THESIS REF. ……..

CONVENTIONAL AND MOLECULAR EPIDEMIOLOGY OF NEW CASTLE DISEASE IN WILD AND DOMESTIC BIRDS IN SELECTED RIFT VALLEY AREAS, ETHIOPIA

MVSc THESIS

BY

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Conventional and Molecular Epidemiology of New Castle Disease Virus in Wild and Domestic Birds in Selected Rift Valley Areas, Ethiopia

MVSc Thesis

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LIST OF ABBREVIATIONS

AI	avian influenza
APMV	avian Paramyxovirus
ATARC	Adami Tullu Agriculture and Research Center
ATJK	Adami Tulu Jido Kombolcha
χ^2	chi square
Ct	Threshold cycle
ELISA	Enzyme Linked Immuno Sorbent Assay
F	Fusion
HA	Haemagglutination
HAU	Haemagglutinating units
HI	Haemagglutination Inhibition
HN	Hemagglutinin neuraminidase
L	Large RNA polymerase
M	Matrix
Mab	Monoclonal antibody
MoLF	Ministry of livestock and fisheries
mRNA	Messanger of Ribonucliec acid
NAHDIC	National Animal Health Diagnosis and Investigation Center
ND	Newcastle disease
NDV	Newcastle disease virus
NP	Nucleoprotein
OIE	Office International des Epizooties
ORF	Open reading frames
PI	Percentage of inhibition
PBS	Phosphate buffered saline
P	Phospho-protein
PPMV	Pigeon paramyxovirus
PCR	Polymerase chain reaction
RBCs	Red blood cells

RT- PCR	Reverse-transcriptase polymerase chain reaction
rRT-PCR	Real time reverse-transcriptase polymerase chain reaction
RNA	Ribonucleic acid
SNNP	Southern Nation Nationality and people
VTM	Virus transport media

ABSTRACT

New castle disease is a highly contagious and devastating viral disease of wild and domestic birds. The cross-sectional study was conducted from February 2016 up to March 2017 on domestic and wild birds in selected rift valley areas of Ethiopia to study epidemiology of Newcastle disease in wild and domestic birds. A total 300 domestic backyard chicken were selected, 150 of them blood sample for serology and 150 pools of five trachea and cloaca swab samples and also from wild birds 60 pool of five faecal droplets and ten swabs from pigeon were sampled. The samples were tested by Haemagglutination Inhibition and molecular characterization was done by real time reverse transcriptase polymerase chain reaction targeting a conserved region of the Fusion gene to specifically amplify virulent *Newcastle disease virus* strains. Laboratory results were analyzed using descriptive statistics, chi-square test, fisher's exact test and logistic regression. The overall sero-prevalence of Newcastle disease in domestic birds was 12.7% (19/150) with statistical no significant difference among the study districts 20% (10/50), 12% (6/50), 6% (3/50) in Bishoftu, Hawassa and Batu, respectively. The samples collected from trachea and cloaca swab tested by real time reverse transcriptase polymerase chain reaction 16.7% (7/30) of New castle disease virus was tested that 20%, 15%, 15% from Bishoftu, Hawassa and Batu, respectively, with statistical no significance differences among the study districts. In the wild birds all faecal droplet samples tested by real time reverse-transcriptase polymerase chain reaction were negative where as 50% (5/10) swab samples from Pigeon were positive to *Avian Paramyxovirus*-1. The questionnaire survey response indicated that interaction of domestic poultry within the market and with wild birds and presence of water bodies has been recognized as a possible source of New castle disease. The current study provides important information on serological, associated risk factors and molecular characterization of New castle disease virus in the study areas, and hence it should be applied prevention and control methods and detail study will be conducted.

Key words: *Conventional, Ethiopia, Molecular, New castle disease, New castle disease virus, Rift valley*

1. INTRODUCTION

Poultry play an important economic, nutritional and socio-cultural role in the livelihoods of poor households in developing countries, including Ethiopia. Village backyard poultry, characterized by traditional production methods and local breeds represents 97% of the total Ethiopian poultry population estimated at 43 million (CSA, 2012); and also has a diverse number of both terrestrial and aquatic bird species and one of the most important in Africa and its diver's habitat type contribute for the immensely diverse avifauna (Ash and Atkins, 2009).

Free range rural chicken provides off farm employment and income generating opportunity and serves as a source of gifts and religious sacrifices (Permin and Hansen, 1998; Regmi, 2001). Besides, poultry rearing is particularly important to women who often own and manage chickens and the income earns is used to support education of children (Eshetu *et al.,* 2001). Despite its role in raising incomes and reducing poverty in local communities, poultry production is hampered by wide arrays of constraints which restrict the potential of village chickens include; low inputs of feeding, poor management, the presence of diseases of various natures and lack of appropriate selection and breeding practices (Ashenafi, 2000; Tadelle and Ogle, 2001).

Newcastle disease (ND) is one of the most important disease due to highly contagious and devastating disease of poultry affecting many domestic and wild *avian* species and causes severe economic losses in the poultry sector (Cattoli *et al.,* 2009). It is caused by the Newcastle disease virus (*NDV*) of *avian Paramyxovirus* type-1 (*APMV*-1) classified under the genus *Avula* virus of family *Paramyxovaridae* (Mayo, 2002).

NDV is an envelope, non-segmented, single-stranded, negative-sense RNA virus with a helical morphology. Its genome has six open reading frames (ORF) in the order of 3′-NP-P-M-F-HN-L-5′. These genes encode for the following proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin neuraminidase

(HN) and the large RNA dependent polymerase (L) respectively. During P-gene transcription, two additional non-structural proteins, the V and the W proteins, are also generated through RNA editing (Steward, 1993).

Pigeon *Paramyxovirus*-1 (*PPMV*-1) is a variant of *NDV*, it is characterized by unique monoclonal antibody (Mab) binding profiles. Based on genomic size and the nucleotide sequences of the F and L genes, *NDV* strains can be categorized as class I or class II (Alexander, 2008).

PPMV-1 was started and disseminated to the world, after its emergence in the Middle East (late 1970s), 32 pigeon carrier of *NDV* spread worldwide and dissemination from pigeon populations into commercial poultry has been documented in Great Britain and Austria (Kommers *et al.*, 2002).

In Ethiopia, the first documented evidence of *ND* was recorded in 1978 when an outbreak occurred in Eritrea, then after Northern part, gradually distributed all over the country through wild birds and related risk factors. Since then, the disease has become endemic in village poultry population and thus it recurs every year inflicting heavy losses (Tadelle and Jobre, 2004).

NDV infects a wide range of domestic and wild bird species worldwide (Madadger *et al.*, 2013). ND is characterized by respiratory, nervous system impairment, gastrointestinal and reproductive problems (Nanthakumar *et al.*, 2000; Tiwari *et al.*, 2004). Sources of infection for *NDV* are exhaled air from infected birds and contaminated feed and water by faecal droplet and body discharges, transmission among birds mostly through aerosol (Li *et al.*, 2009).

NDV is one of the biggest contributors of economic losses to the world's economy (Alexander, 2008; Alexander, 2009). In several developing countries, it is endemic and has greatest impact on villages where people's livelihood depends upon poultry farming (Mohamed *et al.*, 2011; Rezaeianzadeh *et al.*, 2011). Annual losses caused by this disease

worldwide are in millions of dollars (Waheed *et al.*, 2013; Susta *et al.*, 2010). It is an economically important disease and also a major threat to poultry industry (Narayanan *et al.*, 2010). According to variation in strains of *NDV*, the rate of mortality and morbidity in a flock varies from 90 - 100% (Nanthakumar *et al.*, 2000; Haque *et al.*, 2010) along with decrease in egg production (Choi *et al.*, 2010).

In Ethiopia ND is one of the most important diseases that inflicting heavy losses of chickens (Chaka *et al.*, 2012; Mazengia, 2012). However, there is scarcity of information on the status of ND in wild and domestic birds. Hence, it is important to generate information on the status and molecular characterization of *NDV* that circulate in selected rift valley areas, Ethiopia.

Therefore, the general objective of this study was:

- ✓ To study ND in wild and domestic birds by using conventional and molecular epidemiology techniques in selected rift valley areas, of Oromia and SNNP regions, Ethiopia.

The specific objectives of these studies were:

- ✓ To estimate the sero-prevalence of ND in village chickens in the study area.
- ✓ To detect and characterize *NDV* in samples collected from both domestic (chicken) and wild birds by using rRT-PCR.
- ✓ To assess the status of knowledge of society and associated risk factors of ND.

2. LITERATURE REVIEW

2.1. Definition

ND was first recognized in 1926 in Java, Indonesia and in Newcastle Upon Tyne, England. It was, however, reported that there were known outbreaks of the disease in poultry before 1926 with similar clinical signs to ND. *NDV* is synonymous with *avian Paramyxovirus*-1 (*APMV*-1). It has been described by different terminologies including Pseudo fowl pest, Pseudo vogel pest, avian distemper, avian pest, Ranikhet disease, Tetelo disease, Pseudo fowl plague, Korean fowl plague and Avian Pneumo encephalitis (Spradbrow, 2001a).

2.2. Etiology

According to taxonomy of virus, *NDV* belongs to order Mononegavirales, family *Paramyxoviridae* and subfamily *Paramyxovirinae* (Cattoli *et al.,* 2011). The subfamily is divided into five genera: *Morbillivirus*, *Respirovirus*, *Henipavirus*, *Rubulavirus*, and *Avulavirus* (Miller *et al.,* 2009); all the *APMVs* are part of genus *Avulavirus*. It exists in 10 serotypes; *APMV*-1 to *APMV*-10 (Waheed *et al.,* 2013), but all *NDV* isolates belong to serotype 1 (*APMV*-1). Virions are roughly spherical; 150 nm or more in diameter and filamentous (Catroxo *et al.,* 2011). The genome is about 15.2 kb in length (Cao *et al.,* 2013; Zhang *et al.,* 2012) that codes for six structural and two non-structural proteins (Choi *et al.,* 2010). 'Rule of six' should be followed by genome because it should be of polyhexameric length to replicate rapidly. It encodes for six proteins in 3' to 5' direction; these are Nucleoprotein (NP), Large RNA polymerase (L), Fusion (F), Hemagglutinin Neuraminidase (HN), Matrix (M) and phospho protein (P) (Linde *et al.,* 2011; Alhabeeb *et al.,* 2013). The proteins W and V were additionally created within the P gene during transcription of mRNA at editing site by insertion of guanines (Linde *et al.,* 2011; Qiu *et al.,* 2011).

