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ON FARM EVALUTION OF THERMOSTABLE I-2 NEWCASTLE DISEASE VACCINE AND MOLECULAR CHARACHTERIZATION OF NDV IN SMALLHOLDER POULTRY FARMS IN MINJAR-SHENKORA WEREDA AMHARA REGIONAL STATE, ETHIOPIA

BY

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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 Epidemiology

By

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DEDICATION

This MVSc thesis manuscript is dedicated to my mother “Mulu Wuneh”, my uncles “Gidey Wuneh, Abeba Wuneh and Roman Wuneh” for the love and protection given to me when I grow up. You all are my reason to my success.

STATEMENT OF AUTHOR

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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LIST OF ABBREVIATIONS

AAF	Amnioallontoic Fluid
APMV	Avian Paramyxo Virus
CI	Confidence Interval
CMI	Cell Mediated Immunity
cRBC	Chicken Red Blood Cells
ECE	Embryonated Chicken Eggs
EFSA	European Food Safety Authority
EID_{50}	Embryonic Infection Dose
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
FTA card	Flinders Technology Associates
HA	Haemagglutination
HI	Haemagglutination Inhibition
IBDV	Infectious Bursal Disease Virus
ICPI	Intracerebral Pathogenicity Index
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IVPI	Intravenous Pathogenicity Index
LMP	livestock Master Plan
Mabs	Mouse Monoclonal Antibody
MDT	Mean Embryonic Death Time
*m*RNA	messenger Ribonucleic Acid
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NVI	National Veterinary Institute
OIE	Office des Internationale Epizooties
RNA	Riboneuclic Acid

LIST OF ABBREVIATION (*Continued*)

RT- PCR	Reverse Transcriptase Polymerase Chain Reaction
SD	Standard Deviation
SPF	Specific Pathogen Free
US	United States
VN	Virus Neutralization
VNNDV	Velogenic Neurotropic Newcastle Disease Virus
VVNDV	Velogenic Viscerotrropic Newcastle Disease Virus

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ABSTRACT

Infectious diseases including Newcastle disease are considered among important factors responsible for huge losses to poultry farmers and vaccination has been considered effective and affordable control option. Conventional vaccine delivery methods are not suitable for smallholder and rural poultry producers. This study was carried out with the aim of evaluating the efficacy of NDI_2 vaccine delivered via drinking water and litter spray compared to standard eye drop method under smallholder farmers' condition between December 2016 and May 2017. Molecular diagnosis of Newcastle disease was also conducted using RT-PCR targeting the L gene of Newcastle disease virus RNA. Twenty households rearing 154 chickens (82 indigenous and 72 exotic breeds) were selected purposively. The selected households were randomly assigned to one of the four treatments groups: placebo, drinking water, eye drop and spray. Blood samples were collected and HI assay was conducted on individual samples. Impressions of organ samples were collected from suspected Newcastle disease cases on FTA cards for molecular analysis. At baseline, there was no significant difference in antibody titre among the experimental groups. After the first and booster vaccinations, statistically significant ($P < 0.001$) variation was observed in mean (log_2) antibody titre between the vaccinated groups and the unvaccinated control group. Multiple pairwise comparison of mean antibody titre showed that the three experimental groups had significantly higher antibody titer than the control group after the first and booster vaccinations. Interestingly there was no statistically significant difference in antibody titre among the vaccinated groups. After the first vaccination, the proportion of chicken with antibody titre $\geq log_2^3$ was 74 %, 90% and 93% in the group vaccinated via drinking water, eye drop and spray respectively. Whereas in the control group it was 48%. After booster vaccination, the proportion of chicken with antibody titre $\geq \mathbf{log_2^3}$ was 90 %, 90% and 93% in the group vaccinated via drinking water, eye drop and spray respectively. Whereas in the control group it remained 45%. The vaccinated group showed 100 % survival while the control group showed only 40% survival after challenge infection. Out of 11 representative samples analyzed 3 samples (27.27%) were positive for NDV RNA. The results obtained from this study shows that NDI_2 vaccine administered via drinking water and litter spray under smallholder farmers' situation provoked protective antibody

level similar to the eye drop method. Use of NDI_2 vaccine needs to be considered by the veterinary and livestock authorities to prevent ND outbreaks and large-scale evaluation of the NDI_2 vaccine delivery routes have to be carried out.

Key words: *Newcastle disease, I_2 vaccine, Smallholder/village chicken, Immune response, Molecular characterization*

1 INTRODUCTION

Chicken are considered to be important livestock resources that can break the vicious cycle of poverty and malnutrition in developing countries. They are cheap sources of quality animal protein. In Ethiopia chicken are integral parts of agriculture and are reared by every household. The chicken of the country are featured by high genetic diversity (Goraga *et al*., 2001), morphological diversity (Duguma, 2006) and variable performance (Duguma, 2016). The country is endowed with over 42.9 million chickens, the majority (95 %) of which is kept in village scavenging systems (Wilson, 2010). Poultry production plays a major role in the economy of all regions of the country (Mazengia, 2012). Chicken play paramount role in the well-being of women and children. Most of the family chicken are owned and managed by women. They are important sources of cash income for women and some of the cash collected from sale of family chicken is used to cover school fees for children (Hailemichael *et al*., 2016). Chicken are also important for the society in general. They are consumed in every household during holidays and festive. Improving the efficiency of family chicken production will have significant impact on well-being of women and children, who are vulnerable to malnutrition. As the demand for food rises due to a growing population, it will be critically important to develop methods to produce more food with greater efficiency. As a result, the livestock master plan of the Ministry of Livestock and Fisheries targeted upgrading of village chicken production to improved family poultry. The aim is to raise chicken meat production from 2.9 thousand tons in 2015 to 10.2 thousand tons in 2020. Similarly, the egg production is aimed to rise from 258 million to 894 million during the same period (LMP, 2015). The number of specialized poultry production units are also planned to increase several folds. Introduction of improved exotic breeds and their crosses is crucial to achieve the targets.

Better knowledge of chicken health is vital for better welfare, productivity and optimum financial return. Specifically lowering the incidence and prevalence of infectious diseases such as Newcastle disease (ND) is needed. But infectious diseases including ND are considered to be the most important factors responsible for reducing both the number and productivity of chickens (Tadesse *et al*., 2005). The expansion of small scale poultry farms

in the rural Ethiopia could be intercepted as a result of widespread occurrence fatal cases of ND. Intensification of production and uncontrolled marketing can aid the spread of ND between and within poultry farms.

Various strains and pathotypes of ND virus have been identified in poultry throughout the world (Alexander, 1986). In Ethiopia for example, more than 15 outbreaks of ND were confirmed in 2016 whereas as hundreds of outbreaks were confirmed in 2015 (Unpublished data). However, the strains involved have not been identified. In endemic areas spread of ND between smallholder/village poultry and commercial poultry farms is inevitable. It has been suggested that village chicken may serve as a reservoir to disseminate ND virus to the nearby commercial poultry farms (Bell and Mouloudi, 1988). The control of ND in village chicken, therefore, not only reduces the impact of the disease within the village but may also prevent spread to nearby poultry commercial farms. Historically, annual ND outbreaks with high mortality are thought to have deterred the potential of owners to rear village chicken (Allan *et al.,* 1978). It has been clear that ND has the potential to impede up grading of poultry production and counter house development. Understanding of the epidemiology and effective control approaches of ND is a priority development area.

Effective control of ND is needed to improve the livelihood of smallholder farmers particularly women, which is key activity in poverty reduction strategies in developing countries (OIE, 2004). Vaccination has been considered effective and affordable control option in several countries (Palya, 1991). It has been widely used in commercial poultry farms. The demand for vaccine against ND has grown in smallholder farmers and scavenging poultry farmers. The conventional vaccines and their application methods, however, are not suitable for smallholder and rural poultry production systems (Alders and Spradbrow, 2001). An innovative alternative for poultry producers under these production systems was developed in Australia from heat stable strains of ND virus designated I2 (Spradbrow *et al*., 1977). This vaccine has been found to be suitable for rural poultry producers and is cheaper than conventional ND vaccines (Tu *et al*., 1998). Its efficacy has been proven in Asian and some African countries in the absence of cold chain (Spradbrow, 1993). Despite expansion of smallholder poultry production, information on the use of ND

I_2 vaccine in rural poultry production in Ethiopia is scarce. Specifically, route of vaccine delivery with optimal protection level has not been explored. This study was, therefore, carried out with the following objectives:

- To evaluate the efficacy of ND I_2 vaccine delivered via various routes in poultry under smallholder production system
- To identify the strains of ND virus circulating in the area using RT-PCR and sequencing

2 LITERATURE REVIEW

2.1 Poultry Production in Ethiopia

The poultry sector in Ethiopia can be characterized into three major production systems based on genotype of chicken reared and inputs utilized, these are large scale commercial production system, small-scale production system and village or backyard production system (Bush, 2006). The large-scale commercial production system is highly intensive production unit over 10,000 chicken reared under maximal inputs. This system heavily depends on imported exotic breeds that require intensive inputs such as feed, housing, health, and modern management systems. It is estimated that this sector accounts for nearly 2% of the national poultry population. This system is characterized by higher level of productivity and it is entirely market oriented. They are mostly situated in major cities. Although the practice of better biosecurity is expected to reduce chicken mortality to acceptable level of 5% (Bush, 2006), empirical evidence in the field shoed that the commercial poultry farms in Ethiopia experienced higher mortality. Small-scale poultry production system is characterized by medium level of feed, water and veterinary service inputs and minimal to low bio-security. Most small-scale poultry farms obtain their feed and foundation stock from large-scale commercial farms (Nzietchueng, 2008). There are few studies about diseases affecting poultry in this production system. Kinung'hi *et al*. (2004), however, showed that infectious diseases are responsible for mortality, reduced weight gain, egg production and market value of chicken. Village/backyard poultry production system is characterized by little or no inputs for housing, feeding (scavenging is the only source of diet) and health care with no bio-security, high off take rates and high level of mortality. As such, it does not involve investment beyond the cost of the foundation stock, a few handfuls of local grains and possibly simple night shades, mostly in the family dwellings. Mostly, indigenous chickens are kept although some hybrids and exotic breeds may be kept under this system (Dawit *et al*., 2008).

2.2 History of Newcastle Disease

Newcastle disease (ND) was first reported in 1926 on the island of Java, Indonesia. A year later the disease was recognized in poultry flocks in Newcastle-on-Tyne, England and found to be due to a virus (Alexander, 1980; Doyle, 1927). Mouth exudates from infected birds were found to be infectious after filtration with different types of filters used to remove bacteria. In addition, no bacteria could be cultured aerobically or anaerobically from organs of infected birds. Doyle suggested this disease be named for this city in which it was identified. Reports from Central Europe exist suggesting earlier ND episodes, but due to the lack of definitive evidence ND is considered to have been first recognized in 1926 (Halasz, 1912). In the past ND has been referred to as avian pneumoencephalitis, Korean fowl plague, Tetelo disease, Ranikhet disease, avian distemper, avian pest, pseudo-poultry plague, atypische Geflugelpest, pseudovogel-pest, and pseudo-fowl pest (Alexander, 2003). In 1927 Doyle reported that in Europe and Asia the birds with ND presented with respiratory and nervous signs, small hemorrhages in internal organs in 20% of the birds, along with high mortality rates (Doyle, 1927). In 1944 Beach reported that an isolate from a flock with pneumoencephalitis, a disease that had been prevalent in California poultry flocks for the previous nine years, was neutralized by Newcastle disease immune serum (Beach, 1944). The clinical signs in the California birds differed from the symptoms observed in European outbreaks in that the respiratory and nervous signs were more mild and the mortality was low (Beach, 1944). Over the following decades, the international transport of psittacine birds and poultry (initially by boat and eventually by air transport) along with the commercialization of the poultry industry assisted the spread of the virus around the world (Lancaster, 1975).

