflect a lack of resources for disease surveillance and control in poultry production system. In addition, the diagnostic coverage of poultry diseases in Ethiopia is limited to the extent that, even from commercial farms, only a few cases are brought to National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta or the National Veterinary Institute (NVI), Bishoftu. Most poultry disease outbreaks, particularly in more remote parts of the country, remain undiagnosed and dead chickens are simply discarded (Chaka *et al.*, 2012).

2.4.Diagnosis

Newcastle disease can be diagnosed based on history, clinical sign and laboratory test. Newcastle disease clinically resembles highly pathogenic avian influenza so during outbreak rapid and accurate diagnosis is important to control and prevent dissemination of disease. Laboratory diagnosis for NCD includes virus isolation, serological (enzyme-linked immune sorbent assays (ELISA), immune diffusion test, agar gel precipitation and molecular test (Reverse transcription polymerase chain reaction (RT-PCR). Newcastle diseases mainly diagnosed by heamaglutination inhibition test Isolation of the NCD virus is definitive diagnosis of NCD (seif*et al.*, 2005).

2.4.1. Diagnosis based on clinical sign

The clinical sign of NCD is depends on age, immune status of the host, tissue tropism and virulence of virus strain. Sudden highmortality in a flock in the absence of premonitory clinical signs occurs when susceptible species are exposed to highly virulent strain. In susceptible flocks the mortality rate in fully can reach 100%.

Figure 5: Torticollis, nervous symptoms, Newcastle disease.

Sources: Backyard chicken.com,2015

The incubation period of NCD is usually about five days. In chicken's nerve, respiratory and digestive sign may occur. The major clinical sign observed in Newcastle disease are: greenish white diarrhea, with ruffled feathers; depression in the birds and a state of prostration, a condition known as torticollis (the head turned to one side and other neurological sign like paralysis of leg and wing). NCD is acute disease can cause death within 2 to 3 days (Hasan*et al*., 2012).

2.4.2. Diagnosis based on pathological lesion

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

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

Virulent strains can cause hemorrhages of the blood vessels in multiple organs especially the intestinal tract. The serosal and mucosal surfaces show marked necrosis in intestinal lymphoid aggregates. Necrosis can be seen in the cecal tonsils, and hyperplasia of monocytes is evident in the liver and other organs. The germinal centers of the spleen and thymus show marked focal vacuolation and lymphocyte destruction. Hemorrhages can also occur in the heart, gallbladder, skin, and eyelids leading to conjunctivitis. Petechiae of the wattle and combs and facial edema are commonly seen during infection. Diagnosis should not be based on path gnomonic lesions or clinical signs because these types of symptoms and lesions are not specific to any strain of NDV. Some lesions may be seen with infection of low virulent strains, and symptoms may be similar to those seen with more virulent strains. Pathology is a useful tool to guide disease diagnosis, but it cannot be used solely to diagnose ND considering these types of lesions were not unique to NDV infection (Hines and Miller, 2012).

2.4.3. Diagnosis based on Laboratory techniques

i.Sample for laboratory diagnosis

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

ii.Virus isolation

Because of their extreme sensitivity and convenience, ten to twelve (10-12) day-old specific Pathogen free (SPF) embryonated eggs are readily used for cultivation of NDV. This is done by inoculation on to the chorioallantoic membrane or into the allantoic sac. Virulent ND viruses' can also be propagated in cell culture. Samples for isolation include antibiotic treated cloacal and tracheal swabs or trachea and bone marrow. For cultivation in eggs, finely ground tissues, organs and faecal samples treated with antibiotics are centrifuged at 1000g for 10 minutes and 0.2ml of the supernatant inoculated into the allantoic sac. The eggs are incubated at 37^{o}C and examined

daily by candling. While eggs dying on the first day are discarded, those, which die thereafter up to day 7 of incubation, are chilled at 4^{o}C and the allantoamniotic fluid harvested. The presence of the virus is detected by hemagglutination test (Alexander, 2003).

iii. Serological technique

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

Haemagglutination inhibition test

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

Enzyme linked immunosorpent assay

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

is conjugated to the anti-chicken antibodies so when anti-NDV antibodies are present and bound to the NDV antigen, the enzyme bound to the anti-chicken antibodies catalyzes a color change in the well. This can be read by viewing the plate or quantitatively using a spectrophotometer. Serial dilution of the anti-NDV antibody test serum can be used to determine the titer (Saif*et al.,* 2008).

Molecular technique

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

Polymerase chain reaction

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

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

Reverse transcription polymerase chain reaction

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

This is rapidly becoming the assay of choice. Several important steps need to be considered in developing an RT-PCR protocol. The first aspect is the RNA extraction. This needs to be an efficient process that can extract RNA from the samples even when it's in low concentrations and eliminate contaminants that will degrade the RNA. Another important aspect is the gene being targeted and the choice of primers. This can have a profound effect on the efficacy of the assay. Poorly design primers can result in mispriming and the amplification of nonspecific products or the formation of primer dimmer (AbdElsalam, 2003).

2.5.Prevention and Control

2.5.1. Vaccination

Vaccination is the most successful tool for prevention of ND. Non usage of ND vaccine in rural areas is one of the factors for outbreak of ND. For the prevention of ND in chicks, the birds should be vaccinated against ND. The vaccine against the local strain of ND virus prevalent in the respective areas may be used for vaccination against ND. The improper vaccination may result in the outbreak of ND. Routine vaccination of rural poultry flocks against ND can help preventing ND. The local ND vaccine commonly available in the market helps protect the chicks against local strains. Based on the age of birds and manufacturers recommendations and guidelines should be followed for vaccination. Different routes of vaccination are; eye drop

(intra-ocular), intra-nasal (spray), sub-cutaneous and drinking water. In case of use of ND vaccine in drinking water, use of medications and sanitizing agents in drinking water must be discontinued 24 hours before drinking, and must be resumed after 24 hours following vaccination. Non-chlorinated water should be used for vaccination (Mustafa and Ali, 2005).

i. Preventing vaccine failure

Vaccine failure results in the outbreak of ND. In spite of vigorous and regular vaccination, ND is still havoc to the poultry flocks and outbreaks have occurred even in vaccinated flocks. Vaccine failure results from, inadequate method of vaccination, vaccination during concurrent incubation period of ND, stress of extreme weather conditions (winter or summer), transportation of birds after vaccination, etc. Poor quality of vaccine is also one of the factors that can cause vaccine failure Poor vaccine quality is due to poor manufacturing standards, low quantity of antigen (immunogen), use of expired vaccines, faulty application in birds, lack of proper storage, exposure to direct sunlight, poor vaccine handling during transportation and non-maintenance of supply cold chain(Numan*et al.,* 2005).