In virus particles, NP is the most abundant protein which provides the *NDV*s core helical nucleo-capsid structure. It is the main regulator in replication of viral genome (Kho *et al.*, 2004). The genomic RNA is associated with NP, P and L proteins to form RNP complex, which serve as template for RNA synthesis (*Kho et al.*, 2003). NP is found to be highly immunogenic, as it induces antibody responses in chickens (Ahmad Raus *et al.*, 2009).

Model for infectious *NDV* particle ploidy and size, *NDV* particles with a single functional genome are the most prominent species, and increasingly larger particles packaging two, three, or more functional genomes exist along a declining population gradient (Peter, 2016) (Figure 1).

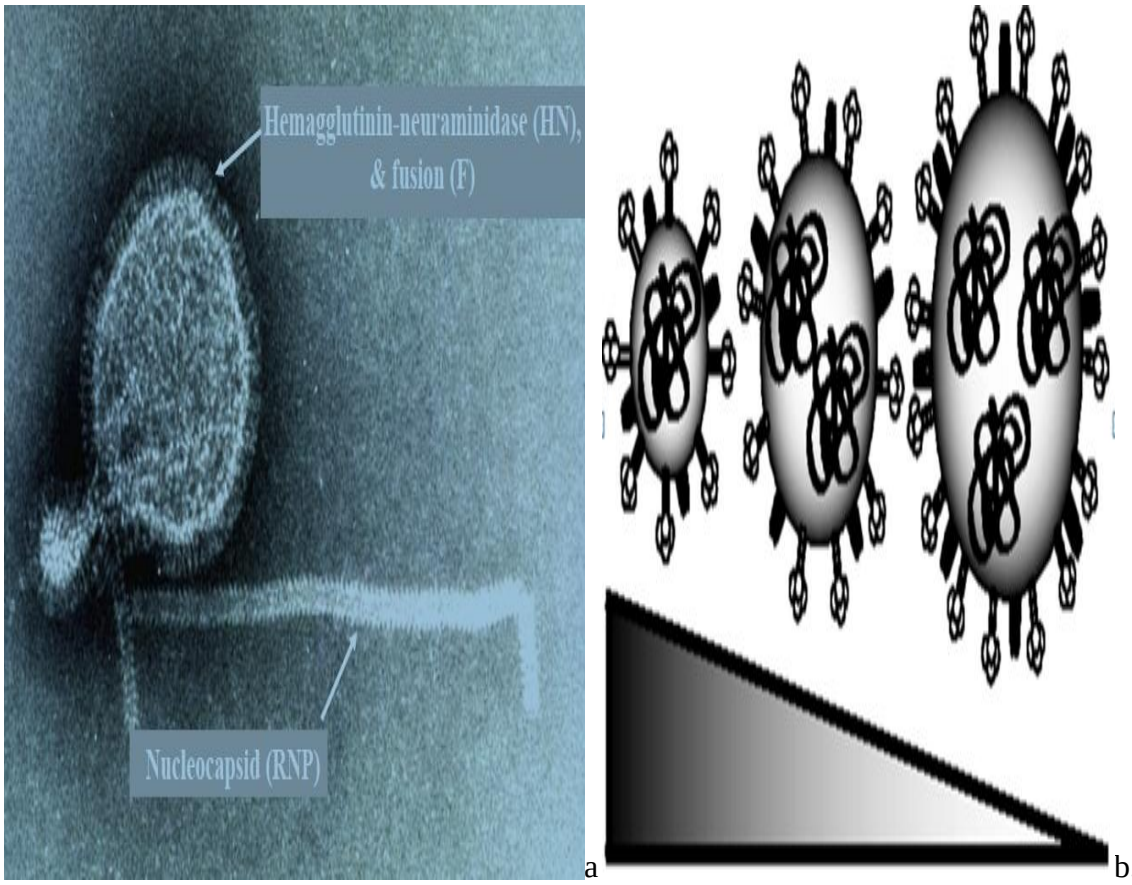

Figure 1: Virulent *NDV* virions isolated from birds (Peter, 2016).

2.3. Epidemiology

2.3.1. Distribution

The epizootics of ND in poultry continue to occur in Asia, Africa, Central and South America while in Europe, sporadic epizootics occur (Naveen *et al.,* 2013). It was reported consistently from all continents of the globe (Munir *et al.*, 2012). Major panzootics of ND have been recorded from different parts of the world. The very first panzootic started in 1926 in Southeast Asia from Java, Indonesia and in Europe from Newcastle-upon-Tyne, England (Seal *et al.,* 2000; Arifin *et al.,* 2011), and it remained till late 1950s (Qiu *et al.,* 2011). The second panzootic began in Middle East in late 1960s and spread to other countries till 1973. The third drastic panzootic caused by neurotropic form of *NDV*, termed as pigeon *Paramyxovirus* type-1 virus, appeared in Middle East about in the late 1970s. In 1981, ND reached Europe then spread rapidly throughout the globe (Mase *et al.,* 2002). The latest and fourth pandemic emerged by late 1980s in Far East, South Africa, and Europe (Qiu *et al.,* 2011). A sporadic form of ND exists in Pakistan throughout the year; only a limited number of outbreaks are reported annually (Munir *et al.,* 2012a). In Southeast Asia, it is endemic and a cause of huge economic losses to commercial poultry (Munir *et al.,* 2012b).

ND is one of the major problems in village chickens in most parts of Ethiopia (Tadelle and Jobre, 2004; Mazengia, 2012). It has become endemic in poultry population and recurs every year inflicting heavy losses (Tadelle and Jobre, 2004). The main movement of chicken marketing is from periphery to the center (rural to towns) which favors the spread of diseases all over the country (Dessie and Ogle, 2001). However, there is acute lack of information on bio-security situation and roles of poultry marketing practices in diseases dissemination in the country (Shewantasew *et al.,* 2012).

ND is an endemic in the village chicken population in Ethiopia. A number of studies have been conducted to determine the prevalence of ND in the rift valley area and surrounding of Ethiopia. Various prevalence's had been reported for *NDV* antibodies in previous studies, ranging from 5.6% (Belayheh *et al*., 2014) to 47.6% (Zeleke *et al.,* 2005).

2.3.2. The Spatial distribution of Newcastle disease in Ethiopia

ND is widely distributed in all areas of Ethiopia, although the level of the disease prevalence may show significant variations across the different farming systems and agro-ecological zones of the country. The national picture of ND status by summarizing the outbreak data reported to Ministry of Livestock and Fishery (MoLF) /formerly said Ministry of Agriculture and Rural Development (MoARD) from 2011 - 2015 was presented in Chart 1. The highest rate of outbreaks from March to May is suggested to be associated with high rate of chicken marketing for Easter (Spradbrow, 2001). According to secondary data reports from MoLF, ND outbreak was reported 205 times from 2011 up to 2015 among this, many reports from Oromia region followed by Amhara, Addis Ababa, SNNP, Benishangul gumuz and Tigray region by percentage 74.6%, 11.7%, 10.5%, 6.3%, 3.9%, 2.9%, respectively but the rest regions have not report of ND outbreak.

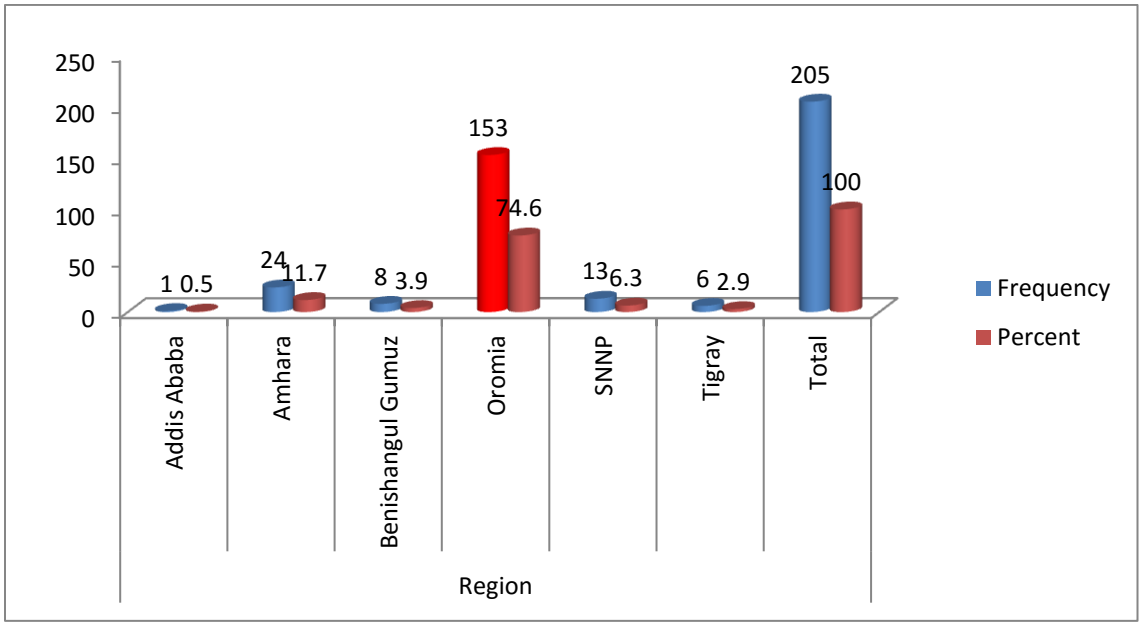

Chart 1: Outbreak reports of New castle disease in poultry from different region of Ethiopia. The graph was drawn using data obtained from Ministry of Livestock and Fisheries (MoLF).

2.3.3. Transmission and source of infection

NDV can infect many species of birds and it spreads primarily through direct contact between healthy and infected birds by airborne transmission of the virus. It can be transmitted through faecal droppings and secretions from the nose, mouth and eyes of infected birds and spreads by contaminated water feed and during transport (Li *et al.,* 2009). Mechanical transfer of infected faeces occurs by rodents, insects, dogs, fleas, or scavenging animals (Ullah *et al.,* 2004). Infection takes place by virus inhalation, ingestion or by contact with conjunctiva. It may vary from subclinical with no mortality to severe infection, with 100% mortality. Chickens infected with virulent *NDV* may die without showing any clinical sign of illness though young chickens are more susceptible and show sign sooner than older ones. ND more spread in village is probably through human agents (Ashraf and Shah, 2014). An outbreak of ND is unpredictable and discourage villager from paying proper attention to the husbandry and welfare of their chickens (Spradbrow, 2001).