There have been four recognized panzootic episodes of ND. The first one began in 1926 and took sixteen years to spread (Lancaster, 1975). The second began in the late 1960's and spread more rapidly, only taking four years to spread across the globe (Alexander, 2003). As the third panzootic spread in the early 1970's, there was an increase in the number of countries affected by ND and also a change from the virulent viruses that typically caused neurological symptoms with moderate mortality to viruses that caused

intestinal lesions and higher mortality in unvaccinated birds (Lancaster, 1975). In 1970 and 1971 the United States (US.) was affected with outbreaks of ND in California, Florida and Texas in which these more virulent viruses were isolated (Butterfield and Graves, 1975). By the mid-1970s many birds were vaccinated for ND and, therefore, did not die upon infection. This complicated the control of ND in the U.S. after it was determined that government sponsored vaccine teams were unintentionally spreading the disease as they vaccinated seemingly healthy, but infected flocks. The infected birds shed the virus via oral secretion and fecal matter (Alexander, 2001). Since 1973, the U.S. has restricted the importation of exotic birds by requiring that they be quarantined and tested for NDV before entering. The last panzootic mostly affected pigeons whose symptoms were more neurological without respiratory signs (Alexander, 1988a). By 1981 the virus had spread around the world and in England poultry flocks were infected by feed contaminated with pigeon feces and carcasses.

2.3 Characteristics of ND virus

Newcastle disease virus is also known as avian paramyxovirus serotype-1 (APMV-1) (Alexander, 2003) and is a member of the genus Avulavirus (Fauquet and Fargette, 2005) in the Paramyxoviridae family. It is a negative-sense; single stranded, non-segmented, 15.2 kb enveloped RNA virus that replicates in the cytoplasm (Alexander, 2003; Lamb, 2007). The envelope is a lipid bi-layer and is acquired from the host cell that the virus has infected (Rifkin and Quigley, 1974). NDV is composed of six genes and their corresponding six structural proteins, listed from 3’ to 5’: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the large RNA polymerase (L) (Hamaguchi *et al*., 1983). RNA editing of the P protein produces two additional proteins, V and W (Collins *et al*., 1982). When one guanine residue is added to the conserved editing site of the mRNA by the RNA dependent RNA polymerase (RNAP), the V protein is produced after transcription. The addition of two G residues results in the production of the W protein. It is believed that the RNAP adds the G residues in a similar manner that stuttering adds four to seven uracil bases. The carboxy-terminus portion of the V protein has been shown to have anti-interferon activity, which allows the virus to reduce this

response of the host's innate immune system (Park *et al*., 2003). The function of the W protein is unknown (Huang *et al*., 2003).

The HN and F are glycoproteins that allow binding and fusion to host cells. The HN is a homo-tetrameric, type II integral membrane attachment protein that binds to the host cell receptors and is able to agglutinate red blood cells (Villar and Barroso, 2006). Infection of birds can be confirmed by testing sampleinoculated egg fluids for hemagglutination, followed with hemagglutination-inhibition assays using serum with NDV antibodies. The neuraminidase portion of the HN prevents the progeny virions from clumping to the surface of the cell from which they are being released, and may possibly prevent the virus from being trapped in secretions of the host that contain cell receptors that the virus recognizes (Villar and Barroso, 2006). The neuraminidase activity of the HN protein allows red blood cells (RBC) to eventually elute from the virus when the host receptors are degraded by the HN protein (Alexander, 2003). It has been shown that NDV isolated from wild birds may agglutinate RBC from different species than those isolated from poultry (Ito *et al*., 1999). Ito and co-workers showed that, in general, isolates from wild birds were able to agglutinate horse, pig, mouse, human, cow and chicken RBC, while the chicken isolates were mostly only able to agglutinate chicken and cow RBC. This suggests that the receptors may be slightly different and that this difference may affect how easily that virus can be transmitted from a wild bird to a chicken. There is also evidence that the HN protein plays a role in cell to cell and virus to cell fusion by enhancing the activity of the F protein (Lamb, 1993).

The HN protein of a few avirulent isolates such as Ulster 2C/1976, D26/1976 and V4 Queensland/1966, all class II/genotype I viruses, and Alaska 196/1998 (class I) is made in a precursor format of HN0. Posttranslational cleavage is needed to remove a forty-five-residue region of a glycosylated extension on the carboxy terminus that is thought to impair the function of the HN (Gotoh *et al*., 1988; Miller *et al*., 2007; Scanlon *et al*., 1999). These HN0 HN proteins are longer, having 616 amino acid compared to either 577 or 571 amino acids for the HN proteins that are active and do not need to be cleaved (Sakaguchi *et al*., 1989). HN0 can be cleaved by trypsin and by other proteases (chymotrypsin, thermolysin,

elastase) that do not cleave the fusion protein of these low virulence viruses (Nagai and Klenk, 1977).

The trimeric F protein allows fusion of the virus to the host cell and is a type I integral membrane protein with the transmembrane domain located in the C-terminus (Lamb, 2007). The F protein is always produced as a precursor molecule, F0. The F0 of a low virulence virus containing a single basic amino acid at the fusion cleavage site is expressed on cell surface and relies on exogenous proteases to be cleaved which occurs primarily in the respiratory or gastrointestinal tract (Rott, 1979).

F0 of a virulent virus having multiple basic amino acids is cleaved as it is transported through the *trans* Golgi at the N-terminus into F1 and F2 rendering the virus activated and infectious facilitating systemic replication (Rott, 1988). This is typically facilitated by host cell proteases, but can also be performed by bacterial proteases (Nagai *et al*., 1976). For cleavage of the F0 to be complete the host also needs to provide a second protease specifically a carboxypeptidase to remove basic amino acids (Lamb, 2007). The type of host proteases able to cleave the different F0 molecules determines if the virus can replicate systemically, as seen with virulent viruses, or primarily in the respiratory or gastrointestinal tract, as seen with the milder viruses (Rott, 1979). The less virulent 15 viruses having one basic amino acid at their fusion cleavage sites can be cleaved by only trypsin and trypsin-like proteases. The location of these proteases limits the location of where the virus can spread in the body.

The matrix protein (M) is largely conserved and associates with the inner surface of the viral membrane (Pantua *et al*., 2006; Peeples and Bratt, 1984). The NP, P, and L proteins form the ribonucleoprotein complex, which is the template for RNA synthesis (Yusoff, 2001). The NP of NDV is herringbone-like structure (Alexander, 1988b). Newcastle disease virions are pleomorphic, and may be round with a diameter between 100 to 500 nm or filamentous in shape with a diameter of 100 nm (Alexander, 2003).

2.4 Genetic Diversity

Although all NDV are members of APMV-1, antigenic and genetic diversity is recognized (Aldous *et al*., 2003; Alexander, 1998a). For example, antigenic diversity can be seen in Table 2.2 by the different binding patterns to monoclonal antibodies produced by viruses in different genotypes: Ulster vaccine virus (class II, genotype I), B1 vaccine virus (class II, genotype II), CA02 virulent viscerotropic virus (class II genotype V), Pigeon84, a pigeon APMV-1 variant (class II genotype VIb), and AK196, an avirulent class I waterfowl virus.

Aldous *et al*. (2003) have identified at least six distinct lineages or groupings of NDV based on nucleotide sequence. A more traditional classification using full-length sequence to relate the viruses isolated over time has been reviewed by the Lomniczi laboratory (Czegledi *et al*., 2006) and shows two major divisions represented by class I and class II, with both classes being further divided into nine genotypes (Kim *et al*., 2007a). Class I strains commonly isolated from apparently healthy waterfowl, shorebirds and birds from live bird markets are genetically distinct and phylogenetically distant from the commonly isolated vaccine viruses and the rarer outbreak viruses isolated in the United States (Kim *et al*., 2007a). Genotyping techniques were not yet developed when B1 and LaSota vaccines were developed. The U.S. *isolates* of NDV identified in the 1940s and the vaccines used today to control ND are Aldous lineage 2 (class II genotype II) viruses.

APMV-1 viruses have at least three genome lengths; 15,186, 15,192 and 15,198 nucleotides (Czegledi *et al*., 2006). Class I viruses are avirulent in chickens (except for one known virulent virus), typically recovered from waterfowl (Family Anatidae), and recently have been divided further into nine genotypes (1-9) based on a 374 base-pair portion of the F gene (Alexander *et al*., 1992; Kim *et al*., 2007a). Class II viruses are divided into nine (I-IX) genotypes.

Genotype III viruses were mostly isolated before 1960 in Japan, but have been isolated sporadically in Taiwan in 1969 and 1985 and Zimbabwe in 1990 (Yu *et al*., 2001).

Genotype IV viruses were the predominant viruses isolated in Europe before 1970 (Czegledi *et al*., 2006). Genotypes V, VI, VII, and VIII contain only virulent viruses. Genotype V viruses emerged in South and Central American in 1970 and caused outbreaks in Europe that same year.

Genotype VI emerged in the 1960s and continued to circulate as the predominant genotype in Asia until 1985 when genotype VII became more common (Mase *et al*., 2002). Genotype VI is further divided into sub-lineages VIa through VIg with VIb being commonly isolated from pigeons. Genotype VII was initially divided in to two sublineages: VIIa, representing viruses that emerged in the 1990s in the Far East and spread to Europe and Asia and VIIb, representing viruses that emerged in the Far East and spread to South Africa (Aldous *et al*., 2003). The two sub-lineages of VII were then further divided into VIIc, d, and e and represent more isolates from China and South Africa (Wang *et al*., 2006). Genotype VIII viruses have been circulating in South Africa since 1960s (Abolnik *et al*., 2004).

Genotype IX is a unique group that contains the first virulent outbreak virus from China from 1948 and members of this genotype continue to occasionally be isolated in China (Wang *et al*., 2006). The genotypes that are considered "early" (1930-1960) I, II, III, and IV contain 15,186 nucleotides. Viruses that emerged "late" (after 1960), V, VI, VII, VIII, and IX contain 15,192 nucleotides. Genotype IX viruses were initially isolated in 1948, an exception to the "late" grouping. Class I viruses are the longest of the APMV-1 genomes at 15,198 nucleotides.

2.5 Phenotypic characterization of Pathogenicity of ND virus

Newcastle disease affects a wide range of domestic and wild avian species; however, the severity of the disease varies greatly, spanning from peracute disease with almost 100% mortality to subclinical disease with no lesions. Such variability makes it impossible to pinpoint ND as a single clinicopathologic entity. Based on severity of clinical disease, the strains of NDV were originally classified into 4 pathotypes, known as Doyle, Beach, Beaudette, and Hitchner forms (Alexander, 1998). At present, pathotypes are more

commonly classified based on pathogenicity from least most pathogenic: “asymptomatic enteric,” “lentogen” (formerly Hitchner), “mesogen” (formerly Beaudette), and “velogen.” The velogens have been further divided into “viscerotropic” (formerly Doyle; velogenic viscerotropic NDV (VVNDV) or “neurotropic” (formerly Beach; velogenic neurotropic NDV [VNNDV]) according to their ability to cause primarily visceral or nervous signs (Alexander, 2003).

Additionally, some laboratory testing in embryos or chickens using standard pathogenicity parameters can be done, including MDT (mean death time), IVPI (intravenous pathogenicity index), and ICPI (intracerebral pathogenicity index). MDT, originally described in 1955, relies on the ability of virulent viruses to kill embryos faster than the less virulent viruses (Hanson and Brandly, 1955). The MDT is the time to death, measured in hours, after inoculation of embryonated eggs (if the embryos die in less than 60 hr, it is classified as a velogen; if the embryos survive for more than 90 hr, it is classified as a lentogen; anything in between is a mesogen) (OIE, 2008). The ICPI is currently used to differentiate endemic lentogenic viruses from more virulent mesogenic and velogenic viruses. The IVPI is used to distinguish mesogenic strains from the more virulent velogenic strains (Alexander, 2003).

The IVPI test involves scoring illness (0 = normal; 1 = sick; 2 = paralyzed or nervous signs; 3 = death) after intravenous inoculation of 6-week-old chickens. The IVPI scores are computed similarly to ICPI and range from 0 to 3. Velogenic NDV have IVPI scores between 2 and 3, mesogenic between 0.0 and 0.5, while lentogens have 0 (Alexander, 1998). However, there are no IVPI cut-off values to define notifiability to the international community the IVPI is not in widespread use today (Cattoli *et al*, 2011). At present, the definitive in vivo assessment of virus virulence is based on the ICPI test, which is regarded as the most sensitive and widely used test for measuring virulence (OIE, 2008).