The vaccine failure may also result due to mycotoxins and drug induced modulation, stress to birds, concurrent infectious agents, malfunctioning of host defense mechanisms or presence of high titers of maternal antibodies titer. Preventing vaccine failure through monitoring quality of vaccine and host immune system can prevent ND in poultry flocks (Muhammadamin and Qubih, 2010).

ii. Boosting immune system of birds

Immunity level (antibodies titer) of breeder flocks against ND virus must be checked and maintained at regular intervals through vaccinations. Low serum antibody titers against ND are often found in even in vaccinated flocks. Any type of stress in birds can break the immunity level of bird and serum antibody titer level may decrease. The low serum antibodies titers cannot protect birds from ND. Unsuitable vaccination schedule, improper vaccination techniques, impaired immune competence due to immunosuppressive substances in feed or immunosuppressive diseases like Infectious bursa disease (IBD) are the possible factors for low level of serum antibodies against ND. The low level of antibodies against ND in non-vaccinated

chicks of early ages is also due to low levels of maternally derived antibodies from parent flock transmitted through egg yolk and may not protect chicks form ND. Heat stress and water deprivation result in production of steroids and cause immunosuppressant. Impaired immune system of birds is also a risk factor for high incidence and prevalence of ND in chicks. Immune stimulation of a bird leads to increased antibody production, increased cellular immune responses and increased macrophage phagocytic ability which results in enhanced resistance to bacterial and viral infections. Deficiency in essential nutrients in feed also poses risk of ND. High stocking densities, over-crowding and intensive poultry production result in breakdown of immunity and is also one of the factors for severe outbreaks of ND. Use of immune booster products enhances immune response against ND in immune suppressed birds. For boosting the immune response of birds the feed may be supplemented with vitamin E / Selenium and minerals (Emad and Amjad, 2007).

iii. Passive immunization

The ND in breeder flocks can be controlled through passive immunization by use of hyperimmune serum (HIS). The use of HIS has protective effect in ND affected chicks. HIS has been successfully used to decrease the morbidity and mortality in affected birds. It is found that mortality and morbidity rate is low in chicks passively immunized with HIS while mortality is high in the chicks not passively immunized with HIS during ND virus infection (Pansota*et al.*, 2013).

2.6.Public Health

Humans are among the many species that can be infected by NDV in addition to avian species. NDV may cause conjunctivitis in humans, when a person has been exposed to large quantities of the virus. Mostly, Laboratory workers and vaccinators are affected. The use of personnel protective equipment and biological safety cabinet has reduced the exposure of laboratory workers. Infection is rarely seen in the workers of a farm; moreover persons handling or consuming poultry products do not appear to be at risk (Nolen, 2003).

The conjunctivitis usually resolves rapidly, but the virus will be shed in the ocular discharges from 4 to 7 days. In some cases, mild, self-limiting influenza like disease with fever and headache has also been reported in humans. There is no evidence found to support human to human transmission but the potential for human to bird transmission exists (David and Daniel, 2003).

3. MATERIAL AND METHODS

3.1.The study area

The study was conducted in Ethiopia institute of agricultural research center in Worer Research Center, Afar region, Ethiopia. The climate can generally be described as arid to semiarid, with maximum and minimum temperatures varying from 25 to 42 °C and 15.2 to 23.5 °C, respectively, and an average annual rainfall of 560 mm. Amibaraworeda is located between Latitude: 9° 39' 59.99" N and Longitude**:** 40° 19' 60.00" E. The driving distance from MelkaWerer to Addis Ababa is 291 km(WAS, 2008).

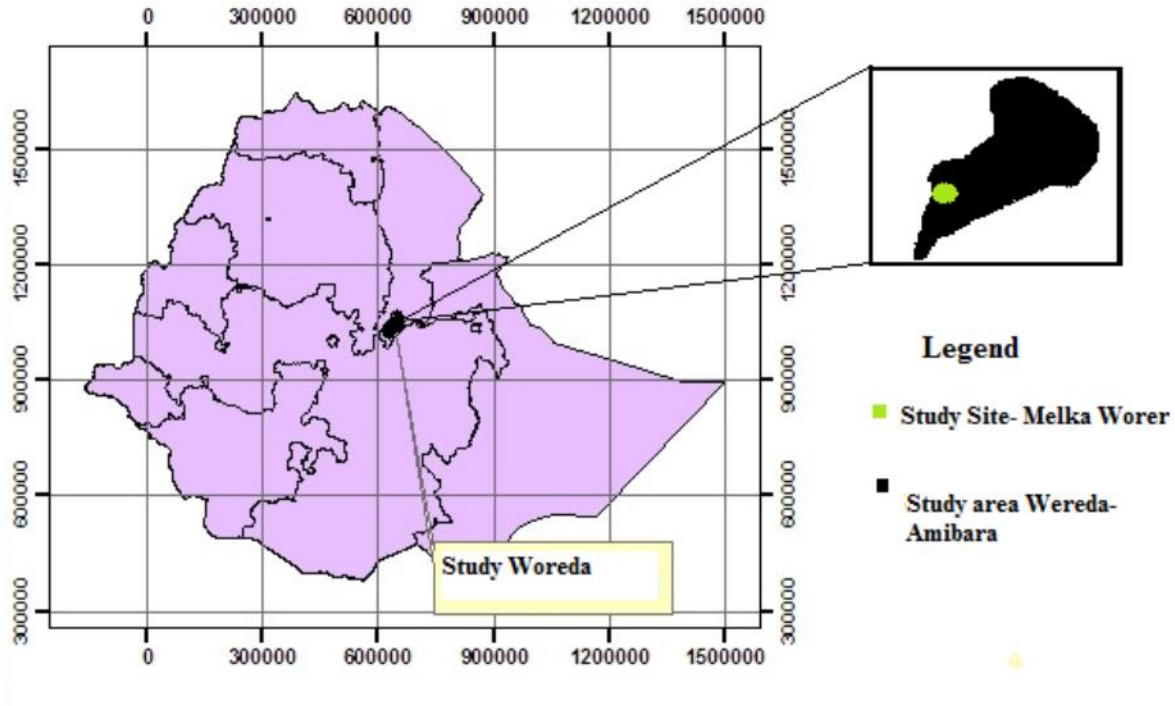

Figure 6: Schematic representation of study area

Sources: WAS, 2008

May and June are the driest months, whereas July through September is the main rainy season. The cattle population of Afar State is approximately 1,620,147, with 131,832 of them found in

Amibara DistrictThe CSA estimated that farmers in the Afar Regional State had a total of 327,370, cattle (representing 0.84% of Ethiopia's total cattle), 196,390 sheep (1.13%), 483,780 goats (3.73%), 200 mules (0.14%), 12,270 asses (0.49%), 99,830 camels (21.85%), 38,320 poultry of all species (0.12%), and 810 beehives (less than 0.1%). The CSA estimated on the basis of a survey performed in December 2003 Over 90 % of the population depends largely on the milk from cattle, sheep, goat, and camels as their staple food.Based on the 2010 Census conducted by the Central Statistical Agencyof Ethiopia (CSA), this woreda has a total population of 63,378, with an area of 2,007.05 square kilometers;Amibara has a population density of 31.58. While 28,137 or 44.40% are urban inhabitants, a further 6,555 or 10.34% are pastoralists (CSA, 2010).

3.2.Study chickens

A total number of 140 day-old (Lohman brown) chicks with the history of parent stock vaccinated against Newcastle disease (ND) was purchased from Alema farm, Bishoftu, Ethiopia, Chicks were reared in poultry farm of Ethiopian institute of Agricultural Research (EIARO) Worer Agricultural Research Center (WARC). There were attendants to provide feed and water and clean the equipments. The birds were provided with formulated ration obtained from one of the government owned animal feed industry (Kality). The flocks were fed daily and given tap water ad lib.