In countries where poultry are kept exclusively in bird proof housing, the ability of the feral birds to invade affected flocks and transfer the disease will be minimal, whereas birds kept on open range are more likely to be infected with strains carried by feral birds (Onapa *et al.,* 2006).

2.3.4. Molecular basis of pathogenesis

The genome of *NDV* encodes for six major structural proteins. Viral replication, transcription and translation occur in the cytoplasm of the host cell, while virus particles are assembled in plasma membrane by budding (Zanetti *et al.,* 2003). Important pathogenic marker of *NDV* exists in F protein (Madadgar *et al.,* 2013). Disulphide linkage is present between F1 and F2. These proteins enable the virus to attach to the host cell membrane (Wen *et al.,* 2007). At cleavage site, F0 protein has two pair of basic amino acids that can be cleaved by the host proteases (Pham *et al.,* 2005). Highly virulent *NDV* has three or more basic amino acids, which are lysine (K) or arginine (R) present at

113 - 116 residues and phenylalanine (F) at position 117 (OIE, 2012). Cleavage of F0 protein is due to the presence of these basic amino acids in virulent *NDV* (Boostani *et al.*, 2013). It has been found that avirulent viruses have 112G/E-K/R-Q-G/E-R-L117 and virulent viruses have 112R/K-R-Q-K/R-R-F117 amino acid sequence at cleavage site (Pham *et al.*, 2005). Most of the pathogenic *APMV*-1 viruses for chicken have sequence 112R/K-R-Q/K/R-K/R-R116 (Choi *et al.*, 2010). OIE accepts F cleavage sequence as determinant of primary virulence (Wise *et al.*, 2004).

Class-I *NDV* which has a genomic size of 15,198 nucleotides (Liu, 2009), is occasionally isolated from wild aquatic birds and domestic poultry, it is mostly avirulent to chickens. Class-II *NDV*s comprise the majority of virulent *NDV* strains and some avirulent *NDV* strains (Alexander, 2008). Class II *NDV*s are further subdivided into 11 genotypes (I-XI) (Cattoli *et al.*, 2010). Early sublineages of Class II *NDV*s that occurred before the 1960s (genotypes I to IV) have a genomic size of 15,186 nucleotides, whereas late Class-II *NDV* sublineages (genotypes VI to XI) have a genomic size of 15,192 nucleotides. Class-II *NDV*s under genotype VI and VII are further subdivided into eight (a-h) subgenotypes (Aldous *et al.*, 2003) proposed the creation of lineages and sublineages in classifying *NDV*s to make it possible to rapidly type future virus isolates on the basis of their nucleotide sequence and make inferences about their origins (Cattoli, 2010).

Different *NDV* strains vary greatly in pathogenicity. It can be broadly grouped into five pathotypes on the basis of clinical signs in infected chickens. ND may manifest as viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric (Tsunekuni *et al.*, 2010). Other factors, such as host species, host immune status and age, environmental stress, co-infection with other organisms, viral dose and route of exposure, may also influence the severity of the disease (Toshihiro, 2013).

2.3.5. Host infected

NDV infects a wide range of domestic and wild bird species worldwide (Alexander, 2009). More commonly affected species include chickens, turkeys, ducks, pigeons,

(Zhang *et al.,* 2011) guinea fowl, Japanese quail and many wild birds of all ages (Nanthakumar *et al.,* 2000). The most susceptible avian species to this disease are chickens (Rezaeianzadeh *et al.,* 2011) and also some mammals like humans, cats and dogs. Class I *NDV*s are occasionally isolated from wild aquatic birds and domestic poultry and are mostly avirulent to chickens. Class II contains viruses that have been isolated from multiple wild birds and poultry species. Most viruses within this group are virulent and cause significant economic losses to poultry industry worldwide (Miller *et al.* 2009).

ND in case of wild birds

Isolates of *NDV* have been obtained frequently from wild birds, especially migratory feral waterfowl and other aquatic birds. Most of these isolates have been of low virulence for chickens and similar to viruses of the "asymptomatic enteric" pathotype. Occasionally, virulent viruses have been detected in wild birds, but usually these were in birds found dead near infected poultry. The most significant outbreaks of *NDV* in feral birds have been those reported in double crested cormorants (*Phalacrocorax auritus*) in North America since the 1990s (Alexander, 2009).

NDV was responsible for the outbreaks of ND in the UK in 1997 Alexander *et al. (*1999) had most likely been introduced by migratory wild birds. This virus responsible for the outbreaks in free-living pheasants in Denmark in 1996 was closely related, as were isolates from a goosander in Finland in 1996 and, perhaps significantly, a cormorant (Figure 2) from Denmark in 2001 (Jorgensen *et al.,* 1999; Alexander *et al.,* 1999). Re-emergence of a genetically very closely related virus in pheasants in Great Britain and France in 2005 and the close proximity of the French (Loire Atlantique) farm to a lake led to the speculation that this virus may be established in some species of wild birds in Europe as cited in (Alexander, 2009).

Figure 2: Migratory cormorant birds (Aldous *et al.,* 2003).

2.4. Clinical finding

Clinical signs are dependent on factors such as the virus strain, host species, age of the host, and co-infection with other micro organisms, environmental stress, and immune status (Alhabeeb *et al.,* 2013). In chickens, the general symptoms are loss of appetite, listlessness, abnormal thirst, weakness, and drop in egg production, air sacculitis, tracheitis and conjunctivitis. Respiratory signs can include sneezing, gasping for air, nasal discharge and coughing, whereas a clear intestinal symptom is a greenish watery diarrhea. Nervous symptoms may consist of paralysis of wings and/or legs, twisting of head and neck or complete paralysis (Bhaiyat *et al.,* 1994). Layers show drop in egg production and misshapen soft egg shells. In acute and severe cases (like neurotropic velogenic strain), death is very sudden and birds die without showing any clinical signs. Dead birds have hemorrhagic or necrotic lesions in mucosa of intestine, cecal tonsils, proventriculus and gizzard. Swollen kidneys and liver and deposition of urates are also common lesions (Hadipour *et al.,* 2011).

2.5. Diagnosis

2.5.1. Clinical examination and post-mortem

Rapid and accurate diagnosis of ND outbreak is important because it clinically resembles highly pathogenic avian influenza (AI). Clinical diagnosis based on history, signs and lesions (hemorrhage) on visceral organs may establish a strong index of suspicion but the laboratory confirmation must be done (Khan *et al.,* 2010).

2.5.2. Laboratory test

HA and HI test, virus neutralization test, ELISA, plaque neutralization test and RT-PCR can be used for confirmation of the *NDV* (Chaka *et al.,* 2013). Now rRT-PCR is the most exclusively used method to detect AIVs and *NDV*s (Liu *et al.,* 2011; Haque *et al.,* 2010). rRT-PCR assay is more sensitive, specific and less labor intensives as compare to other conventional methods used for laboratory diagnoses such as virus isolation, Immuno-Fluorescence Staining, Neuraminidase Inhibition and ELIZA (Tang *et al.,* 2012; Shahzad *et al.,* 2011). Other molecular diagnostic tests like rRT-PCR and nucleotide sequence analysis (Table 3) are also important in *NDV* diagnosis (Shabbir *et al.,* 2012).

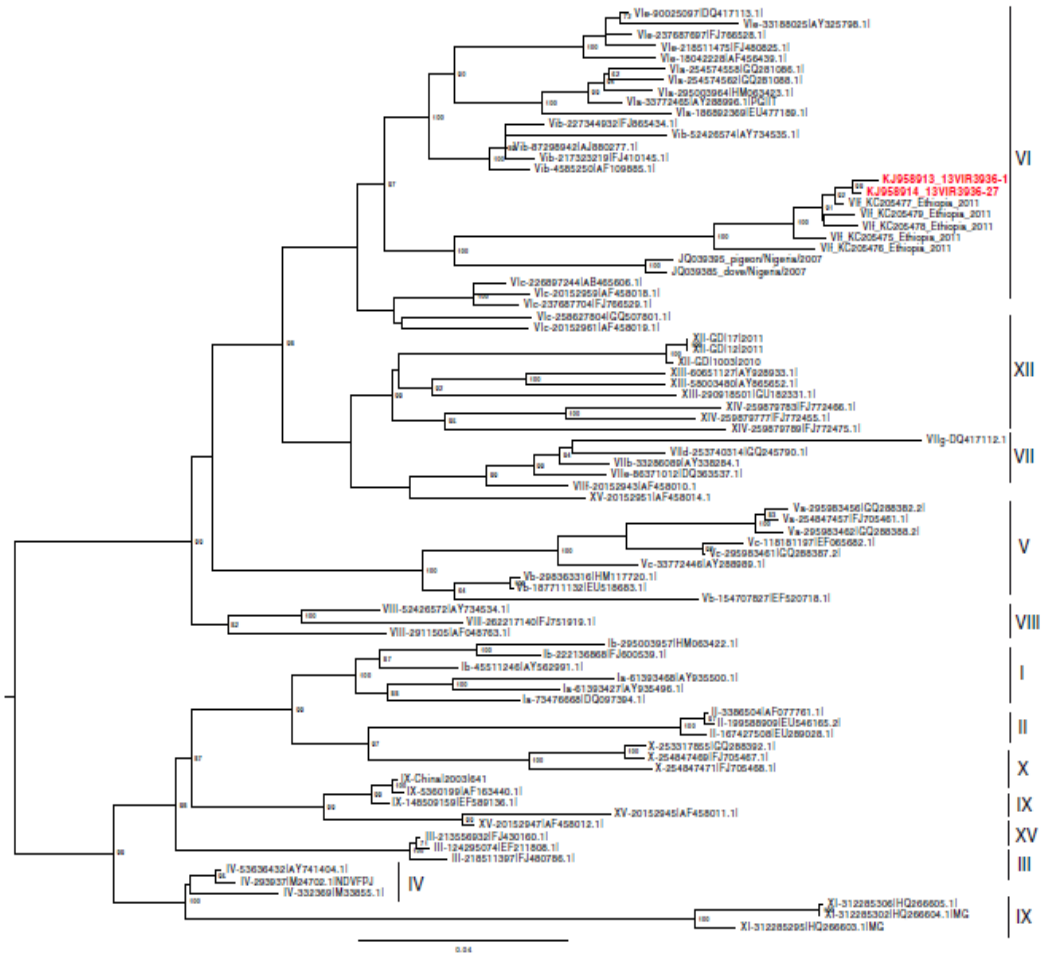

Figure 3: Phylogenetic tree of the complete nucleotide sequence of the F gene for two representative isolates (Mulisa *et al.*, 2014).