The ICPI test is based on scoring sick or dead birds (0 = normal; 1 = sick; 2 = dead) every day for 8 days after inoculation of virus intracerebrally into ten 1-day-old chicks (Terregino and Capua, 2009). The score of the ICPI test is calculated using the mean score per bird,

per observation, over the 8-day period. Scores range from 0 to 2, and any strain with an ICPI ≥0.7 is considered virulent or "notifiable". Additionally, the OIE recognizes specific sequences of the F protein as a qualifier for virulence: "notifiable" are those strains that have, with respect to the amino acid sequence of the F protein, one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at residue 113 (OIE, 2008). The correspondence between these standard tests and pathotypes are reported in Table 1 (Alexander, 2003).

Table 1. Pathotypes of ND virus as determined by In vivo methods

Pathotype	**ICPI**	**IVPI**	**MDT**
Viscerotropic velogenic	>1.5-2.0	2.0-3.0	<60 hours
Neurotropic velogenic	> 1.5-2.0	2.0-3.0	<60 hours
Mesogenic	> 0.7-1.5	0.0-0.5	60-90 hours
Lentogenic	0.2-0.7	0.0	>90 hours
Asymptomatic	0.0-0.2	0.00	>90 hours

2.6 Epidemiology

2.6.1 Susceptible host species

NDV is reported to infect over 200 species of birds, both wild and domestic (Alexander, 2003; Kaleta, 1988). Turkeys (Meleagris gallopavo) are as susceptible to infection as chickens (Gallus gallus), but clinical signs are less severe (Alexander *et al*., 1999; McFerran, 1988). Waterfowl and shorebirds are felt to be the most clinically resistant among the wild bird populations (Kaleta, 1988). Domesticated and feral pigeons (Columba livia) are known to carry virulent NDV and have not only infected poultry but also have been infected by chickens (Pearson *et al*., 1987). Viruses have been isolated from cormorants and anhingas (Anhinga anhinga) and are maintained in kidney tissue for long periods of time (Kuiken, 1999). The most important outbreaks in wild birds have been in double-crested cormorants (Phalacrocorax auritus) (Kuiken *et al*., 1998). Ostriches

(Struthio camelus) are also susceptible to NDV infection (Verwoerd *et al*., 1999). Many animals including reptiles and humans are susceptible to NDV infection (Alexander, 1995). NDV will replicate in human conjunctival tissue and has been implicated in the possible death via pneumonia of an immunocompromised organ transplant recipient (Alexander, 1988c; Goebel *et al*., 2007).

2.6.2 Transmission, spread and maintenance of NDV

Newcastle disease virus is spread horizontally to susceptible birds through the inhalation or ingestion of respiratory secretions and fecal matter from infected birds (Alexander, 1988c). The infective dose of NDV per bird will depend on the virus and the susceptibility of the host. In general, the infective dose of a virulent NDV for a susceptible chicken will be in the range between 10^3 to 10^4 median embryos infectious dose 50 (EID50) (King, 1996b). Virulent NDV, being a systemic infection, can be found in the egg of infected breeder birds, but since infected birds typically have decreased egg production, it is thought that few infected eggs are laid. Because chicken embryos are highly susceptible to infection with resulting embryonic death, it is unlikely that vertical transmission of the virus occurs, although there have been reports of hatchlings born infected with vNDV (Chen and Wang, 2002; Roy and Venugopalan, 2005). In addition, eggs with fissures and cracks contaminated with fecal matter containing NDV may become infected. NDV in the environment from contaminated tissues, and feces can persist for days and can be spread indirectly on contaminated equipment, litter, soil, feed, vaccines and water. Vermin, wild birds, insects, and people all need to be considered as possible routes of exposing poultry to NDV. In addition, having members of a flock come into a house together and leave at the same time (all in-all out) and excluding water sources that have exposure to wild birds are a necessary part of biosecurity. NDV has been recovered from the air of poultry houses containing infected birds (Delay, 1948), and there is evidence that the virus can be spread from an infected flock to a susceptible flock through the air (Hugh-Jones *et al*., 1973). Transmission of avian influenza (AI) virus has been shown to be dependent on the relative humidity and ambient temperature and it is likely the NDV stability is also affected by

these factors (Lowen *et al*., 2007). Wild birds are known to have a potential role in the transmission of NDV to poultry (Aldous *et al*., 2007).

Cormorants have been implicated in ND outbreaks in turkeys raised in open range environments with access to the same water sources as the cormorants (Heckert, 1993; Heckert *et al*., 1996). Transmission of NDV to poultry through various insects has been investigated since the 1970s (Rogoff *et al*., 1975). Small amounts of CA02 NDV were isolated from flies collected from two residences that had backyard poultry infected in the CA02 outbreak (Chakrabarti *et al*., 2007). Laboratory infected house flies have been shown to be able to carry small amounts of NDV in their intestinal tracts for up to 96 hours (Watson *et al*., 2007). A novel, "upwards vertical", route of transmission of avian polyomavirus was discovered where blowfly larvae transmit the virus to nestlings, which then pass the virus on to the parents (Potti *et al*., 2007). Based upon these findings, it is clear that insect control should be taken into consideration when biosecurity measures are planned for a facility. Vaccinated poultry can shed virus for at least nine days after vaccination (Kapczynski and King, 2005). Parrots have been shown to be able to shed virulent virus sporadically for years (Erickson *et al*., 1977). While virulent NDV has been isolated from various wild birds, the reservoir for NDV is still unknown.

2.6.3 Newcastle disease status in Ethiopia

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 and policy by the Veterinary Services Department of the Ministry of Agriculture and Rural Development (Tadelle and Jobre, 2004). 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). Farmers start to consider, therefore, losses due to diseases as normal and natural (Nasser, 1998) and they fail to report outbreaks to the veterinary authorities.

There are only few studies that had been conducted to determine the prevalence and epidemiology of ND in various parts of the country. According to the research conducted in different parts of the country, (Chaka *et al*., 2012) in two districts of Eastern Shewa Zone, the seroprevalence of ND in the wet and dry seasons was 6.0 % and 5.9 % respectively. Zeleke *et al.* (2005) in southern and rift vally districts, Geresu *et al.* (2016) in Agarfa and Sinana districts of Bale Zone, Sori *et al*. (2016) in Sebata Hawas district, Tadesse *et al*. (2005) in central Ethiopia, Getachew *et al.* (2014) in Kersana-kondalaity district reported a prevalence of 19.78%, 27.86%, 11.34%, 32.22 and 5.6% respectively. Therefore, all the above studies reveal that ND is seriously devastating poultry disease in Ethiopia.

2.7 Clinical Signs

The incubation period for NDV is usually five to six days, but can vary from two to fifteen days and will depend on the species and immune status of the host, and the virulence of the virus (Alexander, 2003). The vast majority of references on NDV in poultry are related to chickens, as this species is the most seriously impacted by NDV (Alexander, 2003). There varying disease forms such that clinical findings in chicken are further divided according to pathotypes. However, the severity of clinical signs does not vary only accordingly to the inherent virulence of the virus, but also according to some host-related factors. These factors are mainly age, route of infection, immune status, and concomitant environmental stress (Alexander, 2003; Kinde *et al*., 2005).

2.7.1 Velogenic viscerotropic Newcastle disease

With VVND, mortality can easily reach 100%, and in experimental conditions, the course of disease is rapid, usually 2-4 days. Clinical signs are first recognizable starting at 2 days' post infection (Kommers *et al*., 2003; Brown *et al*., 1999). The main signs are conjunctival

swelling and reddening centered over the lymphoid patch located in the lower eyelid (Figure 1A), anorexia, ruffled plumage, prostration, weakness, tremors, and diarrhea; labored breathing is variably reported (Kommers *et al*., 2003; Susta *et al*., 2010).

The presence of multifocal hemorrhages seen through the serosal surface of the intestines, multifocal areas of necrosis and/or ulceration of the gut-associated lymphoid tissues and disseminated foci of necrosis in the spleen are highly suggestive of VVNDV infection (Alexander, 2003; Susta *et al*., 2010). The cecal tonsils, which are especially prominent gut lymphoid aggregates located in the proximal portion of the ceca, are often regarded as the "old faithful" lesion for VVND, as they most consistently display hemorrhage and necrosis grossly (Figure 1B). Other common intestinal lesions are multifocal hemorrhages and ulceration in the junction between proventriculus and gizzard, which is a site of lymphoid aggregate development (Figure 1C). Spleens are enlarged and severely mottled, showing multiple foci of white to yellow discoloration (necrosis) in the most severe cases (Figure 1D). (Kommers *et al*., 2003; Wakamatsu *et al*., 2006; Brown *et al*., 1999). Perithymic hemorrhages are occasionally observed (Brown, 1999), and as the disease progresses, there is severe atrophy of thymus and bursa (Kommers *et al*., 2003; Susta *et al*., 2010). Tracheal hemorrhages have been rarely described, but were notable features in many chickens infected with the CA02 isolate, especially in the cranial portion of the trachea, and were the consequence of necrosis in the laryngeal tonsils (Wakamatsu *et al*., 2006). Comb and/or wattle edema are variably present (McDaniel and Orsborn, 1973). Eyelid edema and hemorrhage are consistent findings in animals inoculated via the conjunctival route (Nakamura 2004).

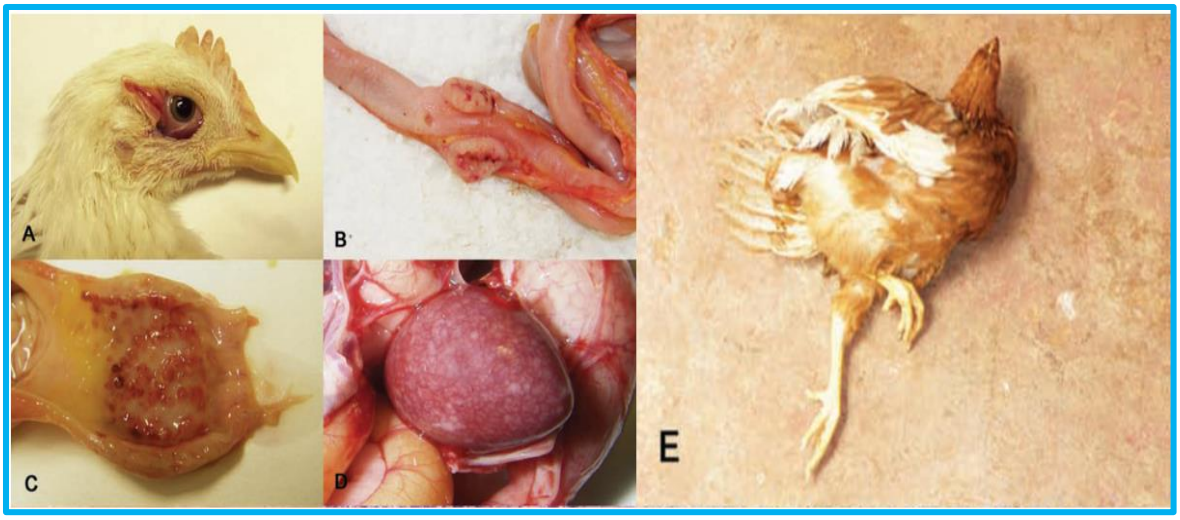

Figure 1: Clinical and pathologic features of Velogenic viscerotropic Newcastle Virus (VVNDV) and VNNDV

2.7.2 Velogenic neurotropic Newcastle disease

Morbidity with VNND often reaches 100%, and mortality is usually 50% (but can rise to 100% in young chickens). The most prominent clinical signs are neurologic and consist of head twitch, tremors, opisthotonus, and paralysis Fig. 1E (Terregino and Capua, 2009). Despite the fact that the neurologic involvement can be dramatic, the animals are characteristically bright and alert, and if able to reach food, will eat. The course of the disease is longer than with VVND, and the neurological signs are most prominent between 5 and 10 days of post infection, which is beyond the point of survival with most VVND strains, where animals often die at 4 or 5 days of post infection (Brown *et al*., 1999).

Gross lesions are often absent, and the involvement of the visceral organs appears to be minimal, although animals euthanized in the early stages of disease may have splenic or proventricular congestion (Brown *et al*., 1999). Despite the neurotropism of these strains, gross lesions in the central nervous tissue are not present (Alexander, 2003; Brown *et al*., 1999). In comparison to VVND, there are no characteristic gross lesions for VNND. In fact, in most cases, gross lesions are completely absent.