3.3.Vaccines

Conventional ND vaccines LaSota, HB1 and Thermostable I-2 produced by the National veterinary institute (Bishoftu, Ethiopia), were used to vaccinate chickens. HB1 and LaSotawere class II genotype II viruses used worldwide as live vaccines, while I-2 was a class II genotype I virus of Australian origin used as a thermostable live vaccine. These vaccines were often distributed on request to large scale and small scale commercial producers but are rarely used to vaccinate rural family chickens in response to outbreaks. The lyophilized LaSota, HB1 and I-2 vaccines with a titre of 10^7 EID50 per dose were used after reconstitution into the recommended dilution. Experimental chickens were vaccinated by the eye drop (0.1 ml) routes.

3.4.Study design and methodology

Complete random design (CRD) method was employed to assign total of 140 mixed sex (Lohman brown) day old layer chickens in seven experimental groups (6 vaccinated and 1 control groups). Each chick was identified using wing tag and randomly assigned to one of the 7 pens. Accordingly, a total of 140 chickens were randomly assigned to 7 pens with 20 chicks per pen. From which treatment group seven was unvaccinated groups. Groups 1, 2, and 3 were primarily vaccinated using HitchnerB1, LaSota, and Thermostable-I2 vaccines of 5 day of age and a (booster vaccine) LaSota, LaSota, and Thermostable- I2 vaccines administered at the age of 21 day, after twenty one day of primary vaccination, again at the age of 54 day immunized by Thermostable-I2, Thermostable-I2, and Thermostable-I2 vaccines, respectively, through in one eye instillation. Whereas, groups 4, 5, and 6 were vaccinated with the same vaccines respectively through two eye instillation following the same schedules.

All vaccinated chickens (group 1-6) were vaccinated with NDV vaccine, via ocular instillation outlined by the manufacturer (National Veterinary Institute/NVI, Ethiopia), using an eye dropper calibrated according to the calibration methods described by Alders and Spradbrow (2001).Group 7 was kept as unvaccinated control (Table 1).

Table 1: Vaccination schedule and route of administration

Groups	Vaccines used			Routes of inoculation
	1st	2nd	3rd	
Group 1	HB1	LaSota	I2	One eye
Group 2	LaSota	LaSota	I2	One eye
Group 3	I2	I2	I2	One eye
Group 4	HB1	LaSota	I2	Two eye
Group 5	LaSota	LaSota	I2	Two eye
Group 6	I2	I2	I2	Two eye
Group 7	Control	Control	control	Control

HB1- Hitchner B1vaccines, I2- thermostable vaccines

The experimental vaccination trial was conducted from February to March 2018 for twelve weeks whereas; virus challenge experiment was conducted in National Veterinary Institutes (NVI) Bishoftu, for fourteen days. During the study periods, Chickens that died or were euthanized at the end of the experiment were examined for evidence of which internal organ of chicken infected by field strain challenged virulent virus.

The chickens from each treatment group were kept separately and followed daily for morbidity and mortality. The chickens were kept in experimental poultry house that is isolated from poultry and other livestock farms under the auspice of the National Veterinary Institute, Bishoftu, Ethiopia. The experimental house is equipped with necessary containment facility.

3.5.Experimental animal Management

The experimental house ceiling, walls and floor of the house were disinfected using 1 % formalin. Clean disinfected deep litter was spread over the floor of bedding. Equipment including drinker, feederand buckets were cleaned, disinfected and introduced to the houses. The house was kept closed before the chickens were introduced.

All groups were housed in separate classes with wire mesh partition. The 140 experimental chickens were reared in pen with infrared bulbs for heating and deep litter for bedding.The chickens were fed on purchased starter commercial ration for 2 months, grower ration and Water was given ad lib. Antibiotic (oxytetracycline), minerals and vitamins mix in a sachet (i.e. Vytlet) was purchased and supplied for 3 days after each bleeding. Chickens showing signs of disease were given 20 %oxytetracycline and amprolium. Mortality was recorded daily.

Chickens were placed into separate sterile cages under strict hygienic and standard management conditions until the end of the post challenge experiment. All experiments were performed in animal facilitiesaccording to Addis Ababa University College of Veterinary Medicine and Agriculture Home Office ethical guidelines on animal welfare.

3.6.Blood collection

Blood for sera separation wascollected after 10 vaccinationdays from all vaccinated groups and at day 5, 15, 20, 31 days of age from unvaccinated controlled group using 2ml syringe, 1-2 ml blood from each chickens and subjected to Haemagglutination inhibition (HI) test for determination of antibody titters. Chicks in all groups werebled by sterile needle method and the collected blood was left overnight at room temperature to clot and then centrifuged at 1000 rpm for 10 minutes. Separated serums were stored at -20°C.

3.7.Antibody detection

Antibody titre against NDV in serum was determined usinghaemagglutination inhibition (HI) test using 4 units of haemagglutinin with two-fold serum dilutions, as recommended by the World Organization for Animal Health (OIE, 2012). The HI test was performed using the I-2 vaccinal strain of NDV, propagated in the allantoic cavity of 9-day-old to 10-day-old embryonated SPF eggs at the National Veterinary Institute, Ethiopia. The virus haemagglutination activity and infectivity could be inhibited by NDV antisera. The results were recorded as log2 values of the highest reciprocal of the dilution that showed inhibition. HI titres $\geq$4 log2 were considered positive results for NDV (OIE, 2013).

3.8.Protection test

Four weeks after booster vaccination, at the age of 90 days, 70 chickens (10 from each treatment group) were randomly selected and brought to the National Veterinary Institute, Bishoftu, Ethiopia. The chickens were housed in experimental poultry house. They were infected with local virulent NDV strain designated Alemaya strain obtained from the NVI, Bishoftu, Ethiopia. It has a mean embryonic death time of 51.1 h, an intracerebral pathogenicity index of 1.84 and an intravenous pathogenicity index of 2.51 (Nasser *et al.,* 2000).Each bird was inoculated with 0.1 mL of the viral suspension containing 10^7 $EID_{50/}$ml via naso-ocular routs. To check the virulence potency of the virus, in vivo tests was performed during the study. The challenge virus

was propagated and titrated in 9-day-old to 11-day-old SPF chicken embryos via the allantoic route. The EID50/ml of the virus was determined as described by Abdi (2016).

3.9.Ethical considerations

Before any attempt to collect sample the protocol was approved by Addis Ababa University College of Veterinary Medicine and Agriculture animal research ethical committee with reference number VM/ERC/20/05/10/2018. Official permission was also obtained from livestock and fishery office of the study areas as well as animal owners. This study was reviewed by the Research Ethics Board of Addis Ababa University College of Veterinary Medicine and Agriculture. Necessary changes and revision were carried out as per the feedback from the committee board before moving ahead to the data collection scheme.