Phylogenetic tree of complete nucleotide sequence of class II Newcastle diseases viruses. Ethiopian isolates included in this analysis (KJ958913_13VIR3936-1 and J958914_13VIR3936-27) were labeled in red. The Nomenclature system used in the phylogenetic tree is based on (Diel *et al.*, 2012).

2.6. Control and prevention

The control of ND in village chickens by strict quarantine or strict bio-security cannot be achieve good results because of the difficulty in controlling the movement of people, chickens and wild birds as well as the control of the seasonal peaks in the marketing of

the chicken. The use of vaccines could be the one method that would be feasible in the control of the disease in village chickens. This method can only be helpful if the most challenging problem of thermo stability is dealt with, as refrigeration is a huge setback in rural areas, the thermo labile, live lentogenic LaSota /46/ vaccine strain is widely used by poultry producers in the commercial sector (Mazengia, 2012).

The other control method would be to provide poultry farmers with written livestock messages that are comprehensive and concise. These extension messages include information such as the importance of segregation of unhealthy birds, the proper disposal of dead birds as well as viscera and feathers that remain if the birds are eaten and ensuring that the meat is properly cooked before consumption. Thus, for the successful control of ND, new vaccination technologies (such as the thermo stable vaccines that have been tried and tested and have scored successes in countries such as Mozambique) and appropriate extension methods will have to be adopted (Spradbrow, 2001).

2.7. Prevalence and economic impacts of ND in Ethiopia

ND is one of the major problems in village chickens in most parts of Ethiopia. It has become endemic in poultry population around study areas (Table 1) and recurs every year inflicting heavy losses (Tadelle and Jobre, 2004; Mazengia, 2012).

Table 1: Sero-prevalence of ND in the rift valley and surrounding area of Ethiopia.

R. No	Study area	Prevalence (%)	References
1.	Rift valley	22.5	Ashenafi (2000)
	Central high land	47.3	
2.	Rift valley and southern; Hawassa	12.9	Zeleke *et al*. (2005)
	Butajira	16.7	
	Alage	35.9	
	Hossana	47.6	
	Over all	19.78	
3.	Adama	38	Tadesse *et al*. (2005)
4.	Rift valley	11	Regasa *et al*.(2007)
5.	ATJK	5.9	Chaka *et al*. (2012).
	Ada'a	6	
6.	ATJK	16.73	Chaka *et al.* (2013)
	Ada'a	32.2	
7.	Kersna Kondaltity	5.6	Belayheh *et al*. (2014)
8.	Rift valley (Bishoftu, Ziway and Tikur wuha)	11.6	Terefe *et al.* (2015)
9.	Ada'a (lume district)	28.6	Desalegn (2015)

2.8. Public health

Humans are among the many species that can be infected by *NDV* in addition to avian species. It may cause conjunctivitis in humans, when a person has been exposed to large quantities of the virus. Mostly, laboratory workers and vaccinators are affected (Alexander, 2000). The use of personnel protective equipment and biological safety cabinet has reduced the exposure of laboratory workers. Infection is rarely seen in the

workers of a farm; moreover persons handling or consuming poultry products do not appear to be at risk (Nolen, 2003). The conjunctivitis usually resolves rapidly, but the virus will be shed in the ocular discharges from 4 to 7 days. In some cases, mild, self limiting influenza like disease with fever and headache has also been reported in humans (Alexander, 2000; OIE, 2012). There is no evidence found to support human to human transmission but the potential for human to bird transmission exists (Alexander, 2000).

2.9. Risk factors associated with Newcastle disease

The interaction of domestic poultry with other animals, particularly wild birds, has been recognized as a possible source of avian disease in domestic flocks Koch and Elbers (2006). It may also allow the disease to be transmitted from domestic poultry back to the wild bird populations. Some production practices, such as free-ranging may increase the chance of such an interaction and therefore the risk of disease transmission (Gilbert *et al.,* 2006; Songserm *et al.,* 2006). Although the role that migratory birds play in the transmission of avian disease, phylogenetic comparison of the viruses obtained from different hosts suggest that migratory birds carry the virus and spread it to domestic birds (Prosser *et al.,* 2011; Liang *et al.,* 2010).

The transportation of poultry or working utensils may facilitate the transmission and spread of avian diseases. The spread of avian diseases has also been associated with the movement of humans between different bird flocks (McQuiston *et al.,* 2005). At the local level, the existence of natural or artificial water bodies appears to be strongly correlated with outbreak probabilities. Several risk factors related to poultry trade movements such as the presence of at least one poultry trader in the village were also identified as drivers of the local dissemination of the virus from one village to other villages (Desvaux *et al*. 2011).

Similarly, the risk of virus circulation increases in markets because of the high density of domestic birds coming from different areas. Farming practices also affect virus circulation. As the previous study indicated that, in Mali the risk of infection is lower on

commercial farms applying health protection measures (bio-security) than on small and traditional farms with little protection (Molia *et al*., 2011). In Ethiopia, ND virus prevalence and exposure levels decrease when backyards are cleaned more regularly and drinking places are protected. According to study in Madagascar, two types of farming were more susceptible to infection of ND virus; small backyard farms that do not apply bio-security measures, and factory farms, which have more frequent contacts with the outside (visits by veterinarians, purchases of food and chicks) Rasamoelina *et al*. (2012). Contact with the outside also plays an important role in traditional farms in Ethiopia, where the infection rate is higher if breeders renew their poultry throughout sourcing, and the exposure level decreases when eggs and chicks come from the farm's own stock (Chaka *et al*., 2012).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in the rift valley areas of Eastern Shewa zone of the Oromia region of Bishoftu and Batu town and Southern Nation, Nationality and People (SNNP) region Hawassa town from November 2016 to June 2017. The areas were regularly exposed to migratory birds because it was situated water reservoir to search their feeds. Birds and their droplets were sampled from urban markets of each selected districts in mid rift valley of Ethiopia. The study areas were located at 7^{o}9' N to 8^{o}45' N and 38^{o}32' E to 39^{o}17'E encompassing about 40-60km width and more than hundred kilometers length bounded by high land plateaus characterized by semi-arid type of climate with an erratic, unreliable and low rain fall, averaging between 500 and 900mm per annual (Figure 4). The rainfall is bimodal with the long rains from June to September and short rains from February to April (ATARC, 2016).

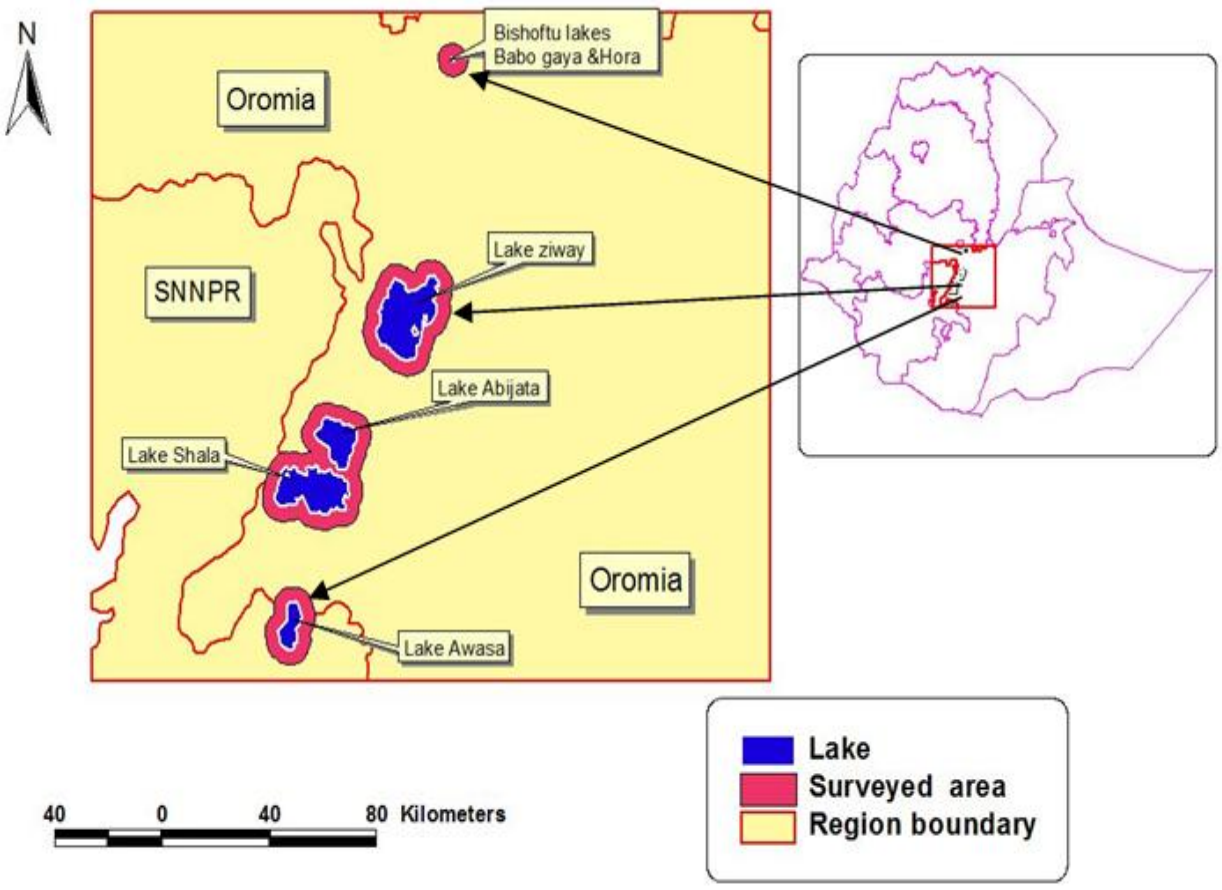

Figure 4: Map showing study areas in rift valley lakes of Oromia and Southern nation nationalities and people, Ethiopia.