2.7.3 Mesogenic Newcastle disease

Mesogenic viruses in field conditions cause mild clinical signs, mainly respiratory. Field outbreaks with mesogenic strains also have been associated with a drop-in egg production and misshapen eggs (Alexander, 2003). Concurrent viral and secondary bacterial infections are thought to be common complications of mesogenic NDV that result in more severe morbidity (El Tayeb and Hanson, 2002; Terregino and Capua, 2009). In numerous animal experiments conducted, mesogenic strain infection will in rare cases result in neurologic signs, similar to those observed with VNND, but much milder, and with lower mortality rates (Kommers *et al*., 2003; Susta *et al*, 2010). Gross lesions with mesogenic strains are minimal (Brown *et al*., 1999). Specific pathogen free **(**SPF) chickens infected with mesogenic strains had mild splenomegaly and some degree of conjunctivitis when inoculated via eye-drop instillation. In the field, infection with mesogenic strains is often associated with secondary bacterial infections, which have their own set of morphologic correlates (Alexander, 2003).

2.7.4 Lentogenic Newcastle disease

It is generally accepted that lentogenic viruses do not cause disease in adult chickens. Some lentogenic isolates in Australia have been associated with respiratory disease in commercial broilers in the field ("late respiratory syndrome") with very low mortality, detectable gross lesions (reddening of the trachea), and chronic non-suppurative tracheitis histologically (Hooper *et al*., 1999).

2.8 Immunity

NDV infection induces active immunity (cell-mediated immunity (CMI), humoral, and mucosal) and allows passive immunity to be transferred to embryos (Beard and Brugh, 1975; Ewert *et al*., 1977; Gough and Alexander, 1973). Cell mediated immunity (CMI) occurs two to three days after NDV vaccination (Ewert *et al*., 1977) and is thought to provide protection to vaccinated poultry early in an infection when birds were found to

have low antibody response (Gough and Alexander, 1973). CMI induced from a live vaccine occurs earlier and stronger than that induced by inactivated vaccine (Lambrecht *et al*., 2004). Humoral immunity is essential for protection to ND with antibodies arising in serum six to ten days after infection, and a peak response three to four weeks later. Neutralizing antibodies primarily bind to virions preventing attachment to cells, which reduces the production of progeny and inhibits viral spread (Al-Garib *et al*., 2003). Neutralizing antibodies are measured using virus neutralization (VN) tests or hemagglutination inhibition assays that correlate well to VN (Alexander, 2003). While antibodies to the HN and F provide protection, those to the internal proteins do not (Reynolds and Maraqa, 2000a).

Antibodies to HN inhibit the ability of the virus to attach to the host cell and are measured by the hemagglutination-inhibition (HI) assay. Antibodies to the F protein block the virus from entering the host cell. Local immunity ascribed to immunoglobulin A (IgA) exposed on mucosal surfaces, helps to limit replication of the virus, but does not clear the viral infection (Al-Garib *et al*., 2003; Russell and Ezeifeka, 1995). Passive immunity from maternal antibodies passed to embryos via the egg yolk may be protective depending on the amount of antibody transferred, and the dose and the virulence of the challenge virus. If present at the time of vaccination with a live vaccine these antibodies will neutralize the live vaccine NDV antigen and can lead to vaccine failure (Heller *et al*., 1977). Other factors that have been shown to impact clinical signs and immunity seen in poultry infected with NDV are nutrition and breed. Too little of a nutrient (Vitamin A) or too much of nutrient (Vitamin E) may lead to sub-optimal immune response (Friedman *et al*., 1998). In the chicken, IgM, IgY (avian IgG equivalent), and IgA are produced as part of the immune response (Jeurissen *et al*., 2000).

2.9 Diagnosis

2.9.1 Serological diagnosis

2.9.1.1 The hemagglutination inhibition test

Fluids from eggs inoculated with specimens such as oropharyngeal and cloacal swabs from live and dead birds can be evaluated for NDV antigen using a hemagglutination assay (HA). This is a non- specific assay that tests samples from inoculated 9 - 11-day old specific pathogen-free (SPF) embryonated chicken eggs (ECE) and requires samples to be kept refrigerated to ensure virus viability (International Office of Epizootics, Biological Standards Commission, 2004). However, multiple viruses can agglutinate chicken red blood cells (cRBC) including any of the sixteen hemagglutination subtypes of influenza A virus, any of the other eight avian paramyxovirus serotypes, some adenoviruses, and some bacteria as well as NDV. Hemagglutination positive samples can then be used in a hemagglutination inhibition (HI) assay with NDV specific serum to positively identify the sample. If the sample has NDV antigen, the NDV antibodies will bind it and agglutination of the cRBC will be inhibited. The HI assay is used worldwide not only to test for NDV antigen, but also to show the presence of NDV antibodies (Alexander, 1998). Because the viruses must be grown in ECE this assay takes 5-7 days, which may not be optimal in an outbreak setting. HI results are also dependent on the amount of virus in the antigen, percentage of and type of red blood cells used and the temperature at which the assays are performed.

2.9.1.2 *ELISA*

Commercial ELISA kits are available to test for NDV antibodies. These assays are convenient proxy for protective antibody, measure antibody levels of both the neutralizing antibodies to the F and HN proteins and the non-protective antibodies to internal proteins that are highly expressed (Snyder *et al*., 1983). A variety of immunohistological assays have been developed to detect NDV antigens in tissue samples, but they cannot

differentiate virulent virus strains from vaccine virus (Kommers *et al*., 2001; Wakamatsu *et al*., 2007). ELISA tests are convenient in that they can be automated, and they can be completed in a few hours. Other tests that can be used to detect NDV antibodies include virus neutralization, single radial immuno-diffusion, plaque neutralization, and single radial hemolysis (Alexander, 2003; Thayer, 1998).

2.9.2 Molecular techniques

2.9.2.1 *RT- PCR*

Reverse Transcriptase Polymerase chain reaction (RT-PCR) are often used for rapid detection of the NDV genome in an outbreak setting (Wise *et al*., 2004b). General primers for the M gene are initially used to screen for NDV genome, and positive samples are then tested for primers made to identify a virulent cleavage site (Berinstein *et al*., 2001). Advantages of the RT-PCR assays are that large number of samples can be rapidly processed in an automated fashion, and they can be used to differentiate virulent from non-virulent strains (Peeters *et al*., 1999). However, primers and probes have to be designed to specific conserved areas of various genes, as mismatches will lead to false negative test results. Unfortunately for NDV there is no universal primer/probe set that can identify all genotypes (Kim *et al*., 2006). Before PCR based assays were created, monoclonal antibodies were used to glean information about the antigenicity of an isolate by comparing the binding patterns to multiple monoclonal antibodies against already characterized isolates (Alexander, 2003; Heckert *et al*., 1996). Some antibodies appear to only recognize virulent viruses or avirulent viruses, but with time, as more viruses are characterized, exceptions to what the monoclonal antibodies are known to bind to are found (Alamares *et al*., 2005). Binding patterns are often compared after viruses are passed multiple times in cell culture or in different hosts to see if the antigenicity changed (Kommers *et al*., 2003).

2.9.3 Virus isolation

Even though molecular techniques are commonly used for the rapid diagnosis of NDV, virus isolation in SPF ECE followed with HA and HI is the gold standard for NDV identification and for the validation of other techniques (EFSA, 2007; Alexander and Senne, 2008). At least five 9–11-day-old SPF embryonated chicken eggs for each sample should be inoculated into the allantoic cavity, then incubated at 35–37°C for 4–7 days and candled daily to check vitality. The mortality of inoculated eggs earlier than 24-hour post-inoculation is generally considered nonspecific, although some very virulent strains if present in high concentrations in the sample may cause embryo mortality as early as 24-hour post-inoculation. Specific embryo mortality more often occurs within 3-5 days' post-infection and is influenced by the virus strain, age of embryo, and inoculum concentration. In general, embryonic death is hastened when younger embryos and higher inoculum concentrations are used. Death of the embryo is often very quick if the virus is inoculated in the yolk sac and amniotic sac, while it is slower if inoculation is via the allantoic cavity (OIE, 2008). more than 85% of ND isolations are made on the first passage, with less than 10% needing one blind passage. Isolation of ND viruses after 2 blind passages is considered very rare (Kouwenhoven, 1993).

The allantoic fluid containing dead embryos, or those chilled at the end of the fourth through seventh day, are tested for hemagglutinating (HA) activity, as hemagglutination is a key feature of ND viruses. However, avian influenza (AI) viruses and other avian paramyxoviruses will also cause hemagglutination, so distinction is essential. If HA activity is detected, the hemagglutinating agents should be identified by means of the hemagglutination inhibition (HI) test, which uses specific sera, or by molecular tests, which may provide information on the pathotype and genotype.

Some APMV-1 strains lose the hemagglutinating capacity when heated at 56°C for 5 min, but retain infectivity for chicken embryos even after 30 min at the same temperature. Influenza viruses, instead, always lose their infectivity before the loss of HA ability. On the basis of the response to heat treatment, it may be also possible to distinguish between

two types of lentogenic viruses. In fact, classical vaccine viruses La Sota or B1 strain can be heat-inactivated while other lentogenic viruses as well as mesogenic and velogenic strains remain infectious after the treatment (Lomniczi, 1975).

In the HI test, some level of cross-reactivity may be observed among the various avian paramyxovirus serotypes. Cross reactivity can be observed between APMV-1 and APMV-3 viruses (particularly with the psittacine variant of APMV-3, commonly isolated from pet or exotic birds) or APMV-7. The risk of mistyping an isolate can be greatly reduced by using a panel of reference sera or monoclonal antibodies (mAbs) specific for APMV-1, APMV-3, and APMV-7. The use of mAbs also permits characterization of antigenic differences within different strains of APMV-1 or even between subpopulations of the same strain (Russell and Alexander, 1983).

As an alternative to isolating viruses in ECE, cell culture may be used. However, viruses that do not have multiple basic amino acids at their fusion cleavage site will not grow without the addition of trypsin to the media, except in chicken embryo kidney cells. Therefore, cell culture is not considered reliable for isolating new viruses, but is a convenient option for growing viruses previously characterized (EFSA, 2007; Zaffuto *et al*., 2008). Some strains of PPMV-1 and some strains of APMV-1 such as the nonpathogenic Ulster strain can be isolated in chicken liver or chicken kidney cells but not in embryonated eggs (Kouwenhoven, 1993).

2.10 Prevention and Control

2.10.1 Vaccination

Vaccines are being used to control and prevent ND. Currently, many inactivated and live ND vaccines are available around the world (Shim *et al*., 2011; Xiao *et al*., 2013). Chickens and turkeys are immunized against New-castle disease. Live virus vaccines are administered by variety of routes and schedules from hatching till grow-out (Cho *et al*., 2008). Killed virus oil emulsion vaccines are administered parentally prior to the onset of

egg production. Although proper vaccination protects the birds from clinical disease but it does not prevent virus replication and shedding, which results in a source of infection (Chukwudi *et al*., 2012).

Therefore, prophylactic vaccination is not used in developed countries (OIE, 2012). In developing countries, there is wide use of vaccines on commercial flocks (Munir *et al*., 2012b). Anti-NDV antibody titers of flocks are continuously monitored and flocks are revaccinated to maintain the protective antibody titers. The breeders and layers are vaccinated against NDV and oil based vaccines are being used prior to onset of egg production for long term immunity (Nadeem *et al*., 2004). Ant- NDV antibody titers of breeder flock is also important to maintain the anti NDV maternal antibody titers of progeny. These maternal antibodies protect chicks from the disease during the first week of life. In spite of extensive vaccination, outbreaks are continuously occurring (Shabbir *et al*., 2012). To overcome these problem poultry producers are using different combinations of live and killed vaccines in a flock.