3.10. Data management and analysis

Statistical Package for social Sciences (SPSS) version 10.0 programmers was used to analyze the data of the study. The mean value and standard deviation (SD) of HI antibody titers were determined and classified according to treatment groups. The post vaccination mean HI antibody titers were compared by General Linear model of SPSS. Proportion of chickens with HI antibody titer$\geq \log 2^3$ between treatment groups was used as a cut-off value to compare and decide the protective level.

ANOVA Duncan's Multiple Range Test (DMRT) was performed to determine the significant differences in HI titters of chickens of groups after primary and secondary and third vaccination. Significant differences set at 5 % alpha and at 95 % confidence interval. The relationship between chicken mortality (%) and % of chicken with HI titer above cut-off value were compared to the mean HI titer and statistically tested by Pearson correlation (Beri, 2005).

4. RESULTS

4.1.Antibody titer of passive immunity

Maternally derived antibody (MDA) were measured on a number of occasions starting from day 5 to day 31 and the titres are presented in Table 2.The layer chicken at five day of age scored (GMT ± SD) 210.00± 62.45 , 87.09± 39.19at 15 day age, 98.70± 33.12 at 21 day age and 78.79±30.91at 31 day.

Table 2: Maternal antibody of unvaccinatedchickens (Lohman Brown) determined by HI test

No. of serum sample	**Age of birds**			
	Day 5	Day 15	Day 21	Day 31
	Titre	Titre	Titre	Titer
1	1:128	1:256	1:64	1:128
2	1:256	1:128	1:64	1:128
3	1:256	1:64	1:128	1:128
4	1:256	1:128	1:128	1:64
5	1:256	1:128	1:256	1:64
6	1:256	1:128	1:128	1:64
7	1:256	1:128	1:128	1:64
8	1:256	1:32	1:64	1:64
9	1:128	1:64	1:128	1:64
10	1:256	1:64	1:256	1:64
Mean± SD	210.00±62.45	87.09±39.19	98.70±33.12	78.79±30.91

4.2.The immune response of chicken vaccinated through single eye instillation

The principal objective of this study was to observe the immune response of live NDV vaccines in layer chickens commonly used in Ethiopia.The result described in table 3 showed that, the mean and standard deviation of HI titres after primary vaccination via single eye instillation in groups 1, 2 and 3 were 64±47.81, 103.96 ± 30.91and 78.79 ± 64.79, respectively and after 10 days of secondary vaccination 168.89±70.10, 256 ± 0 and 238.85 ± 40.47, respectively. Meanwhile, after ten days of the third vaccination the mean and standard deviation were78.01±46.84in group 1, 194.01 ± 66.09 in group 2 and 181.01 ± 67.46 in group 3 (Table 3).

Table 3: The HI titer of chickens vaccinated throughsingle eye instillation

Groups	Vaccine used				Serum HI titer (GMT±SD) (n=10)		
	1st	2nd	3rd	vaccination route	Post-1st	Post-2nd	Post-3rd
1	HB1	LaSota	NDV-I2	one eye	64±47.81	168.89±70.10	78.01±46.84
2	LaSota	LaSota	NDV-I2	one eye	103.96±30.91	256 ±0	194.01±66.09
3	NDV-I2	NDV-I2	NDV-I2	one eye	78.79±64.79	238.85±40.47	181.01±67.46

HI- Heamaglutination inhibition, I2-Thermostable I2 vaccines, HB1- Hitchner B1vaccines, GMT- Geometic means titer, SD- Standard Deviation, n- Numbers.

4.3.The immune response of chickens vaccinated through two eye instillation

Birds of the double eye instillation vaccinated groups 4, 5 and 6 the mean of the titres after primary vaccination were 111.43 ± 26.98, 84.44 ± 42.66 and 90.50 ± 81.58, respectively. Also, after 10 days of the secondary vaccination the mean and standard deviation of the titer were 222.86 ± 53.96, 119.42 ± 84.25 and 181.01 ± 76.62 in group 4, 5 and 6, respectively. Meanwhile,

after 10 days of third vaccination the mean and standard deviation of the titer were 194.01 ± 66.09 in group 4, 78.79 ± 85.06 in group 5 and 128 ± 91.50 in group 6 (Table4).

Table 4: The HI titer of chicks vaccinated through two eye instillation

Groups	Vaccine used				Serum HI titer GMT±SD) (n=10)		
	1st	2nd	3rd	Vaccination route	Post-1st	Post-2nd	Post-3rd
4	HB1	LaSota	I2	two eyes	111.43±26.98	222.86±53.96	194.01±66.09
5	LaSota	LaSota	I2	two eyes	84.44±42.66	119.42±84.25	78.79±85.06
6	I2	I2	I2	two eyes	90.50±81.58	181.01±76.62	128±91.50

HI- Heamaglutination inhibition, I2-Thermostable I2 vaccines, HB1- Hitchner B1vaccines, GMT- Geometic means titer, SD- Standard Deviation, n- Numbers.

Table 5: Comparative HI titres of chickens between single and double eyes vaccination

Group	Vaccine used				Serum HI titer (GMT ± SD) (n=10)			p-value
	1st	2nd	3rd	vaccination route	Post-1st	Post-2nd	Post-3rd	
1	HB1	LaSota	I2	One eye	64±47.81	168.89±70.10	78.01±46.84	0.184
2	LaSota	LaSota	I2	One eye	103.96±30.91	256 ±0	194.01±66.09	0.014
3	I2	I2	I2	One eye	78.79±64.79	238.85±40.47	181.01±67.46	0.054
4	HB1	LaSota	I2	Two eyes	111.43±26.98	222.86±53.96	194.01±66.09	0.428
5	LaSota	LaSota	I2	Two eyes	84.44±42.66	119.42±84.25	78.79±85.06	0.483
6	I2	I2	I2	Two eyes	90.50±81.58	181.01±76.62	128±91.50	0.164

HI- Heamaglutination inhibition, I2-Thermostable I2 vaccines, HB1- Hitchner B1vaccines, GMT- Geometic means titer, SD- Standard Deviation, n- Numbers.

4.4.Protection test against Newcastle diseases virus (challenge-virus)

4.4.1. Protection result of unvaccinated control chicken

No clinical signs of ND were observed in any bird prior to challenge. Protection from virulent NDV challenge was determined by the absence of clinical signs and death during the 15 dpc period. Challenged birds appeared apparently normal for the first 3 dpc in all the experimental groups. The clinical signs observed were a decrease in feed intake, moderate to severe depression, ruffled feathers, respiratory distress with gasping and sneezing, and nervous signs such as wing drop and leg paralysis and prostration and death.

Figure 7: Wing drop and paralysis of leg clinical sign of challenged chicken

Figure 8: Died chicken in challenged pen

The morbidity rates in unvaccinated controlled group (G-7) showed three chickens at fourth day of day post challenge (dpc) and seven chickensalso revealed different symptoms from fourth day after challenged upto thirteen day. Totally from unvaccinated control group, ten chickens wereshowed different clinical signs and the first death recorded on fifth day of dpc. From controlled group totally ten chicken was died.