3.2. Study population

The study animals were selected from rift valley areas of Oromia and SNNP region, Ethiopia; from wild birds especially pigeon and domestic (back yard) chicken were sampled in respective of districts, sex and age. The age was determined based on history from the owners (Belayheh *et al.*, 2014), young 3 - 6 months and adult greater than six months. At the study areas, existence of natural water bodies appears for interaction of domestic poultry with wild birds, large numbers of poultry were brought to the market.

3.3. Study design

A cross-sectional study of ND sero-prevalence was conducted in domestic chickens found in the study areas (Figure 4) from February 2016 to March 2017. Blood, swab and faecal samples were collected from domestic (chicken) and wild birds for serology and molecular characterization. The samples were collected from the markets found in the study areas twice on consecutive market days from each district to obtain enough sample size.

3.4. Sample size determination

The sample size was calculated based on the prevalence of 11.6% as previously reported by (Terefe *et al.,* 2015) using 95% confidence level and 5% error. A previously recommended formula (Thrusfield, 2005) was used to determine the required sample size as indicated below:

$$n = \frac{(1.96)\text{^}2 * \text{Pexp}\ (1 - \text{Pexp})}{(\text{d})\text{^}2}$$

Where, n = required sample size;

d^2 = desired absolute precision;

P_{exp} = expected prevalence.

$$n = \frac{(1.96)\text{^}2 * 0.116\ (1 - 0.116)}{(0.05)\text{^}2}$$

n = [3.8416 * 0.116 (0.884) /0.0025]

n = 0.3939330304/0.0025

n = 157

Hence, a total of 157 birds were needed to be sampled for determination of sero-prevalence and molecular characterization of *NDV*. However, 300 domestic birds were

selected from Bishoftu, Batu and Hawassa poultry markets to increase the precision of study.

3.5. Study methods

3.5.1. Questionnaire survey

A total of 67 volunteer individuals and traders were interviewed by structured questionnaire survey, it was translated to the local language Afan Oromo and Amharic to assess; means of transport their chicken towards market and from the market, cleaning practices of their home, presence of ND outbreak and season of it more observed, age and breed at more pronounced and also outbreak observed from less than 10 km distance from lake and markets, cross contacts of wild and domestic birds were considered. In addition, individual respondents were interviewed their knowledge to causes, transmission, prevention and control methods of ND (with clinical sign of mass mortality, respiratory distress, diarrhea, ocular and nasal discharges, or nervous signs) and veterinary health care in their village of origin as studied by Alexander and Senne (2008).

3.5.2. Sampling methods

A total of 300 unvaccinated and healthy live chickens were sampled using systematic random sampling technique in Bishoftu, Batu and Hawassa districts commercial market by equal distribution, 150 serums and 150 trachea and cloaca swabs were collected. Furthermore ten tracheal and cloaca swab samples were collected from wild birds (specifically from pigeons) with clinical sign found in Batu town for virus detection. In addition, 60 fresh faecal droplets (a pool of five) were collected from three different areas (20 pooled samples per area) namely Bishoftu, Batu and Hawassa at early mornings by purposive sampling technique. All the samples were kept at 4^{O}C until transported to NAHDIC, Sebeta for subsequent experiment. Summary of study design and result was described (Figure 5).

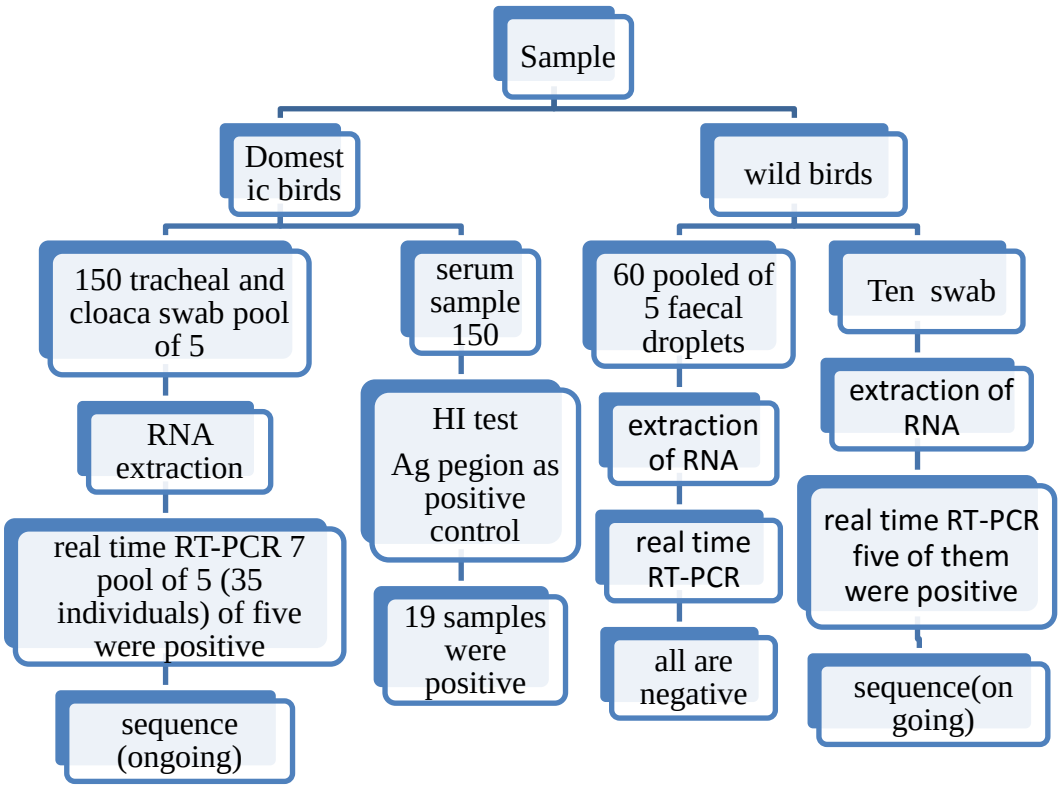

Figure 5: Diagram showing study design and result of Newcastle disease in wild and domestic birds.

3.5.3. Sample collection and test procedure

Two milliliter of chicken blood was aseptically collected from the brachial vein using a 21- gauge sterile hypodermic needle and 3 ml syringes. The syringes were labeled and kept in slanting position till clot formation. Sera was harvested and transferred into a sterile serum bottles. Total 150 chicken were selected thus, 50 chicken from each area tracheal swabs (pool of five) and cloacal swabs (pool of five) were collected and kept in separated cryovial containing 2 ml of freshly prepared viral transport media (VTM) with antibiotic. The swabs were collected in pairs (Tracheal swab pools and cloacal swab pools) collected from the same chicken. Then the samples were placed in ice plastic bags and transport to the NAHDIC, Sebeta, Ethiopia and stored in -80^{o}c until analysis was

started. The swabs were collected aseptically and wild birds faecal droplets were pooled from five dropped site by assuming different birds were dropped, sample suspensions in VTM were homogenized and processed for virus molecular characterization (OIE, 2013).

Haemagglutination for reference Newcastle disease virus strain

HA test was performed in micro plate as outlined by the World Organization for Animal Health. Inactivated antigen for HA test, and positive and negative control sera for HI were obtained from Istituto Zooprofilattico Spermentale delle Venezie (OIE/FAO Reference Laboratory for AI and ND), Padua, Italy. The test was carried out by running two fold dilutions of equal volumes (0.025 ml) of phosphate buffered saline (PBS) and virus suspension (0.025 ml) in V-bottomed microtiter plates. Finally, 0.025 ml of 1% (volume/volume) chicken red blood cells (RBCs) was added to each well and, after gentle mixing, allowed to settle for about 30 minutes at room temperature. The samples were read by holding the plate perpendicular to the bench, by holding it vertically, against a white background and observed the presence or absence of agglutination of the RBC as a protocol of (OIE, 2013).

Haemagglutination Inhibition

The HI test was done followed procedures outlined by the OIE (2013). Inactivated *NDV* antigens (LaSota strain), mono specific antiserum against *NDV*, monoclonal antibody against Pigeon *Paramyxoviruses* and negative sera for HI controls were from Istituto Zooprofilattico Sperimentale delle Venezie (OIE/FAO Reference Laboratory for AI and ND), Padua, Italy.

The test was carried out by running two fold dilutions of equal volumes (0.025 ml) of phosphate buffered saline (PBS pH 7.2) and reference sera (0.025 ml) in V-bottomed microtiter plates (96 wells) followed the plate layout protocol. Four haemagglutinating units (4HAU) of virus (antigen) were added to each well and the plates were left at room temperature for a minimum of 30 minutes. Finally, 0.025 ml of 1% (volume/volume)

chicken red blood cells (RBCs) were added to each well and, after gentle mixed, allow settling for about 30 minutes at room temperature. The HI titer was read from the highest dilution of serum causing complete inhibition of 4HAU of antigen. HI was assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS) was considered to show inhibition (OIE, 2013).

Real time reverse transcriptase Polymerase chain reaction (rRT-PCR)

RNA extraction from swabs and faeces were conducted using Qiagen® RNeasy Mini kit according to manufacturer's instruction. The rRT-PCR reaction was performed using an Applied Biosystems 7500 fast real time PCR thermo cycler. Primer probe set Forward F +4829 5'-GGT GAG TCT ATC CGG ARG ATA CAA G-3', Reverse Primer F- 4939 5'-AGC TGT TGC AAC CCC AAG-3' and Probe F +4894 5'-FAM –AAG CGT TTC TGT CTC CTT CCT CCA-BHQ -3' to specifically detect only pathogenic strains of *NDV*. For F-gene assay, the following amounts of reagents per 25 µl reaction was used: 5 µl of kit supplied PCR buffer (5x), 1 µl of each primer (20 pmol), 1 µl of probe (6 pmol), 0.8 µl of kit supplied deoxynucleoside triphosphates (final concentration: 320 µ M each), 1.25 µl of 25 mM MgCl2 (combined with $MgCl_2$ in kit-supplied buffer, final concentration 3.75 mM) and 1 µl of 13.3 u/µl of RNase inhibitor (Promega, Madison, Wis) and 1 µl Qiagen enzyme mix and 6.95 Rnase free water. For each primer set, the reverse transcription (RT) step was 30 min at 50°C, followed by 15 min at 95°C. The cycling conditions for the *APMV*-1 matrix primers consisted of 40 cycles of 10 s of denaturation at 94°C, 30s of annealing at 58°C, and extension at 72°C for 10s Wise *et al. (*2004). The reporter dye (FAM) signals were measured at the extension step of each cycle, and the threshold cycle (Ct) for each sample was calculated. The samples that have a Ct value <35 was considered positive in F genes based rRT-PCR (OIE, 2013).