Both live and inactivated vaccines have their advantages and disadvantages (Bermudez, 2003; Senne *et al*., 2004). Live vaccines are advantageous because they are inexpensive to produce, are able to be mass applied, induce mucosal immunity, and provide rapid onset of immunity (Marangon and Busani, 2007). Live vaccines are imperfect in that they may cause disease, are inactivated by maternal antibodies, interfere with surveillance and they require proper handling as to not be inactivated before being administered (Senne *et al*., 2004). Inactivated vaccines are easier to store, are able to overcome maternal antibodies, and will not cause the disease for which they are being given to protect (Brugh and Siegel, 1978). The disadvantages of using inactivated vaccines are that they need a large amount of antigen, are expensive to produce, cannot be mass applied and have a slower onset of immunity (Marangon and Busani, 2007).

Turkeys are often vaccinated with ND vaccines with good results, but some morbidity and mortality can be associated from vaccination with live vaccines and upon challenge with a virulent NDV after vaccination (Saif and Nestor, 2002). While ND vaccines are only

licensed for chickens, turkeys and pigeons, ND vaccines also appear to provide good protection in partridges (Family Phasianidae) and Guinea fowl (Family Numididae) (Alexander, 2003). Ostriches respond favorably to vaccination with ND vaccines and are typically given an inactivated LaSota vaccine (Blignaut *et al*., 2000). Protective immune responses are induced in pigeons vaccinated with PPMV-1 vaccines (Kapczynski *et al*., 2006). PPMV-1 viruses are AMPV-1 strains isolated from pigeons that are often referred to as variant due to their propensity to bind to different monoclonal antibodies.

Ideally, the goal of a vaccination program is to produce lifelong immunity with a single vaccine at the earliest age possible without side effects due to mortality, respiratory disease or poor growth. In addition, the vaccine should be administered by mass administration methods, be easy to store, and include markers that allow the differentiation of infected animals from vaccinated animals which could aid quarantine and eradication efforts in an outbreak setting (Veits *et al*., 2006). The discovery of a vaccine that satisfies all of the previously listed attributes does not appear imminent.Initially, inactivated vaccines were used to control ND but were noted to be not as effective due to their inability to produce mucosal responses and to be mass applied (Alexander, 1988a). A transition to live vaccines was made in the 1950s when strains of low virulence were characterized and proven to be efficacious for vaccination against similar circulating viruses at the time (Beaudette, 1949).

In addition to the common live and inactivated vaccines, there have been less conventional vaccines created for NDV that include DNA vaccines, virosomes, ISCOMs, NDV as vectors for vaccines, NDV marker vaccines, pox and herpes vectors for expressing NDV proteins, baculoviral system for expressing NDV proteins and NDV proteins expressed in plant products (Loke *et al*., 2005; Veits *et al*., 2006)**.** Most of these newer vaccines, while efficacious, have not yet been used in commercial settings due to their production costs being more expensive than the current vaccines (Seal *et al*., 2000). All of these above-mentioned vaccines do have the potential to be used as a marker type vaccine to allow the differentiation of infected versus vaccinated animals.

Today the strains of NDV used to produce ND vaccines (B1/1947, LaSota/1946) being class II genotype II viruses are phylogenetically the same as the outbreak viruses isolated in the 1940's, but phylogenetically they are different from strains causing outbreaks of END in North America since the 1970's. Similar to B1 and LaSota in class and genotype, the VG/GA strain, a class II genotype II virus, isolated from a healthy turkey, marketed and used in many countries as the live ND vaccine, Avinew® by Merial, was isolated in 1989 (Seal *et al*., 1995). Since all of the APMV-1 ND viruses share similar antigenic epitopes they are considered to be of a single serotype, meaning the antibodies and cell mediated immunity induced by any one NDV would protect from disease and death after a challenge with any other NDV. It is widely recognized that because all NDV isolates are of one serotype, ND vaccines prepared with any NDV lineage, given correctly, can protect poultry from clinical disease and mortality from a virulent NDV challenge (Kapczynski and King, 2005).

Optimal vaccination occurs at a time after maternal antibody has waned allowing the vaccines to induce a good immunological response but before the birds are likely to be exposed to a virulent strain of NDV. The type of program chosen for each poultry flock will depend on several factors beginning with whether the goal is to protect the birds from infection, or from clinical disease and death. At the same time, it is important not to induce iatrogenic respiratory reactions, which would lead to economic losses. When defining a vaccination program for a flock factors such as the age, the maternal antibody level, the breed, and the presence of concurrent infections are to be taken into account.

2.10.2 Biosecurity

Good biosecurity measures are essential to prevent Newcastle disease in poultry flocks. Commercial flocks should not have any contact with domesticated poultry or wild birds or any pet birds. Workers should avoid contact with birds outside the farm. Biosecurity measures include bird-proof houses, feed and water supplies, minimizing travel on and off the facility, disinfecting vehicles and equipment's that enter the farm. Pests such as insects

and mice should also be controlled. If possible, employees should shower and change into dedicated clothing prior entry into the poultry farm.

3 MATERIALS AND METHODS

3.1 Study Area

The study was conducted between December 2016 and May 2017 in Minjar-Shenkora district, Bolo Silassie kebelle, which is located in North Shoa Zone of Amhara Regional State about 135 km south east of Addis Ababa. The area is situated between 90˚6' and 90˚5' N and 39˚46' and 39˚26' East (Figure 2). The district has a total area of about 229,463 hectares. The altitude of the study area ranges from 1400-2400 m.a.s.l. The meteorological data showed that the district receives bimodal rainfall with annual average rainfall ranging from 162.8mm to 1028mm. The lowest mean annual temperature over ten years was 7.3˚C whereas the highest was 20˚C (Alemayehu, 2015). The district is divided in to three agro-climatic zones namely highland, midland and lowlands. Unpublished data from the district agricultural office showed that 20% of the land area of the district is mountainous whereas 65% is plain. About 10% of the land is gorge with the remaining 5% featured by other topographic appearances. Agriculture is the main stay of the population of the district with teff, barley, wheat, sorghum, chickpeas, maize, field peas, onions, potatoes, pepper and fenugreek being the major crops grown. The wereda has a livestock population number, cattle (95270), Sheep (57603), Goats (74049) and Poultry (168,702), equine (3,826), mule and camel (8131). Extotic poultry (Sasso, kockock and white leghorn) are the domonat adopted breeds in the area (MSWARDO, 2016).

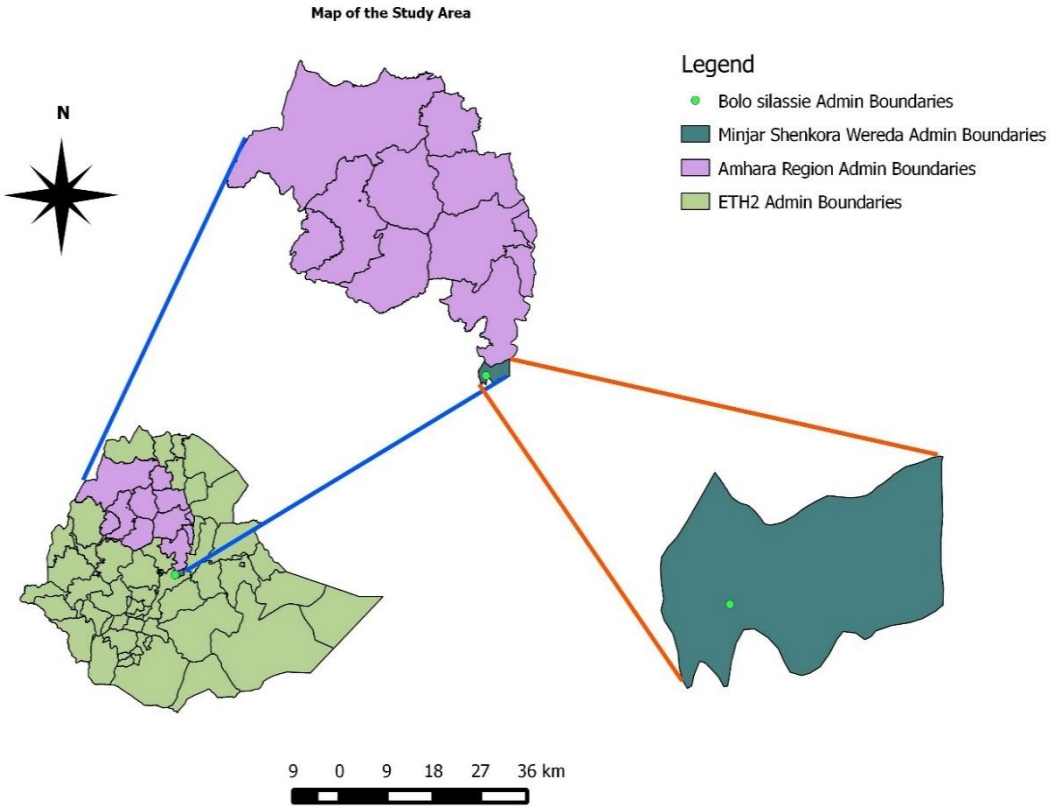

Figure 2: Map of the study area (Quantum Geographic Information System (QGIS) software 2.0.1 version).

3.2 Study Population and Management

The study chicken comprises indigenous ecotypes and exotic chicken breeds (White leg horn, Sasso and kockock). The chickens were kept by smallholder farmers. The farmers practice semi-intensive production system with open housing and wire enclosed small areas and small scavenging flocks of local chickens (Appendix 1). The feed resources for the chickens are household refuse, homestead pickings, crop residues and seeds offered by the flock owners.

3.3 Selection of Study Village and Farmers

The peasant association was selected purposively considering the presence of higher chicken population per household, the accessibility of the peasant association to the road and the consent of the farmers to participate in the study. The selection of peasant association and households was facilitated by livestock experts/extension staff of the district. Meeting with selected households was organized in the selected peasant associations in collaboration with community leaders and extension staffs (Appendix 2). Households were also selected purposively based on the number of indigenous and exotic chicken they owned. Farmers were clearly introduced to the objectives and principles of the study.

3.4 Experimental Design

Twenty households rearing both indigenous and exotic chicken were selected for the purpose of this study. Farmers who owned ten or more indigenous and exotic chickens per household were selected purposively in the experiment. A total of 154 chickens, 82 indigenous ecotypes and 72 exotic breeds owned by the 20 selected households were identified. The selected households were randomized into four treatment groups. Two of the treatment groups have 37 chicken each whereas the other two have 40 chicken each (Table 2). The sampled chicken was individually identified using numbered wing-tags (Appendix 3) and blood sample was collected to estimate the baseline antibody concentration. And the 154 baseline collected blood samples were also screened for IBD infection. The selected households were randomly assigned to one of the four treatments groups: placebo, drinking water, eye drop and spray as depicted below. Each treatment group comprises five households.

Treatment 1: Chicken which received only water (control)
Treatment 2: Chicken which received ND I2 vaccines via drinking water
Treatment 3: Chicken which received ND I2 vaccines via eye drop (positive control)
Treatment 4: Chicken which received ND I2 vaccine via spray

Table 2: Experimental set up

Treatment Groups	No. of Chickens	Breed	No.	Vaccine EID_{50}/ml dose	No. challenged chickens	Challenge EID_{50}/ml dose (IM)
Control	40	Local	23	10^6	10	10^7
		Exotic	17			
D.water	37	Local	20	10^6	10	10^7
		exotic	17			
Eye drop	37	Local	20	10^6	10	10^7
		Exotic	17			
Spray	40	Local	19	10^6	10	10^7
		Exotic	21			

EID_{50}=50 percent embryo infectious dose; No.= number of chickens; D. water= drinking water

3.5 Vaccination and Follow up

Vials of freeze dried ND I_2 vaccine 400 dose (batch 416) and 200 dose (batch 117) with a titer of 10^6 EID_{50} per vial were purchased from National Veterinary Institute (NVI), Bishoftu. The vaccines were transported to the study area using portable refrigerator. The vials were reconstituted with clean and non-chlorinated distilled water (manufacturer's instruction). After reconstitution, the vaccine was delivered to experimental chickens except the control group. The booster vaccination using the same NDI_2 vaccine was given 15 days after the first vaccination. Blood sample was collected on day 15 after the first vaccination and 15 days after booster vaccination were given for antibody titration. The chickens were followed for 3 months before challenge infection was carried out. During the three months of follow up suspected Newcastle disease outbreak and mortality due to the outbreak of the disease in both the vaccinated experimental households and nonvaccinated household farmers in the area were recorded.