4.4.2. Protection result of vaccinated challenged chicken

i. One eye instillation

The morbidity rate in the vaccinated challenged birds was 0 % for (HB1, LaSota and I-2) after three day of dpc from group one and 0% from group two which was administered of (LaSota, LaSota and I-2) at the age of 5, 26 and 54 day, respectively. Similarly, group three,primarily vaccinated by NDV-I2, secondarily boosted by NDV-I2 and also thirdly vaccinated using NDV-I2 vaccines,were no showed clinical sign until the period of fifteen day followed up.Chickensadministered live vaccines through single eye drop were not exhibited either clinical sign or death.

ii. Two eye instillation

In this studythe protection ability of chickens received double dose of vaccines through both eyes (group 4, 5, and 6) were not revealed any typical clinical sign ofNDV as well as death.

iii. *Necropsy results*

At necropsy, all dead birds exhibited typical lesions similar to natural field cases. Macroscopic pathological lesions included oedematous and diphtheric mucosal membrane of the trachea, petechial and necrotic haemorrhages of the proventriculus, intestine, caecum and caecal tonsils, petechial haemorrhage of the heart, and deep-green content of the gastrointestinal tract starting from the proventriculus that ended up with green faeces.

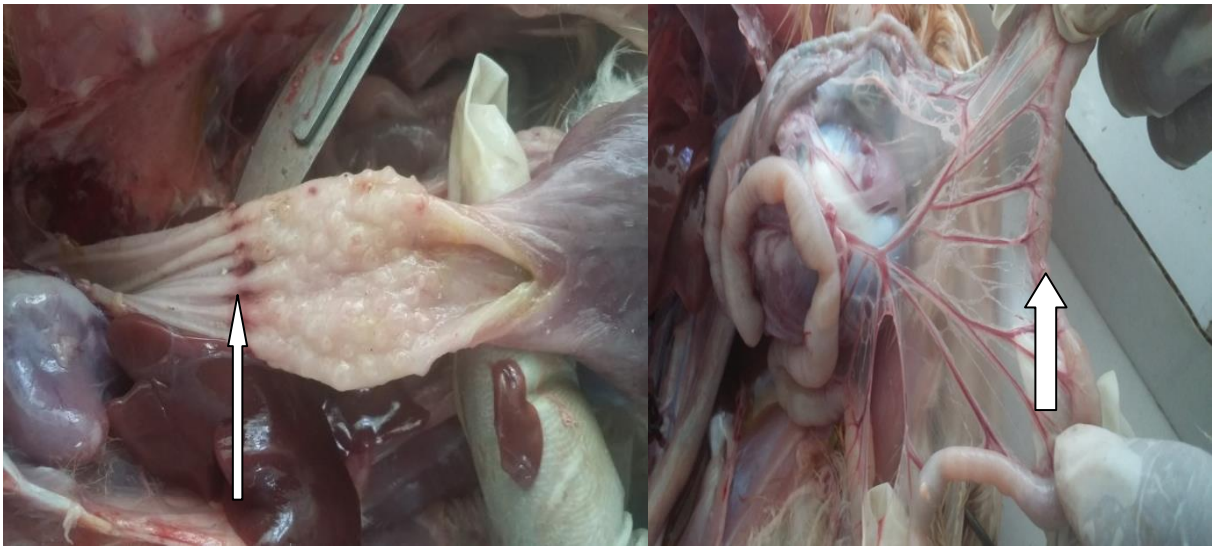

Figure: **9:** Petechial and necrotic haemorrhages of the proventriculus and intestinal tract

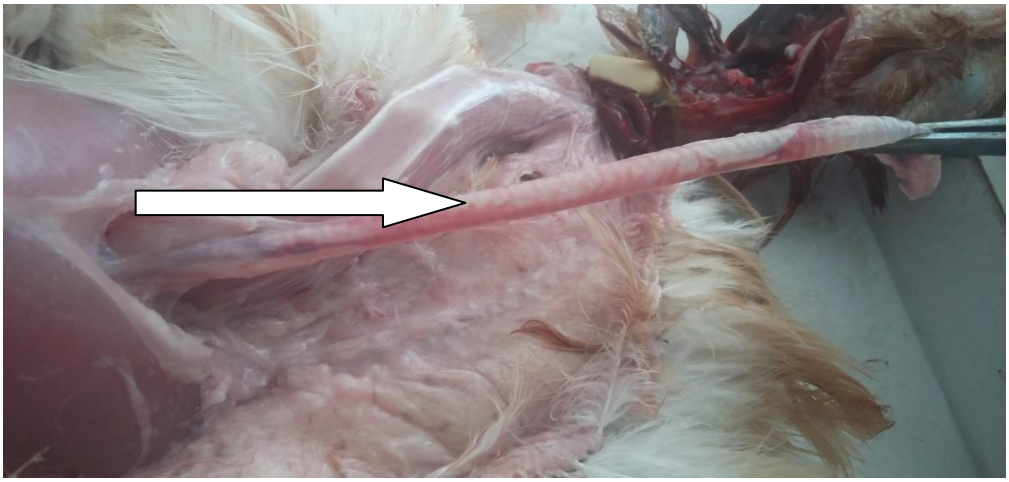

Figure 10: Haemorrhagic throat

5. DISCUSSIONS

The research was designed for evaluation of the immune responses of layer chicken vaccinated using live Newcastle diseases virus vaccines administered through single and double eyes instillation, and also determined the persistence of MDA in layer chickens, which were used as a control of vNDV challenge and as a reference against which the contribution of the vaccine to the overall immunity of the vaccinated chickens was assessed.

There were twenty chickens in the individual pen distributed randomly and totally 40 sample was collected from controlled group and evaluated the persistence of passive immunity from parent to offspring. It was observed that such antibody remained in chicks until the age of day 31, and HI titer ranges from $\log2^{6}$ – $\log2^{7}$, this antibody titer indicated that chicks came from vaccinated parent flock could persisted immunity up to 31 day of age. The finding of this investigation in respect of MDA is closely related with the findings of Mazija*et al.*(2009) who reported that MAD persisted up to 28 days;Mahud*et al.*(2007)also reported a persistence of MDA up to 27 days of age, which may be due to the high MDA titres. Additionally, Mahmud reported that MDA persisted until day 27 of age of chicks.

After ten days of primary vaccination it was clearly demonstrated that chicks vaccinated LaSota strain through single eye produced highest HI titer (GMT=103.96) than chickens vaccinated using Hitchner B1 (84.45) and thermostable I2 strain vaccines (78.79). Whereas chicks vaccinated Hitchner B1 vaccine via both eyes yielded highestHI titer (111.43) than andthermostable I2 strain (90.51) and LaSotavaccines (84.45). From the above result there were no significance differences($p>0.05$).

After ten days of secondary vaccination it was also observed that the antibody titres increased in all vaccinated chickens through one eye instillation as well as two eye instillation (one drop in one eye whereas other drop in second eye). Chickensvaccinated via single eye from Group two, primary vaccinated with LaSota (at the age of five day) and boosted by LaSota vaccines at the age of twenty six day yielded the highest HI titer (GMT=256) than group one and group three. It was indicated significance differences within the groups ($p< 0.05$).Shuaib*et al*. (2003) reported

that secondary vaccination yielded HI titres that were significantly higher than the HI titres after single vaccination.