3.6. Data analysis

The data were entered in to Microsoft excel and coded for analysis. The laboratory investigation results were analyzed using STATA Version 13.0 statistical package (Stata Corp., 2015). Logistic regression was used to determine the degree of risk factors associated with ND and chi square (χ^2) was used to assess the association between outcome and risk factors for value greater than five and fisher's exact test for value less than five. P-value less than 0.05 at 95% confidence interval was considered as statistical significance difference.

3.7. Ethics approval

Sampling from animals were carried out according to the experimental practice and standards approved by the Animal Welfare and Research Ethics Committee at Addis Ababa University Veterinary Medicine and Agriculture, Bishoftu Campus that is in accordance with the international guidelines for animal welfare, with the verification number VM/ERC/08/06/09/2017.

4. RESULTS

4.1. Serology result

The current serology findings indicated that 12.7% (19/150) prevalence of ND in the study districts that titer greater than 64 (4log2) assumed as sero-positive, where it is highest at Bishoftu (20%), followed by Hawassa (12%) and the least prevalence was recorded in Batu (6%).There was statistically no significance difference ($p > 0.05$) among the study districts (Table 2).

Table 2: Sero-prevalence of ND.

		HI test for *NDV*		
Districts	**No. Examined**	**No. Positive**	**Prevalence (%)**	**p- value***
Bishoftu	50	10	20	0.12
Hawassa	50	6	12	
Batu	50	3	6	
Total	150	19	12.7	

* Based on Fisher's exact test.

In this finding, the prevalence of ND by sex was higher in male (14.8%) than female (9.7%). There was statistically no significance difference ($p > 0.05$) by sex (Table 3).

Table 3: Prevalence of ND by sex.

	HI test for *NDV*				
Sex	**No. Examined**	**No. positive**	**Prevalence (%)**	**p-value**	χ^2
Female	62	6	9.7	0.356	
Male	88	13	14.8		0.8537
Total	**150**	**19**	**12.7**		

These findings indicated that the prevalence of ND was similar in old (12.9%) and young (12.2%) shown in Table 4.

Table 4: The prevalence of ND by age.

		HI test for *NDV*			
Age	**No. Examined**	**No. Positive**	**Prevalence (%)**	**p-value**	χ^2
Old	101	13	12.9	0.914	
Young	49	6	12.2		0.0117
Total	**150**	**19**	**12.7**		

The risk of infection of ND in Bishoftu area was 4.2 higher than Batu area. There were statistically no significance difference between study areas ($p > 0.05$), and the risk of infection of ND in Hawassa area was 2.2 higher than Batu area. There were statistically no significance differences between areas ($p > 0.05$). Furthermore, the risk of ND infection in male chicken was 1.8 higher than female chicken and the risk of adult chicken were 1.1 higher than young chicken. There were statistically no significance difference by sex and age ($p > 0.05$) (Table 5).

Table 5: Multivariate logistic regression analysis of serology on ND with different risk factors.

Variables	No. Examined	No. Positive (%)	OR (95% CI)	p-value
Sex				
Female	62	6	**	
Male	88	13	1.8(0.6 - 5.1)	0.28
Districts				
Batu	50	3	**	
Bishoftu	50	10	4.2(1.1 – 6.4)	0.04
Hawassa	50	6	2.2 (0.5 – 9.6)	0.27
Age				
Old	101	13	1.1 (0.4 - 3.0)	0.91
Young	49	6	**	

** = reference category

4.2. rRT-PCR

The current findings of domestic birds swab samples pooled five based on rRT-PCR test on *NDV* (Table 6) was indicated with its Ct. value when read from Applied Biosystems 7500 fast real time PCR thermo cycler (Figure 5).

Table 6: Result of F- gene by rRT-PCR test.

Study areas	**Site of swab collected**	**Ct.value of F-gene**
Bishoftu	Cloaca	20.35
Bishoftu	Trachea	15.35
Bishoftu	Trachea	25.77
Bishoftu	Cloaca	25
Batu	Trachea	30.5
Batu	Cloaca	20
Batu	Cloaca	24.5
Hawassa	Cloaca	26.2
Hawassa	Trachea	31.5
Hawassa	Cloaca	28.1
Mean		24.73

Out of the 30 pooled samples tested based on the rRT-PCR of F gene, 10 isolates were identified as virulent *NDV*. Among these, 4 tracheal swab pools (TS) were positive though the corresponding cloacal swab pools (CS) were negative. Similarly, 6 CS were positive though the corresponding TS were negative. Hence, there were no swabs had shown direct correlation therefore, samples could be collected from both trachea and cloaca from individual animals to arrive on diagnosis using the rRT-PCR.

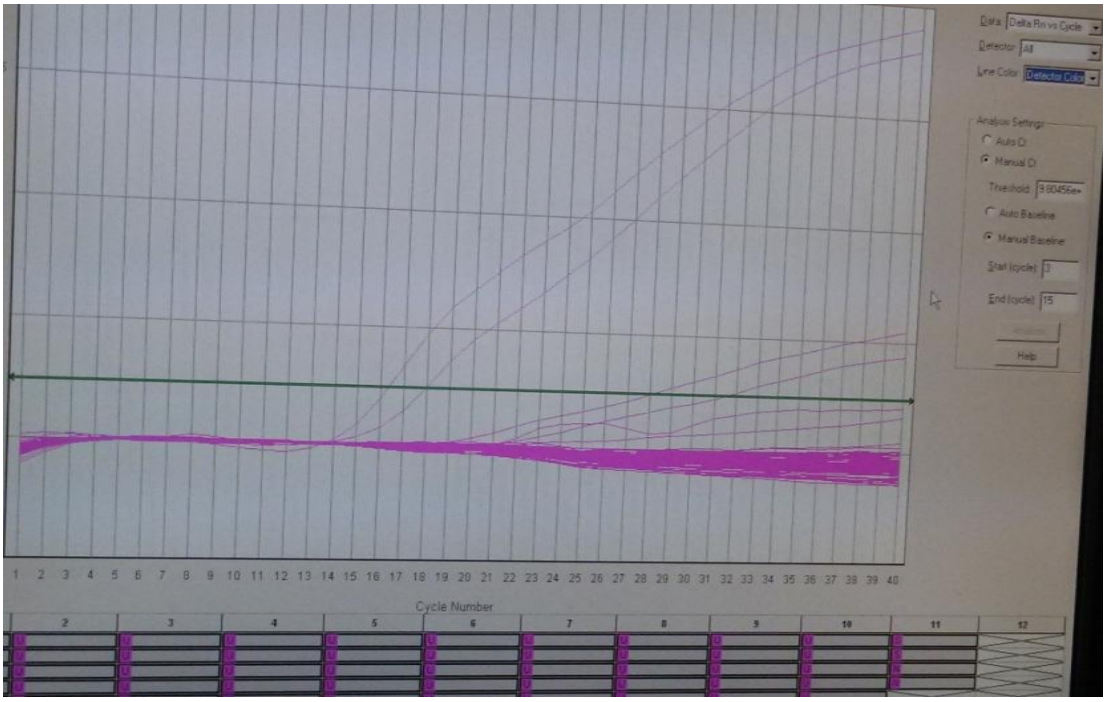

Figure 6: Picture showing result of rRT-PCR with their ct value.

Location wise the 16.7% detection of ND from chicken swabs showed that, it was highest at Bishoftu (20%) but similar in Hawassa and Batu districts (15%) (Table 8). There was statistically no significant difference (p> 0.05) among the study districts.

Table 7: Swab samples from chicken rRT-PCR result and risk of districts.

Districts	**No. Examined**	**No. Positive**	**Prevalence %**	**p-value**
Bishoftu	10	4	40	1.00
Hawassa	10	3	30	
Batu	10	3	30	
Total	**30**	**10**	**33.3**	

Comparison of the detection rate of ND from samples collected from different parts of the birds body showed that it was higher from cloaca site (20%) than tracheal site (13.3%) (Table 10). There was statistically no significant difference ($p > 0.05$) between the two sites compared.

Table 8: rRT-PCR from swab sample of chicken versus site of swab collected.

Site of swab collected	**No. Examined**	**No. Positive**	**Detection rate %**	**p-value**
Cloaca	30	6	20	0.731
Trachea	30	4	13.3	
Total	**60**	**10**	**16.7**	

*Based on Fisher's exact

Location wise, the risk of infection was increased by 1.5 in Bishoftu area than in Batu area (OR = 1.478, 95% CI = 0.28 - 7.87) and the risk of infection was increased by 1 in Hawassa area than in Batu area (OR = 0.975, 95% CI = 0.17 - 5.71) and based on site of swab collected the risk of infection was increased by 1.6 in cloaca than trachea site (OR = 1.602, 95% CI= 0.4 - 6.44) (Table 11) nevertheless, there were no significance difference among all risk factors categories (p value > 0.05).

Table 9: Multivariate logistic regression analysis for ND virus and putative risk factors.

Variables	No. Examined	No. Positive (%)	OR (95% CI)	p-value
Areas				
Batu	10	3 (30)	**	
Bishoftu	10	4 (40)	1.4 (0.27 - 7.41)	0.67
Hawassa	10	3 (30)	1.0 (0.18 - 5.71)	1.00
Site of sampled				
Trachea	30	4 (13.3)	**	0.5
Cloaca	30	6 (20)	1.6 (0.41 - 6.50)	

** = Referance variable.

4.3. Result of wild birds for *NDVs*

All the 60 faecal droplets pooled in five tested by rRT-PCR gave negative. Among ten swab samples obtained from wild birds (pigeon) at Batu area, water bodies area were identified as Pigeon *Paramyxovirus*-1 as they reacted with Mab for Pigeon *Paramyxovirus*-1. Besides, five of the isolates were confirmed positive for virulent *NDV*s by rRT-PCR, mean Ct.value 26 (22.5 – 30.5).