For oral delivery of vaccine via drinking water the vials containing NDI_2 vaccine were reconstituted with distilled water. One vial of the freeze-dried NDI_2 vaccine containing 400 doses was reconstituted in 4000 mL of distilled water following the manufacturer's instructions (1 dose of NDI_2 vaccine was reconstituted with 10 mL of distilled). Each chicken per group received 10 mL of the reconstituted vaccine during the first vaccination while they received 20 mL during booster vaccination (NVI manual) (Appendix 4). Prior to vaccination farmers were informed to withhold water for few hours.

For ocular vaccine administration one vial of the freeze-dried NDI_2 vaccine containing 200 doses was reconstituted in 10 mL of saline solution as recommended by the manufacturer (100 dose of NDI_2 vaccine was reconstituted with 5 mL of saline solution). Individual chicken in the group was provided one drop of the reconstituted vaccine using sterile pippete (Appendix 4).

For the group receiving NDI_2 vaccine by litter spray a mirror cleaner spray was used to spray the vaccine in saline water (Appendix 4). Hundred mL of saline water was used to reconstitute 100 dose of the vaccine (Tadios *et al.*, 2015). Therefore, one vial of the freeze-dried NDI_2 vaccine containing 400 doses was reconstituted in 400 mL of saline water. The chickens were sprayed with the vaccine at dose rate of 1 mL of vaccine per chicken in a cage and the chicken were forced to remain in closed cage for 30minutes.

3.6 Collection of Serum Samples

Blood samples (1-1.5 mL) per chick were collected using 3 mL sterile disposable syringe from the wing vein (Appendix 5) following the standard methods described by (Alders and Spradbrow, 2001). Blood samples were collected from 154 chickens on the first day of the experiment for baseline antibody assay. Then after, blood samples were collected on day 15 after the first vaccination and after 15 days following booster vaccination. Pre-challenge blood samples were collected one month after the booster vaccination was given. The collected blood samples were labeled and allowed to clot overnight at room temperature

and serum were separated. The sera were harvested in to labeled cryovials and stored at -20˚C until HI was carried out.

3.7 Hemagglutination Inhibition (HI) assay

HI assay was conducted in the serology laboratory of NVI. Serum samples collected were heat inactivated at 56 °C for 30 min and stored at -20 °C. The level of anti-ND virus antibodies in serum samples was determined using the HI test as described by OIE (2013) (Appendix 6). The HI test has 98 % specificity and 69-98 % sensitivity (de Wit *et al*., 2005). Two-fold serial dilutions of sera samples were made to estimate the anti-NDV antibody titers. The antibody level for each serum sample was expressed as a log to the base two and recorded. The mean titers were calculated. In this study, we used the published cut off value for the protective HI antibody titer (HI titer $\geq \log_2^3$) for ND vaccination in chickens (Abdi *et al*., 2016; Spradbrow, 1993; Alexander *et al*., 2004).

3.8 Challenge with Virulent Virus

Four weeks after booster vaccination was provided 40 chickens (10 from each treatment group) were randomly selected and purchased and brought to Bishoftu. The chickens were housed in poultry experimental house located in the College of Veterinary Medicine and Agriculture. Local virulent NDV designated Alamya strain was obtained from NVI and inoculated via breast muscle to all chickens (Appendix 7). The challenge strain 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 (Nassir *et al*., 2000). Each chicken was inoculated with 1mL of the suspension of challenge strain containing 10^7 EID_{50}/ml as described by Abdi *et al*. (2016) and Echeonwu *et al.* (2007). The chickens from each treatment group were kept separately and followed daily for morbidity and mortality for one month (Appendix 8).

3.9 Molecular Characterization of NDV

RNA extraction, PCR, and sequencing: Impressions of organ samples were collected from suspected ND cases on FTA cards and stored at room temperature (Appendix 9). The samples were submitted to OIE, FAO and EU Avian Influenza and Newcastle Disease laboratory (Weybridge, New ham, Addlestone, Surray, UK) for molecular characterization. Extraction of viral RNA was done from the cards using QIAamp viral RNA minikit (Qiagen, Venlo, and The Netherlands) following the manufacturers' instructions. Reverse transcription RT-PCR targeting the L gene was used to detect ND virus RNA in the samples. Positive samples were further subjected to sequencing to identify the strain of the virus detected. Sequencing was performed in both orientations using the BigDye Terminator version 3.1 cycle sequencing kit (Life Technologies) and ABI 3130 Avant capillary sequencer (Applied Biosystems).

3.10 Data Analysis

The data collected from the study area was stored in MS excel and analyzed using STATA version 13. Serology data are presented as mean value plus or minus (+/-) standard deviation (SD) of HI antibody titers of the treatment groups. The variation in mean antibody titres among the four experimental groups was analyzed using one way ANOVA. When difference was observed among the groups bonfferoni multiple pair-wise comparison was used and the effect of co-infection of IBD and breed on ND anti body titre were analyzed using linear regression. Significance is reported at $P<0.05$.

3.11 Ethical Consideration

Ethical clearance for this study was obtained from Addis Ababa University, College of Veterinary Medicine and Agriculture (AAU-CVMA), Minutes of animal research ethics and review committee. A seven-page request for explanation of the purpose of carrying out the study and all possible care planned to reduce animal suffering due to sampling and

virulent virus challenge was given to the committee. After the committee evaluated the significance of this research, approval was given (Appendix 11)

4 RESULTS

4.1 Serological Response

At baseline the mean antibody titre against NDV was slightly higher than the protective level in all the treatment groups (Table 3, Figure 3). The overall baseline mean ($log_2 \pm SE$) antibody titre of the 154 study chickens was 3.9 ± 0.21. At baseline, there was no statistically significant difference ($P > 0.05$) in antibody titre among all the experimental groups. After the first and booster vaccinations, statistically significant ($P < 0.001$) variation was observed in mean (log2) antibody titre between the vaccinated groups and the unvaccinated control group (Table 3).

Table 3: Pre-and Post-vaccination mean ± SE antibody titres of chickens

Treatment Group	**M ± SE HI antibody titre (log2) chickens vaccinated by different methods at different days**			
	N	**Day 0**	**Day 15**	**Day 31**
Control	40	3±0.41	4.45±0.58	4.4± 0.60
D. water	37	4.7±0.39	7±0.51	7.6± 0.38
Eye drop	37	4.1± 0.38	7.2±0.41	7.1± 0.41
Spray	40	3.7±0.46	8.05±0.43	7.7± 0.41

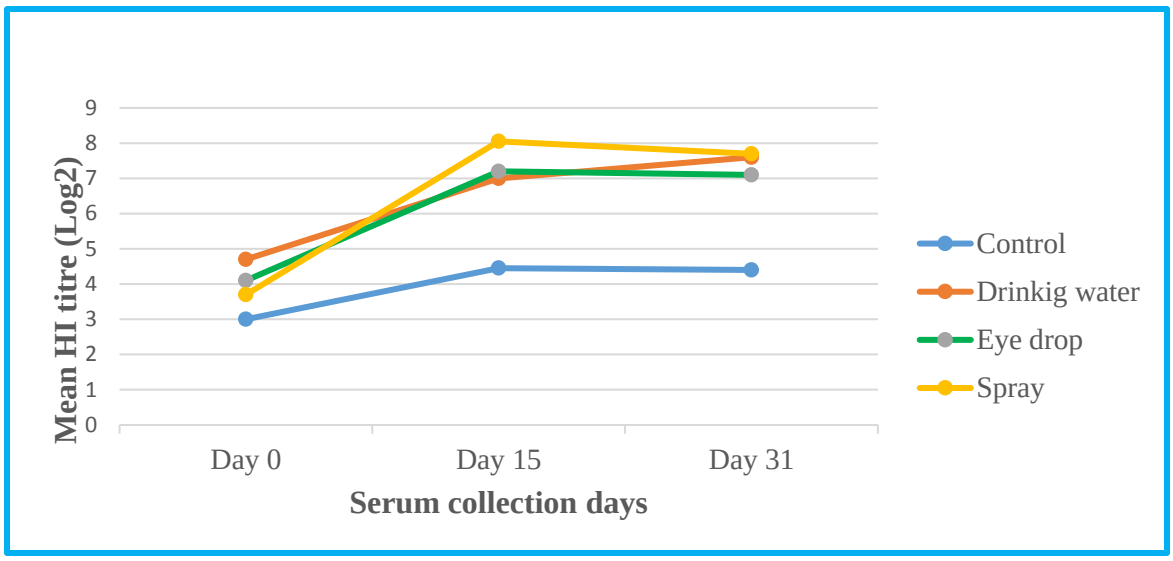

Figure 3: Mean (log_2) antibody titre of chickens at different times during the experiment

Multiple pairwise comparison was further carried out to discern the existence of variation in antibody titre among the experimental groups after the first and booster vaccinations were provided. At day 15 after the first vaccination the three vaccinated groups had significantly higher antibody titre than the control group (Table 4). However, there was no statistically significant difference in antibody titre among the vaccinated groups although the group that received the NDI_2 vaccine via spray had highest titre followed by the eye drop group. Similarly, after booster vaccination was provided the vaccinated groups had significantly higher antibody titre than the unvaccinated groups whereas there was no statistically significant difference among the vaccinated groups (Table 5). Highest antibody titre was observed in chickens vaccinated by spray followed by those that received the vaccine via drinking water.

Table 4: Results of multiple pair-wise comparison of log_2 HI antibody titter in experimental chicken at day 15 after primary vaccination

Treatment Group-1	Treatment Group-2	MD	SE	P- value	95% CI
D.water	Control[a]	2.55	0.705	0.002	0.66 - 4.43
Eye drop	Control[a]	2.82	0.705	0.001	0.93 - 4.70
Spray	Control [a]	3.6	0.691	0.001	1.75 - 5.44
Eye drop	D.water[b]	0.27	0.718	1.000	-1.65 - 2.19
Spray	D.water[b]	1.05	0.705	0.831	-0.83 - 2.93
Spray	Eye drop[b]	0.77	0.705	1.000	-1.10 - 2.66

Groups with different letters significantly differ from each other; MD = mean difference; SE = standard error; D. water = drinking water

Table 5: Results of multiple pair-wise comparison of log_2 HI antibody titre in experimental chicken at day 15 after booster vaccination

Treatment Group-1	Treatment Group-2	MD	SE	P-value	95% CI
D. water	Control[c]	3.22	0.662	0.001	1.45 - 4.99
Eye drop	Control[c]	2.73	0.662	0.001	0.96 - 4.50
Spray	Control[c]	3.32	0.649	0.001	1.58 - 5.06
Eye drop	D.water[d]	-0.48	0.675	1.000	-2.29 - 1.31
Spray	D.water[d]	0.10	0.662	1.000	-1.66 - 1.87
Spray	Eye drop[d]	0.66	0.662	1.000	-1.18 - 2.36

Groups with different letter significantly differ from each other; MD: mean difference; D. water = drinking water

4.2 Effect of Co-Infection with Infectious Bursal Disease Virus and Breeds

The sera samples collected at the baseline was screened for IBD infection. The effect of infection with IBDV on the anti-NDV antibody titre was analyzed using linear regression and the result showed that on average IBD positive chicken had lower anti-NDv antibody titres (Table 6). This difference was, however, not statistically significant. The mean HI titre (log_2) of exotic chicken was 0.32 units higher on average than that of the indigenous chicken although it was not statistically significant.

Table 6: Effects of infection with IBD and breed on anti-NDV antibody titre

Variable	Coefficient	Std. Err.	p-value	95% CI
IBD infection				
Yes	-0.3501494	0.613	0.569	-1.561 - 0.861
Breed				
Exotic	0.3259234	313	0.299	-0.290 - 0.941

4.3 Percentage of Chickens with HI Titre above Log_2^3

At baseline 37%, 52%, 51% and 50% of the chicken in the control, drinking water, eye drop and spray group respectively had antibody titre $\geq log_2^3$. After the first vaccination was provided the proportion of chicken with antibody titre $\geq log_2^3$ rose to 74 %, 90% and 93% in the group vaccinated via drinking water, eye drop and spray respectively (Table 7). Whereas the proportion of chicken with antibody titre $\geq log_2^3$ in the control group was 48 %. After booster vaccination was given the proportion of chicken with antibody titre $\geq log_2^3$ was 90 %, 90% and 93% in the group vaccinated via drinking water, eye drop and spray respectively. The proportion of chickens with antibody titre $\geq log_2^3$ remained 45% in the control groups.