In other hand, secondary vaccination using live Newcastle diseases virus vaccines were produced the highest antibody titer within double eyes vaccinated chickens of group four, and the result was described not significant differences compared with primary vaccination result ($p>0.05$).

Vaccinated chicken were immunized for third time using thermostable I2 (third vaccination) vaccines at 54 day of age, which yielded low antibody titer as compared with secondary vaccination but induced much more protective antibody than primary vaccination through one eye instillation, but there were not significance difference between the groups ($p>0.05$).Thermostable Newcastle disease virus (NDV) vaccines have been used widely to protect village chickens against Newcastle disease, due to their decreased dependence on cold chain for transport and storage.

The new vaccination schedule was conducted in this study that were group three from one eye instillation and group six from double eyes immunized using only thermostable I2 vaccines at the day of 5, 26, and 54 age. The result revealed that the geometric titer at primary vaccination was 78.79 ± 64.79, and the result of second vaccination was 238.85 ± 40.47, here , thermostable I2 vaccines as compared with HB1 with prime vaccination result of titer indicated that 79, 64 of GMT of thermostable I2 and HB1 vaccines respectively again, on the post-secondary result showed that those chicken immunized by thermostable vaccine increased up to 239 GMT but those primarily vaccinated HB1, then boosted by LaSota, scored 169 GMT.

This result from study of new schedule brings crucial information that thermostable I2 vaccines were important for rural households, whereas the third vaccination on 54 day of age both vaccinated using Thermostable I2 vaccines, again treatment group three was scored geometric mean titer of 181 whereas treatment group one was scored geometric mean titer of 78.Overall, from group 1-group 3 vaccinated with one eye drop, group two was produced the highest antibody titer and differed significantly ($p < 0.05$).

A comparative analysis of all the vaccines of different strains revealed that LaSota strain produced higher immune response than those of other strains HB1 and Thermostable I2 strain in one eye instillation. This finding is strongly supported to the findings of Islam*et al*. (2008) who reported that LaSota strain provided superior antibody production after vaccination compared to HB1. Sasipreeyajan (2016) also observed that LaSota is much more immunogenic than the Hitchner B1. However, William *et al.* (2004) did not find any significant difference in immunizing capacity, protection and stress between F strain and B1 strain.

The overall vaccinated chicken population which receive vaccination according to the new schedule in the study produced (100%) protective antibody titter (≥1:16) against NDV. The highest number of protected population in chicken vaccinated three times (5, 26 and 54) due to the fact that booster dose vaccination of NCD was applied on chickens (Al Garib, 2003). Based on the result of this study NCD average logarism antibody level response, vaccination of 5 days old chicken against NCD using the vaccine of Hitchner B1 (at the age of 5 days) and laSota strain vaccine at the age of (26, days of age) and thermostable I2 (at the age 54days)produced from national veterinary institute, Bishoftu, Ethiopia formed protective antibody level. Protective antibody level ≥1:16 was detected in chickens vaccinated based on the newly designed program.

In comparing LaSota strain produced low immune response in double eye drop of group five (instilled in both eye) of this study,primary received LaSota strain and yielded (84.45± 42.66) and secondly boosted by the same vaccines LaSota was produced (119.43±84.25) and at the age of 54 day immunized by thermostable I2 vaccines resulted (79±85).compared and evaluated with group four that primary vaccinated usingHitchner B1 and boosted using LaSota strain yielded (111±27), (222.86 ± 53.96) on secondary vaccines, and also (194±66) from third vaccination, the result demonstrated it was the least scored among double eyes vaccinated schedules. However, there was not significant different between the groups ($p = 0.083$).

In facts, LaSota produced moderate vaccinal reactions, especially in immunologically naive birds and is not usually recommended for primary vaccination. In theory, LaSota was unsuitable for vaccinating a multiage population, including young chicks which were inevitably seen in the

village situation. This was because the virus spreads and it was not practical to isolate the adults from the chicks.

The most commonly used NCD vaccination program is giving vaccine to chicken at 0, 18, 72, 132,192 and 216th day old and between four month intervals, but the finding of this study showed that vaccination of chickens at 5, 26, 54th days of age was as protective as commonly used vaccination schedule (0, 18, 72, 132 and 216 days). This finding strongly supported with the findings of Anebo*et al.* (2014). So that, from economic point of view, the new vaccination schedule reduces cost of vaccine (transport and handling cost), labor cost and time. The less frequently that chicken were vaccinated the more efficient the strategy (Habte*et al.,* 2014).

Birds of vaccinated groups did not respond equally to vaccination in the same manner and individual variation in the humoral immune response was observed within the same group of birds at the same interval following primary, secondary and third vaccination, and variation at different ages of chickens which corresponds with the findings Rhman*et al.* (2004) who stated that individual variations in the production of HI antibody response might be due to individual variations in the production of HI antibody response and the presence of variable passive immunity in chicks or to varying degree of sensitivity of immune mechanism to antigen.

Chickens vaccinated primarilyLaSota, secondary LaSota, and thirdly thermostable I2 vaccines through single eye drop were the best vaccination schedule and yielded the highest immune responses, whereas, chickens immunized primarily HB1, secondarily LaSota and thirdly themostable I2 vaccines through double eyes drop were the best vaccination schedules and yielded the highest immune responses.On other hand, the antibody response of, vaccination schedules of, I2,I2 and I2 strain at the age of 5,26, and 54 days through single eye instillation, were produced the best antibody titer.

The study found that booster immunization with vaccines produced in Ethiopia from vaccine strains used worldwide provided chickens with full protection from overt clinical disease caused by the challenge virulent NDV. Neither signs nor mortality were reported in any of the chickens that had received a prime-boost vaccination regimen. This study finding were consistent with the

reports of Nasser *et al.*(2000) and Degefa*et al*.(2004), who demonstrated that chickens vaccinated through the ocular route using vaccines produced in Ethiopia were fully protected from disease caused by virulent NDV. Similarly, Kapczynski and King (2005) and Van Bove*et al.* (2008) stated that booster and subsequent vaccination scheme provides protection against clinical disease and mortality after infection with virulent NDV provided that vaccinated birds have high antibody titres.

All vaccinated chickens in this study produced protective antibody titres and a positive correlation were made between the presence of positive HI antibody titres at day of challenge and protection from disease in the booster and subsequent vaccination scheme. Other researchers were reported that live ND vaccines provided better protection, and were preferred for priming birds as they produced local immunity in the mucosal membrane of the conjunctiva, thus providing immediate protection on subsequent exposure with field virus challenge (Alexander *et al.,* 2004; Jeon*et al.,* 2008). Moreover, Alexander *et al*.(2004) and Alexander andSenne (2008) demonstrated that vaccine programs adopting the method of progressive vaccinations, which involves successive booster vaccines with increasingly virulent strains were provided the best protection.

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, the present experimental study confirms that NDV vaccines produced in Ethiopia can induce high humoral antibodies to protect chickens against the prevalent disease. However, virulent NDV field strains currently circulating in poultry can easily infect and spread among vaccinated and unvaccinated poultry populations and can readily cause disease and major outbreaks in susceptible flocks. The study found that, to protect chickens from ND and prevent them from acting as viral reservoirs, it is important to maintain solid and potent immunity through efficient and involving successive booster dose vaccination programmers.