4.4. Questionnaire result on ND

The questionnaire survey indicated that among 67 voluntary owners interviewed; 61.19% (41/67) of them transport their chicken carrying by hand. About 89.55% (60/67) of the people interviewed clean chicken's room daily and 46.26% (31/67) reported as out-break of ND was observed during short rainy season followed by long rainy season 28.35% (19/67), while only 7.46% (5/67) and 2.98% (2/67) reported the occurrence of ND outbreak in dry cool season and throughout the year respectively and the rest 14.9% (10/67) were not respond. Health management wise, 35.82% (24/67) treat their chicken by traditional means, 37.31% (25/67) use veterinary clinic, 25.37% (17/67) not use any

medication and the remaining 1.5% (1/67) were using both veterinary and tradition medication methods.

The society has different knowledge on cause, transmission, prevention and control methods of ND. Accordingly, 53.73% (36/67), 20.89% (14/67), 1.5% (1/67) had low, medium and high information respectively while 23.88% (16/67) did not respond.

Out of 67 respondents, 52.23% (35/67) and 47.76% (32/67) respond that ND occurrence was more pronounced in layers and local breed birds respectively. On the other hands, 70.14% (47/67) of them replied as ND outbreak was observed in those chicks which had contact with wild birds. Others respond that the outbreak was observed within 10 km distance from market 73.13% (49/67) and lake 83.33% (55/67) respectively (Table 12).

Table 10: Result of questionnaire survey.

Category of variable	**N= 67**	**%**
Way of transportation		
Carry by hand	41	61.19
By cart	14	20.89
By car	12	17.91
Did you clean chicken's room daily?		
Yes	60	89.55
No	7	10.44
Did you encounter ND outbreaks		
Yes	66	98.5
No	1	1.49
Season of ND outbreaks		
At short rainy season	31	46.26
Long rainy season	19	28.35
Dry cool season	5	7.46
Through out of the year	2	2.98
How could treat your chickens?		
By tradition	24	35.82
Veterinary clinic	25	37.31
Mixed	1	1.49
Not use any medication	17	25.37
Knowledge about cause, transmission, prevention & control of ND		
Low	36	53.73
Medium	14	20.89
High	1	1.49
Not respond	16	23.88
Age of poultry at ND more pronounced		
Starter	20	29.85
Layers	35	52.23
Broilers	1	1.49
Mixed	9	13.43
Not respond	2	2.98
Breed at ND more pronounced		
Local	32	47.76
Exotic	22	32.83
Cross	3	4.47
Did you observe out-break within 10 km distance from lake		
yes	56	82.58
No	11	16.42
Out-break of ND in chicken contact with wild birds		
Yes	47	70.14
No	0	0
Did you observe out-break (< 10 km distance from market)		
Yes	49	73.13

5. DISCUSSION

In the present study, it was confirmed from individual respondents and veterinarians of the districts during sampling that none of them had vaccinated their chickens for ND. Hence, the 12.7% presence of antibodies to ND in backyard chicken was considered to be evidence of exposure to natural infection. This is comparable with previous reports by Zeleke *et al*., 2005 (11.9%) from Hawassa, Terefa *et al.,* 2015 (11.6%) and Regasa *et al.* 2007 (11%) from central rift valley areas and Chaka *et al.* 2013 (16.7%) from ATJK. But, it is lower than previous reports of Ashenafi *et al.* 2000 (22.5%), in the rift valley area, Desalegn, 2015 (28.6%) from eastern Shewa and higher prevalences than reports of Belayheh *et al*. (2014) from Kersna Kondaltity (5.6 %), Chaka *et al*. (2012), from ATJK and Ada'a during wet (5.9%) and dry seasons (6%), respectively. This difference result may be from methodology of study, year of study and sample size.

The present study revealed that ND distribution among study areas were observed 20%, 12% and 6% at Bishoftu, Hawassa and Batu, respectively. Nevertheless, the variation was not statistically significant (P>0.05) among the districts. The result of the study was also in agreement with the findings by Zeleke *et al.* (2005), a study conducted at rift valley and southern Ethiopia and Regasa *et al.* (2007) who reported the absence of variation among the current study rift valley areas. This could be due to the wide distribution and similar exposure of ND in domestic birds in the rift valley areas.

The study also showed that there was higher prevalence of ND in male chickens (14.77%) as compared to females (9.67%). This finding was in agreement with the previous study conducted by Tadesse *et al.* (2005), Zeleke *et al.* (2005) and Jibril *et al. (*2014) who reported the absence of variation in the risk of exposure of male and female chicken to ND. However, as previous reported a slightly higher prevalence of the disease in males than females. The higher prevalence of the disease observed in the present study could be due to higher number of male chickens involved in the study during sampling than females.

To confirm the specific serotype circulating in the study area, rRT-PCR was done at NAHDIC, Sebeta. The rRT-PCR result indicated that 16.7% of trachea and cloaca swab samples collected from 30 pooled domestic chickens to be positive for ND. The swabs of domestic chicken collected from Bishoftu, Hawassa and Batu was tested by rRT-PCR and the result was 40%, 30% and 30%, respectively. This finding was also in line with the studies of Terefa *et al.* (2015) who reported a total of 8.4% prevalence of the disease in swabs collected from Batu, Bishoftu and Tikur wuha, of which the prevalence in Bishoftu and Batu was 12% and 2%, respectively. Moreover, Mulisa *et al.* (2014), at Addis Ababa open market, also reported that 30.14% (44/146) positive samples, 29 of which were virulent strains belonging to sub-genotype VIf.

Besides, the rRT-PCR result also signified a higher positivity of CS samples 60% (6/10) as compared to TS samples 40% (4/10). But this result contradicts with the finding of Chaka *et al.* (2012) who reported higher positivity of TS than CS. This increased positivity of TS in the present study could be due to the presence of viscerotropic velogenic virulent *NDV* in the study areas. However, there is no positive result was obtained both from Ts and CS of an individual chickens; implying that the sampling of both TS and Cs samples from a single chicken will be maximized the chance of identifying the virus from carrier chickens.

Furthermore, the study also connoted the presence of a virulent NDV from swab samples and fecal droplets. Accordingly, swab sample obtained from wild birds, particularly pigeons revealed a 50% (5/10) detection rate of the virus by rRT-PCR. This finding also agrees with the results of previous studies conducted by Damena *et al.* (2016) at Ziway area which revealed a circulation of virulent strains of *NDV* in pigeons

The faecal droplets of wild birds collected from the ground at study areas were observed to be negative by rRT-PCR. This was in agreement with study by Zeynalova *et al.* (2015) from Azerbaijan State Veterinary Control Service (SVCS) who also reported negative result from environmental samples of fecal droplets excretions of wild birds. This might

be due to inability of the *NDV* to survive on harsh environmental conditions which negatively affects the survival of the virus on the fecal droplets. However, Ashraf and Shah (2014) reported that any excretions, faeces and eggs laied by clinically infected chickens and all parts of the carcass during acute infection can act as sources of infection. Therefore, from this information it can be deduced that, though environmental samples of faecal droplets are safer and easily to collect for veterinarians, the likelihood of obtaining positive test results is not rewarding. But it avoids the need to directly handle and sample live birds in terms of welfare and Zoonotic disease. Hence, this approach has been validated as a method for virus detection from swab by rRT-PCR as studies reported by (Indriani *et al.*, 2010).

To assess the level of perception and associated risk factors for the occurrence of *NDV* in domestic and wild birds in the study area, a questionnaire survey was conducted on 67 volunteer individuals. Consequently, about 98.5% of the respondents observed the frequent occurrence of ND, the clinical signs such as, sudden death, diarrhea, nasal and ocular discharge and nervous signs. The survey result also stated that none of the individual respondent knows about disease transmission from wild birds but they know the local name of ND as “fengl” or “fenkil” and its time of occurrence during short rainy seasons as well as predators that were responsible to transmit this disease from village to village. Among these respondents, 73.13% and 83.33% of them were living < 10 km far away from the market and their surrounding lake. Hence, the presence of many lakes in the rift valley areas of Ethiopia appears to be strongly correlated with the likelihood of ND outbreak. This may be due to the seasonal migration of wild birds in search of feed especially during the time of scarcity; which predisposes domestic chickens to the *NDV* originated from wild birds Desvaux *et al*. (2011).

The study also revealed the interaction of domestic poultry in the market and with wild birds as a possible source of ND. Schelling *et al.* (1999) and Koch and Elbers (2006) reported that a virulent *NDV* was isolated from both wild and domestic birds. In this regard, the chance of the traditionally managed free ranging backyard chickens to acquire

NDV infection from wild birds and vice versa could play a significant role the bilateral transmission of the virus between wild birds and domestic chickens.

Furthermore, 61.19% of the respondents carried a number of chickens to and from the market by their hand and this could be related with the occurrence of the disease. Around 89.55% of the respondents clean their house once per day. Nearly 35.82% and of the respondents treated their sick birds by local and traditional medication where as 37.31% of them consulted with their local veterinarians when birds get sick and 1.5% of them used both traditional and veterinary services. However, the remaining respondents 25.37% did not use any medication for sick chickens. This result was in line with the findings of Samson and Endalew (2010) who reported that the majority of the household did not use medications for their sick birds in the mid rift valley area. The finding was indicated that the majority of household that rear chickens did not use treatments for their sick chicken; it has been supported by the works of Halima (2007) northern parts of Ethiopia. The probable reason for this problem could be associated with lack of information and neglected follow up of the chickens. The chickens that were presented to the market from different areas favor the chances of disease transmission among birds as they might be the main source of infection. Clinically healthy chickens harbors the virulent virus that was responsible for the severity of ND among bird flock was evidenced by studies conducted by Jibril *et al.* (2014) though, chickens clinical healthy could be the source of infection for the flock of the poultry. This study was conducted that in live birds markets the persistence and spread of *NDV* through exposed birds from multiple sources having a higher tendency of circulating the virus and may serve as a source of infection to house hold chickens when re-introduced in to the flock.