Table 7: The proportion of chickens with HI titres $\geq \log_2^3$ among the four experimental groups

Treatment Group	Number of chickens (%) with HI log2) ≥ 3.0			
	No.	**Day 15**	**No.**	**Day 31**
Control	35	17(48%)	35	16(45%)
D. water	31	23(74%)	30	27(90%)
Eye drop	33	30(90%)	33	30(90%)
Spray	31	29(93%)	34	31(93%)

The analysis of data on the suspected outbreak of Newcastle disease showed that the average number of chickens per household were dropped from 12.5 to 4.08 and 21.06 to 18.5 in smallholder farmers who did not vaccinate their flock and vaccinate their flock respectively (Table 8).

Table 8: Number of chicken owned and survival status in the study area

	No. owned	**No. died**	**No. alive**	**Survival**
Unvaccinated				
Total households	151	93	49	**32.45%**
Average/household	12.58	7.75	**4.08**	
Vaccinated group				
Total households	337	41	296	**87.83%**
Average/household	21.06	2.56	**18.5**	

4.4 Results of Molecular Diagnosis

Organ and tissue samples collected from suspected cases of ND encountered during this study (depicted in figure 4 below) were analyzed using molecular techniques. The results of the analysis showed that three samples (one bursa, one lung and one proventriculus) were found to be positive for NDV RNA from 11 samples analyzed making the proportion

of NDV RNA detection 27.27% (Table 8). Sequencing of the positive samples is underway to identify the strain of NDV.

Table 9: Results of molecular analysis of samples collected from suspected cases of NDV

Sample reference	Farm	Organ	NDV PCR result
AS8	Farm 1	bursa	**positive**
ET1	Farm 2	eye lid fluid	negative
AE1	Farm 3	trachea	negative
AG1	Farm 3	lung	**positive**
AG2	Farm 3	trachea	negative
AG2	Farm 3	proventriculus	**positive**
MB187	Farm 4	lung	negative
MB180	Farm 4	intestine	negative
MB 180	Farm 4	lung	negative
MB189	Farm 4	intestine	negative
MB189	Farm 4	lung	negative

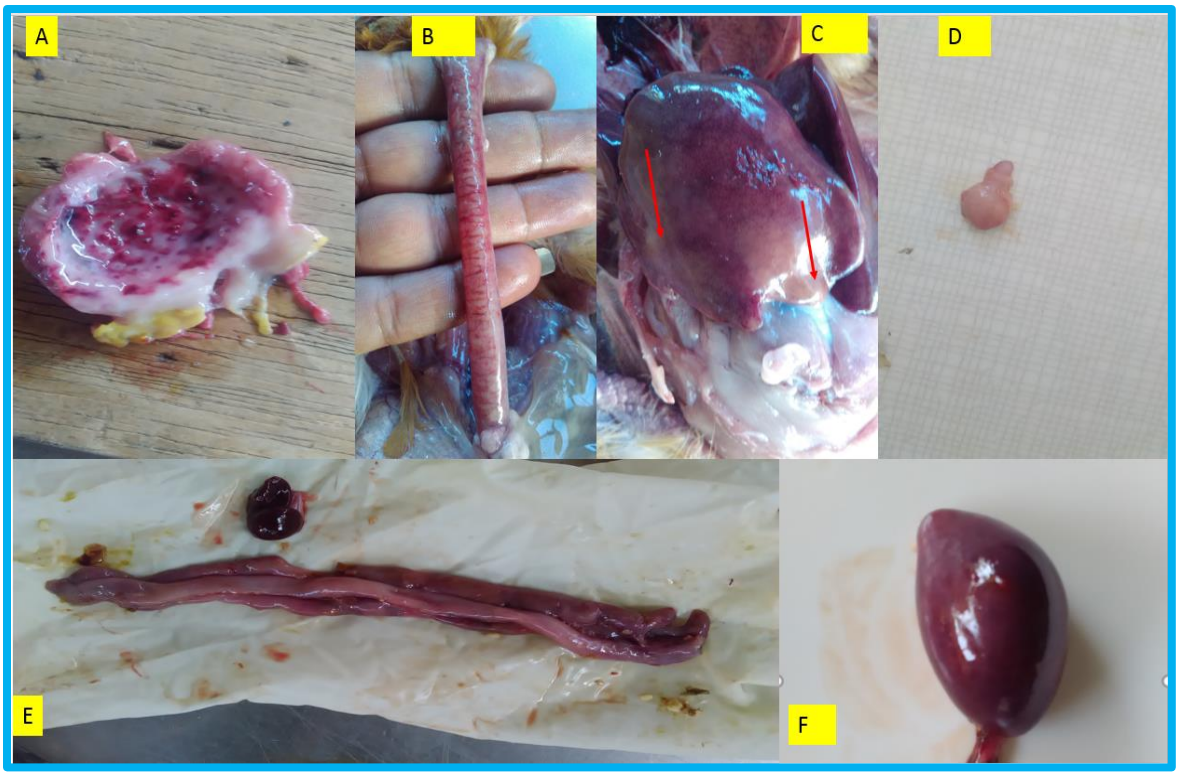

Figure 4: Post mortem lesions of suspected ND cases encountered during the study: hemorrhagic proventriculus (A), hemorrhagic trachea (B), mottled liver (C), enlarged bursa (D), Inflamed caecal tonsil (E) splenomegaly (F)

4.5 Results of Challenge Infection

The results of challenge infection with virulent virus showed that 100% of the chicken in the vaccinated groups survive while 60% of the control groups died showing typical clinical signs of ND (Table 10). However, two chicken from those vaccinated via drinking water had shown clinical signs of ND making morbidity of 20% but all recover before the termination of the study (Appendix 11). The clinical signs observed in infected chickens in control group were listlessness, increased respiration, weakness, prostration, greenish watery diarrhoea, paralysis of legs and wings (Appendix 10). Post mortem examinations revealed lesions indicative of NDV infection such as hemorrhagic proventriculus, hemorrhagic trachea and hemorrhagic caecal tonsil and splenomegaly (Figure 5).

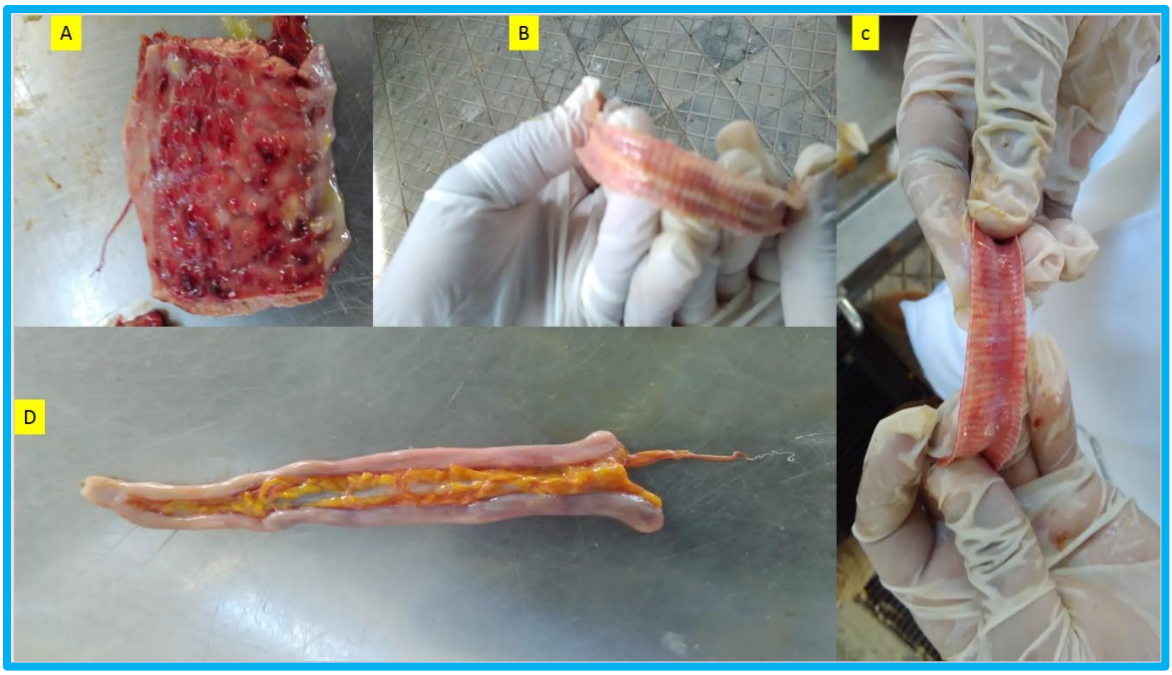

Figure 5: Post mortem lesions encountered in chickens died after challenge with virulent virus: hemorrhagic proventriculus (A), hemorrhagic trachea (B & C) hemorrhagic caecal tonsil (D).

Table 10: Morbidity, mortality and survival rates in chickens vaccinated via different routes and challenged with Alemaya local strain of NDV

Treatment group	N	Mean HI Titre ± SD	No. Morbidity	Death Total	Mortality%	Survival%
Control	10	1.6 ± 0.16	6	6	60	40
D. water	10	9 ± 0	2	0	0	100
Eye drop	10	9 ± 0	0	0	0	100
Spray	10	9 ± 0	0	0	0	100

5 DISCUSSION

Newcastle disease (ND) is a highly virulent disease of poultry which can devastate the entire flocks in short period of time. This was demonstrated by several outbreaks affecting several flocks in several areas during this study. The results of post mortem examinations (figure 4) and molecular analysis conducted on representative samples (Table 9). That means control of ND is needs to be a priority issue if achievement of household food security and national poverty reduction is the target of developing countries like Ethiopia. Vaccine delivery that is suitable for smallholder farmers is important since biosecurity issues cannot be thought in rural settings. Easily adoptable ND vaccine delivery systems that provide good level of immunity is a way forward to alleviate poverty (Duguma, 2006). In this study, different NDI_2 vaccine delivery routes were compared with the recommended route of vaccination (eye drop method).

The results of this study showed that chickens vaccinated with NDI_2 via drinking water and spray elicited anti-NDV antibody titre that is $\geq \log_2^3$, which is considered protective to virulent field virus challenge (Spradbrow, 1993; Alexander, 2001). That is, the level of protection as shown by the antibody titre, conferred by the two routes of vaccination is comparable to the eye drop method of vaccination. This shows that smallholder farmers can choose among the methods of vaccination that suits his/her particular farming system and needs. Chickens vaccinated with NDI_2 by the three routes of vaccination mounted higher antibody titres than the control group. Elsewhere it has been shown that chickens vaccinated via eye drop and oral route elicited good protection level even though the booster vaccination 2-4 weeks later was needed for oral delivery (Bell *et al.*, 1995; Alders and Spradbrow, 2001). The proportion of chickens with antibody titre $\geq \log_2^3$ after the first and booster vaccinations in the group vaccinated via drinking water and spray is comparable to that of chickens vaccinated using eye drop method. Particularly the proportion of chickens with antibody titres greater than $\log_2^3$ after the booster vaccination was provided was greater than 90%. This observation is in consent with the earlier reports of Nasser *et al*. (2000) and Abdi *et al.* (2016) in Ethiopia. Similarly, it agrees with the reports made elsewhere in the world such as the reports of Wegdan *et al*. (2015) who used

eye drop method and drinking water to delivery NDI$_2$ vaccines. The protection level observed in chickens vaccinated by litter spray is in agreement with earlier reports of Tadios *et al*. (2015). This shows that oral delivery of NDI$_2$ vaccine via drinking water, which can be carried out by farmers themselves and litter spray with locally available materials can provoke sufficient immunity comparable to eye drop method. It has been shown that oral administration of ND vaccines primarily provokes mucosal immunity (Jayawardane and Spradbrow, 1995). This is important to confer protection against NDV, can be acquired either by inhalation or ingestion or both (Alexander, 1988). The high level of protection offered by vaccination via litter spray could be due to higher chance of getting the vaccine virus through natural routes of infection such as the eye and nostrils.