It was observed that ND vaccination could achieve a high antibody titre within a short period of time; however, a single vaccination, as often practiced in rural poultry in response to disease onset, was insufficient and demanded successive booster doses to provide adequate and prolonged protection of flocks. Despite protecting birds from sickness and death, ND vaccines produced in Ethiopia would not fully protect birds from viral infection, replication and shedding in vaccinated birds against the currently circulating virulent virus strain.

It is also suggested that vaccination programmers in village poultry should be initiated strategically to protect chickens during the active period of NDV infections and administration of live vaccine through ocular route is very important even if it is costly and labor intensive but has great advantage by equal dropping of virus dose to individual chicken so it interns elicits immediate local immunity and humoral immunity, which able to clear and prevent replication of virus.

Therefore, it may be concluded that the maternal derived antibodypersisted up to 31 day of age wasimportant to protect chicks at early infection against virulent NDV and primary vaccination at day 5, followed by a booster dosing at age of 26, thirdly at 54 day of chickens, younger age, may be followed for better immune response and protection against ND in layers chicken.The current study showed that ocular vaccination of chickens based on the newly designed schedule induced protective antibody level that can protect birds from NCD outbreak. When birds were vaccinated

frequent boosted dose with homologous vaccines were acquired protective antibodies. The net result of the study revealed that both, one eye and two eye vaccinated chickens, produced protective antibody, so that, they can survive in case of outbreak and the major way to prevent loss of egg production as well as decrease young mortality in layer chickens.

Therefore, depending on the above conclusion the following recommendation forwarded:

- Regular vaccinations should be supported by strict biosecurity measures in poultry farms to prevent introduction of the NDV.

- So, emphasis should be givento use of thermostable I2 vaccine in village chickens in reducing the mortality and improving their productivity and Vaccination programs should be continual and sustainable.

- It is paramount that the Newcastle Disease vaccination strategy should be redesigned and continued to apply both on commercial and village chickens to decrease the loss associated with mortality of chickens caused by Newcastle disease.

- To prevent mortality and decrease production loses, they should always ensure that the HI antibody titres of their layers do not drop below 16 (2^4) or 2 log $^{4.}$ They should always monitor the antibody response of the layers chickens after every vaccination for confirmation of adequate response by veterinary professionals.

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8. ANNEXES

Annex 1: Procedure for serological techniques

Haemagglutination and Haemagglutination inhibition tests

Materials

Thawed serum samples in racks, V-bottom 96 micro well plates and covers, PBS, 1 percent washed red blood cells, V-bottom reagent trough, 25 µL single and multichannel pipettes and tips, Microwell plate recording sheet, Newcastle disease virus antigen diluted to 4 HA units per 25 µL, Standard positive and negative serum, water bath to run at 56^{oc} centrifuge, centrifuge tube, measuring cylinder, embryonated egg, 70 percent alcohol solution, Cotton wool, Forceps and/or small scissors, 37^{oc} incubator.

Haemagglutination (HA) Test

1. Dispense 50 l PBS into a row of 12 wells in a micro titer plate for each antigen used in the test.
2. Add 50 l undiluted allantoic flied (antigen) to the 1st well of each corresponding row.
3. Serially dilute the antigen (first through 11th well) with a multichannel micro pipette set to deliver 50 l. In the 11th well positive control; the 12th row, containing only PBS, will serve as a RBC control.
4. Add 50 l 1% erythrocyte suspension to each well and shake the plate to thoroughly reactants.
5. Cover the plate with micro titer plate sealing tape and incubate at room temperature until a distinct button has formed in the cell control well (usually takes 20 to 30 min).
6. Record results as follows: Wells with complete Haemagglutination are recorded as "+" (positive HA); wells with a distinct button formation are recorded as "-" (negative HA); wells with partial button formation (fuzzy margins, or donut-like appearance) are recorded as "I" (incomplete HA). When interpretation between complete and incomplete inhibition is doubtful tilt the micro titer plate at about a 45 degree angle for 20-30 sec and

look for a “tear drop” appearance of erythrocytes in the wells with complete inhibition; wells with partial inhibition will not “tear drop.”

Haemagglutination inhibition test

Procedure

- ✓ Fill in recording sheets to record how samples will be dispensed into micro well plates.
- ✓ Calculate the number of plates required and number each plate. Dispense 25 l of PBS into each well of the plates.
- ✓ Shake each serum sample and dispense 25 l into the first well and the last (control) well of a row of a microwell plate.
- ✓ Use a multichannel pipette to make two-fold serial dilutions along the row until the third last well from the end. The second from the last and last well is the serum control. Do not dilute these well.
- ✓ Add 25 l of the 4HA dilution of antigen to each well excluding the control wells in the last column.
- ✓ Gently tap the sides of the microwell plates to mix the reagents. Cover plates with a lid. Allow to stand for 30 minutes at room temperature.
- ✓ Add 25 l of 1 percent washed red blood cells to each well including the control wells in the last two columns.
- ✓ Gently tap the sides of the microwell plates to mix the reagents. Cover the plates with a lid. Allow to stand at room temperature for 45 minutes.
- ✓ Read the settling patterns for each serum sample. Read the control serum well first then read the patterns in the other wells.
- ✓ Record the pattern observed in each well on a micro well plate recording sheet. Determine the endpoint. This is the point where there is complete inhibition of haemagglutination.
- ✓ Record the antibody level for each serum sample.

Annex 2: ELISA test

Materials

Coating butter, Phosphate buffered saline (PBS), Washing buffer, bovine albumin serum, and positive control, negative control, Conjugate buffer, and Substrate buffer, Reaction stopping solution, Spectrophotometer with 550nm filter, 37oc incubator, and distilled water.

Assay procedures for ELISA

1. Remove the pre-coated plates from their sealed bags and record sample and control locations on a 12*8 templet sheet. Each sample should be run in duplicate for the optimum results. The positive and the negative controls should always be run in duplicate.
2. Add 50µl of the undiluted controls and diluted samples to the appropriate wells. Diluted samples should be retained at the $+4^{oc}$ until successful results are confirmed. Cover the plate with an adhesive covers and incubates at +37oc for 30 minutes. Mix by gently tapping the side of the plate.
3. Remove adhesive cover and the plate 4 times with wash buffer (300µı) per well, invert and tap firmly on the absorbent paper.
4. Add 50µl of enzyme conjugate reagent to each well.Mix by gentle tapping the side of the plate.
5. Cover the plate with adhesive covers and incubates at +37oc for 30minutes.
6. Remove adhesive cover and wash the plate 4 times with wash buffer (300µl) per well, invert and tap firmly on the absorbent paper.
7. Add 50 µl ELISA substrate reagent to each well. The reagent must be at room temperature to achieve maximum color development. Mix by gently tapping the side of the plate.
8. Cover the plate with adhesive cover and incubate at 37oc for 30 minutes. Color development is pale pink, which depend on addition of ELISA stop solution.
9. Remove adhesive cover and add 50µl ELISA stops solution to each well. Mix on a plate shaker to obtain full color development.
10. Wipe the under surface of the plate free of dust with a soft tissue paper. Read the plate using a micro titer plate reader at 450nm having a first blanked on air. in order to obtain

optimum results the plate should be read immediately after adding the ELISA stop solution.