6. CONCLUSION AND RECOMMENDATIONS

Newcastle disease is prevalent in the study districts in both wild birds and domestic chicken. It was also confirmed by serological and molecular, rRtT-PCR, diagnostic techniques that the disease is endemic in the mid rift valley, Ethiopia. The occurrence of ND in the rift valley areas was due to involvement of varied species of adopted and migrant wild birds to search their feed around water bodies; that could facilitate contact with local scavenging back yard chickens. The free ranging of backyard birds presents a high risk for *NDV* transmission between wild birds and poultry in both directions, from wild to domestic and vice versa. On the other hand, sero-prevalence of *NDV* suggested that there were widespread exposure of backyard poultry in rift valley areas from the market. In addition, exposure to wild birds, introduction of new birds, to existing flock, mixing with neighboring poultry, feeding of poultry waste and carcass of died birds in the market and also aerosol contact among chickens infected by ND without manifesting clinical sign, lack of effective vaccination and bio-security in the village back yard chicken producers and also traditional way of poultry production could aggravate the morbidity, mortality and loss of production. Therefore, based on the above remarks, the following points are forwarded:-

- Cost effective and integrated team work in both domestic and wild birds towards disease prevention and control should be designed and implemented with the concerned stakeholders.
- Routine surveillance of ND both in wild and domestic birds and early reporting of outbreak should be encouraged.
- Mixing of domestic and wild birds should be restricted.
- Routine vaccination of back yard chickens should be implemented by giving awareness to back yard chicken owner.
- Detail study should be conducted on different wild and domestic birds found in the rift valley areas.

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8. ANNEXES

Annex 1: Geographical positioning system data collection in selected Rift valley areas.

Location	North (latitude)	East (longitude)	Altitude

Annex 2: Format used for collection of sample and information on the risk factors.

Date of collection…….

Region…….. Zone…….

Districts	Location	Owner name	No. of poultry	Sample codes	Age	Sex	Breed	Managment	Presence of wild birds	Distance from market	Outbreak reports

Detail questionner format.

Category of variable
Way of transportation
Carry by hand
By cart
By car
Did you clean chicken's room daily?
Yes
No
Did you encounter ND outbreaks
Yes
No
Season of ND outbreaks
At short rainy season
Long rainy season
Dry cool season
Through out of the year
How could treat your chickens?
By tradition
Veterinary clinic
Mixed
Not use any medication
Knowledge about cause, transmission, prevention and control of ND
Low
Medium
High
Not respond
Age of poultry at ND more pronounced
Starter

Layers
Broilers
Mixed
Not respond
Breed at ND more pronounced
Local
Exotic
Cross
Did you observe out-break (distance from lake)
<10km
10-20 km
>20km
Out-break of ND in chicken contact with wild birds
Yes
No
Did you observe out-break (< 10 km distance from market)
Yes
No

Annex 3: Procedure of RNA Extraction for rRT-PCR preparation

There are four basic procedures.

1. Lysine
2. Binding
3. Washing
4. Elusion

1. Lysine (AVL) :

Lysine solution buffer = n*.56 ml and

Carrier RNA = buffer of lysine * 10 µl

140 µl of sample and 560 µl of AVL mix in tube

Incubate by 15-25°C for 10 min and mix by vortex mixer

Centrifuge in micro centrifuge tube by 800rpm

2. Binding by ethanol

560µl ethanol adds and centrifuge together

Take 630 µl of mixed solution and add into Q Amp- mini spin column

Centrifuge by 8000 rpm for 1 min

Take out the mini spin column and keep in a clean 2 ml collection tube

Centrifuge by 8000 rpm for 1 min

Discard the tube contain filtrate

Repeat this procedure

3. Washing by washing solution

Add 500 µl buffers AW1in Q Amp mini spin column

Centrifuge 8000rpm for 1 min

Add 500 µl buffer AW2 in Q Amp mini spin column

Centrifuge 8000rpm for 1 min

4. Elusion (AVE)

Add 60 µl AVE commercial available in Q Amp mini spin column with in new micro centrifuge tube.

Incubate in 15- 25 °C for 10 min

Centrifuge by 8000 rpm for 1min

Remove Q Amp mini spin column

Deep freeze -80 °C

The primer required for *NDV* identification by rRT-PCR:

Rnase free waater = 6.45 *N

Buffer = 5* N

$Mgcl_2$ = 1.25 * N

dN Tps = 0.8 * N

Primer F = 0.5 * N

Primer R = 0.5 * N

probe = 1 * N

Inhibitor = 1 * N

Enzyme mix = 1* N

Sum/N =17

Let; N= number of total sample

Annex 4: Procedure of HI used.

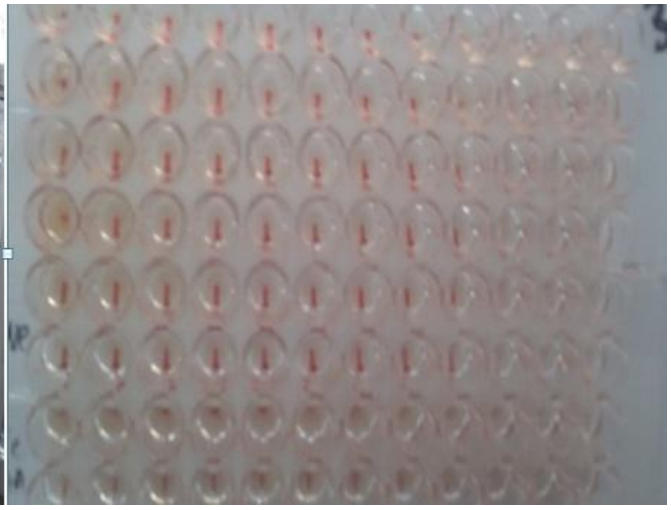
1. Dispense 0.025 ml PBS into all wells of a plastic microtitre plate with V-bottomed wells, except the first well of the 4-HAU control row, generally H1–H6.
2. Place 0.025 ml of each reference antiserum in the first wells of the first column of the plate (A1–G1) and use the last row (H) to titrate the 4 HAU and for the RBC control.
3. Use a multichannel micropipette to obtain two-fold dilutions of all sera across the plate and discard the last 0.025 ml.
4. Add 0.025 ml of diluted fluid containing 4 HAU in each well from row A to row G.
5. 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.
6. 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).
7. Mix by tapping gently and place the plate at +4°C for 40 min or at room temperature for 30 min.
8. Add 0.025 ml 1% RBCs to all wells.
9. Mix by gentle tapping and place at 4°C or at room temperature. Plates are read after 30–40 min, when the RBCs control has settled. This was 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 greater than 4log2 (> 64) was assumed Sero-positive with a homologous antigen (Ag). The 4HAU control ensures that the correct amount of Ag has been used in the test.

Annex 5: Picture showing pigeon swab sample collection procedure.

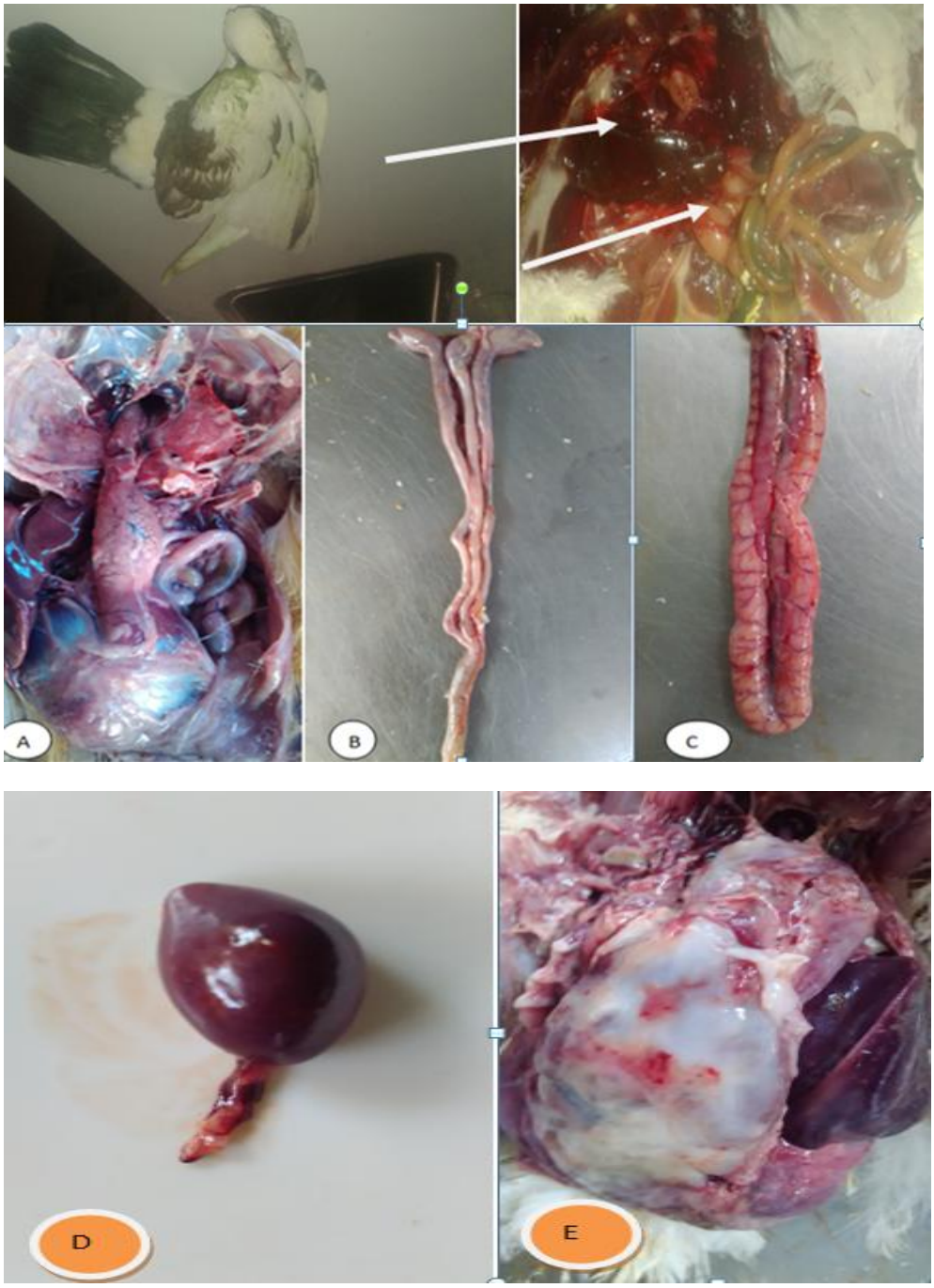
Annex 6: Pictures showing during sample collection.

During collecting faecal droplets of wild birds. (a).During swab sample collection from chicken in the market (b).

Annex 7: Picture during serology of *NDV* by HI.

Annex 8: Picture shown Lesion detected from different visceral organs during post mortem examination of birds infected by ND.



Lesion indicates the visceral organs of Pigeon (A), Caecal lesion (B), Tracheal lesion (C), Hepato megally (D) and Liver cirrhosis (E).