The 100% survival observed in chickens vaccinated via drinking water, litter spray and eye drop method compared to the control group in which 40% survival was observed, indicate that vaccination with NDI$_2$ vaccine can reduce mortality at least by 60%. This has important implication in terms of food and financial security for smallholder poultry producers. This can be further justified by the drop in average number of chickens per household from 12.5 to 4.08 in smallholder farmers who did not vaccinate their flock whereas the drop is only from 21.06 to 18.5 in farmers who vaccinated their flock. This means vaccination against ND with NDI$_2$ vaccine via suitable delivery route can significantly reduce mortality and maintain flock size and ultimately contribute to household income. Women and children can particularly benefit from such vaccinations. In agreement with our findings Nasser *et al.* (2000) and Musa *et al*. (2015) reported 100% protection in chickens vaccinated via drinking water and eye drop following challenge with virulent virus. Abdi *et al*. (2016) also reported 100% protection in chickens vaccinated with NDVI$_2$ via drinking water following challenge with same strain used in this study. Tadios *et al*. (2015) also reported 80% survival following challenge with the same strain in chickens vaccinated by spray which is lower to the current finding. Bell *et al*. (1991) also reported the efficacy of the V4 vaccine strain, which is similar to NDI$_2$ strain applied via spray resulted in seroconversion and protection against virulent challenge. In Vietnam, after extensive laboratory and village trials, it has been officially recognized as the ND vaccine for village chickens (Tu *et al*., 1998). In Tanzania, it has been shown to offer

protection for at least two months after vaccination (Wambura *et al*., 2000). Field records in Mozambique indicate that I_2 ND vaccine provides approximately 80 percent protections in the face of an outbreak (Alders and Spradbrow, 2001).

Chickens infected with IBD had lower antibody titre than those which were not infected although the difference in the antibody titre was not statistically significant. This has implication on the control of ND through vaccination. Literature reveals that infection with IBDV results in the destruction of immature B lymphocytes in the bursa with ultimate immunosuppression (Faragher *et al*., 1974). The occurrence of IBD needs to be taken into account by the veterinary and livestock authorities as it can interfere with vaccination programs. Such interference of ND control programs by vaccination due to occurrence of IBD has already been documented in commercial poultry production systems (Giambrone *et al*., 1976).

Indigenous chicken ecotypes in the study area had similar antibody titre with exotic chicken breeds even if the exotic ones had higher anti-ND antibody titre on average. Notwithstanding the small number of households and chickens included in the study, it shows that the NDI_2 vaccine provoke similar level of antibody production in indigenous chickens and exotic ones. In consent to this finding Nega *et al*. (2011) reported that NDI_2 vaccine provokes high antibody response (90.4%) in four different Indigenous chicken ecotypes in Ethiopia. This important finding for poultry producers and veterinary personnel since information on the efficacy of ND vaccines in indigenous chicken is scarce. Regular vaccination of village chickens with NDI_2 vaccines can reduce the circulation of virulent ND viruses and their spill over to commercial farms.

6 CONCLUSION AND RECOMMENDATIONS

The results obtained from this study shows that NDI_2 vaccine administered via drinking water and litter spray under smallholder farmers' situation provoked protective antibody level similar to the eye drop method. Chicken vaccinated via drinking water and litter spray demonstrated 100% survival similar to the eye drop method after challenge with virulent NDV. The level of protection conferred was similar in indigenous and exotic chicken types reared by smallholder farmers in the area Therefore, smallholder farmers can choose the suitable vaccine delivery routes to prevent outbreaks of ND. It can make a vital contribution to the improvement of household food and financial security in smallholder farmers.

Based on the above conclusion the following recommendations are forwarded:

- The use of NDI_2 vaccine needs to be considered by the veterinary and livestock authorities to prevent ND outbreaks
- Large-scale evaluation of the NDI_2 vaccine delivery routes have to be carried out
- Further study on the epidemiology of NDV and efficacy of vaccine on various strains needs to be carried out.

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8 LIST OF APPENDICES

Appendix 1: Smallholder poultry farmers housing system of the study households

Open housing and wire enclosed small areas (A) free scavenging (B)

Appendix 2: Meeting with farmers of selected households on the objectives of the study

Appendix 3: Numbered wing tag of experimental chickens

Appendix 4: Conducting ND I_2 vaccination via different route of administrations in selected households

Eye drop (A), spray (B), water (C)

Appendix 5: HI test procedure

- Dispense 0.025 ml PBS into all wells of a plastic microtitre plate with V-bottomed wells, except the first well of the 4-HAU control row, generally H1–H6.
- Place 0.025 ml of each reference antiserum in the first wells of the first column of the plate (A1–G1) and use the last row (H) to titrate the 4 HAU and for the RBC control.
- Use a multichannel micropipette to obtain two-fold dilutions of all sera across the plate and discard the last 0.025 ml.
- Add 0.025 ml of diluted allantoic fluid containing 4 HAU in each well from row A to row G.
- In the first two wells of the last row (4-HAU virus titration control: H1–H6) of each plate, dispense 0.025 ml of diluted samples containing 4 HAU and make two-fold dilutions from the second well to the sixth well (H2-H6). Discard the last 0.025 ml.

- Add 0.025 ml of PBS in all wells of the virus control and 0.050 ml of PBS in the RBC control wells (H7–H12).
- Mix by tapping gently and place the plate at +4°C for 40 min or at room temperature for 30 min.
- Add 0.025 ml 1% RBCs to all wells.
- Mix by gentle tapping and place at 4°C or at room temperature. Plates are read after 30 min, when the RBCs control has settled. This is done by holding the plate in a perpendicular position to the bench, in other words by holding it vertically, against a white background and observing the presence of tear-shaped streaming at the same speed as that occurring in the RBCs control wells. Results In the first three wells (H1–H3) of the 4-HAU control, haemagglutination must be observed. In well H4, a partial haemagglutination (half of a tear-shaped drop) and in wells H5 and H6 no haemagglutination should be seen. Wells H1–H6 correspond to 4 HAU, 2 HAU, 1 HAU, 0.5 HAU, 0.25 HAU and 0.125 HAU respectively. The virus is identified on the basis of the correspondence with the reference antiserum, which inhibits its haemagglutinating activity. In case of identity, the titre of the reference antiserum with the virus under examination should be equal to or ± 1 dilution of its titre with a homologous antigen (Ag).

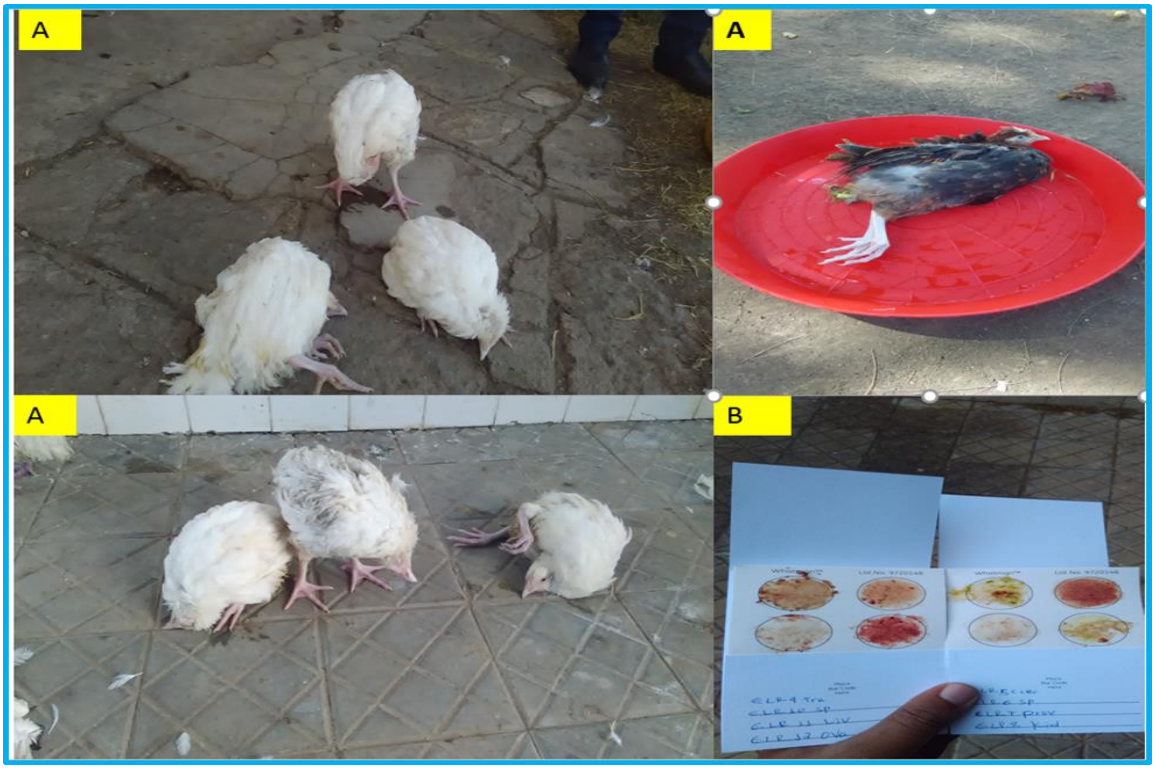
Interpretation: The last dilution where there is complete inhibition of haemaglutination represents the titiration of the serum. Full haemagglutination indicates negative result. The cut of titre for NDV is 1:16 hence, sedimentation of ARBCs at a dilution of ≥ 1:16 indicate positive reaction.

Appendix 6: Blood collection from wing vein and harvested serum samples

Appendix 7: Conducting on station virus challenge with local strain injecting in to breast muscle

Appendix 8: Experimental chickens purchased for virus challenge kept in separated pen

Appendix 9: ND suspected chickens collected for impressions of post mortem organs

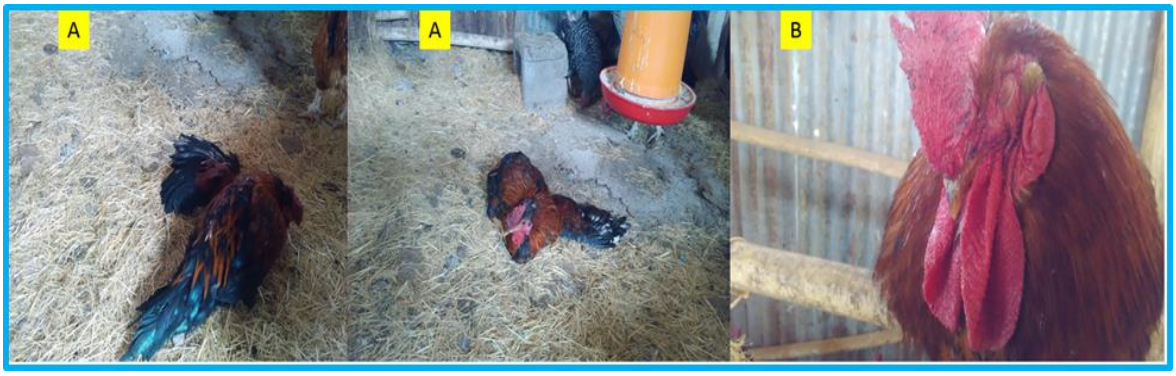


Chickens showing torticollis and leg paralysis (A), tissue stained in to FTA card (B)

Appendix 10: Chicken in the control group died showing typical clinical signs of ND after virus challenge

Fluffed plumage (A, B), paralysis of legs (C, D), listlessness (E, F)

Appendix 10: Two chickens from drinking water treatment group shown clinical signs of ND after virus challenge



Wing and legs paralysis (A), listlessness (B)

Appendix 11: Ethical clearance certificate

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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/24/05/09/2017

Name of Applicant: Kibrom Mebrahtu **(DVM,** MSc fellow)

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

Title of the project: On farm evaluation of thermostable 1-2newcastle disease vaccine and molecular characterization of NDV in smallholder poultry farms in Central Ethiopia

Date of application:	**08/03/2017**
Nature of the project:	**mildly invasive-leading to euthanasia**
Target animal species:	**domestic chicken**
Number of animals involved:	**154 on field and 50 on station**
Study area:	Central **Ethiopia**

Minutes No. and date of review: **VM/ERC/05/09/017, 04/05/2017**

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

Dr. Dinka Ayana
Dean
College of Veterinary Medicine and Agriculture

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

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Please quote Our Ref. No. When replying

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