Annex 3: Chemical reagents preparations

Preparation of 1% chicken RBC suspension in PBS Reagents

Fowl RBC's (1% suspension in PBS, pH 7.2), Newcastle disease virus containing 4 HA units/0.05ml, normal serum (collected from non-vaccinated birds) and anti-Newcastle disease serum (collected from birds vaccinated) with Newcastle disease vaccine).

1. Blood will be collected from five chickens aged 2-6 weeks old in equal volumes.
2. The cells will be centrifuged at 1500rpm for 10minutes and the supernatant
3. Fluid will be discarded.
4. The cell will washed and centrifuged thrice with 40 volumes of PBS (7.2).
5. Resuspend the packed RBC in PBS to give 1 % (v/v)
6. Suspension is kept at 4^{oc} until needed

Preparation of the buffer saline (PBS) PH 7.2

Reagents

Sodium chloride----------8.00g

Potassium chloride----------0.20g

Disodium hydrogen orthophosphate----------2.312g

Potassium dehydrogenate orthophosphate----------0.20g

Distilled water----------1000ml

Elsevier's solution

Reagents

Citric acid C (OH)(COOH)(CH2.COOH)2.H2O ----------0.055g

Sodium Citrate Na3C6H5O7.2H2O ----------0.8g

D-Glucose C6H12O6 ----------2.05g

Sodium chloride NaCl ----------0.42g

Distilled water to make up to ----------1000 mL

Method

1. Weigh out reagents into a conical flask.
2. Dissolve of distilled water and make up to100 mL
3. Dispense into sterile 10 mL bottles.
4. Sterilize by autoclaving at 116oC for 10 minutes. Use slow exhaust.
5. Allow to cool, then tighten the lids and label the bottles.
6. Store in the refrigerator

Annex 4: Newcastle diseases vaccines used during study time

National veterinary institutes, Bishoftu, Ethiopia, produced and distributed Newcastle diseases vaccines to country those are used in this study. LaSota vaccine, Thermostable I2 vaccine and HB1 vaccines

Figure 11: Live Newcastle disease vaccines

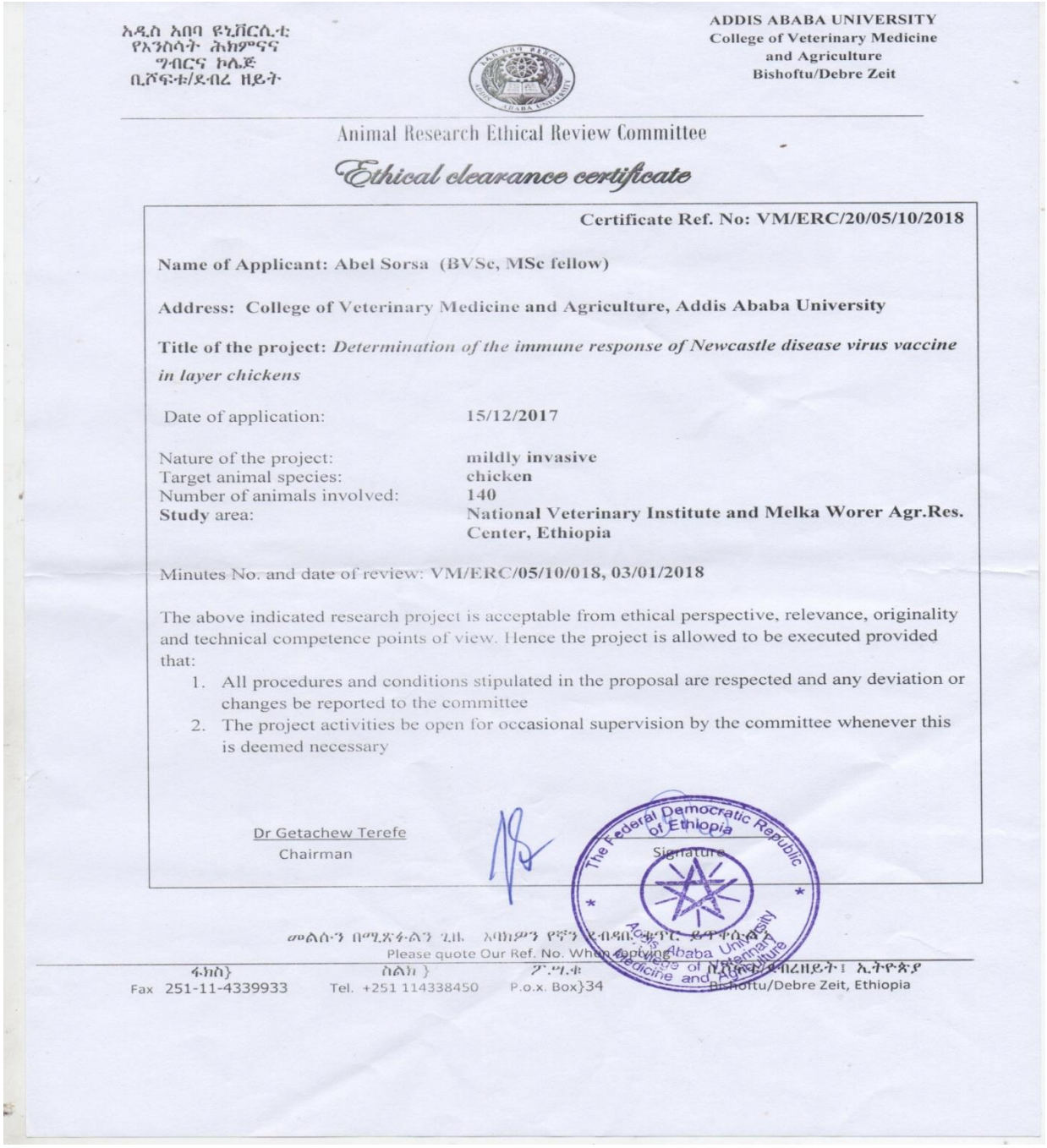
Annex 5: Vaccines administration thoroughocular route

Application of the vaccine by eye-drop methods is probably the most effective for live lentogenic vaccines. When using an eye-dropper, hold it in a vertical position. Eye-droppers are calibrated according to the size of the drop forms and the dropper should be held in a vertical position.

Figure 12: Application of vaccines through eye drop methods

Figure 13: Experimental chicken: (LohmanBrown).

Annex 6: Ethical clearance certificates



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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/20/05/10/2018

Name of Applicant: Abel Sorsa (BVSc, MSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Determination of the immune response of Newcastle disease virus vaccine in layer chickens*

Date of application: 15/12/2017

Nature of the project: mildly invasive
Target animal species: chicken
Number of animals involved: 140
Study area: National Veterinary Institute and Melka Worer Agr.Res. Center, Ethiopia

Minutes No. and date of review: VM/ERC/05/10/018, 03/01/2018

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University College of Veterinary Medicine and Agriculture

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Bishoftu/Debre Zeit, Ethiopia