MOLECULAR CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS AND ITS ASSOCIATED FACTORS IN INDIGENOUS CHICKEN FROM SELECTED REGIONS IN KENYA

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Molecular Characterization of Newcastle Disease Virus and its Associated Factors in Indigenous Chicken from Selected Regions in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this project to the Almighty God whose grace is always sufficient.

To my husband George, our two sons Addis and Keylan, my sister Sheila and my Mama Lonicah Apopo. This belongs to all of us.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

AOAV-1	*Avian ortho-avulavirus 1*
APMV	*Avian paramyxovirus*
BLASTN	Basic local alignment search tool-Nucleotides
cDNA	Complementary deoxyribonucleic acid
CL	Cloacal swab
CVL	Central veterinary laboratories
DVS	Directorate of Veterinary Services
IC	Indigenous chicken
KALRO	Kenya Agriculture and Livestock Research Organization
KCSAP	Kenya Climate Smart Agriculture Project
KNBS	Kenya National Bureau of statistics
LBM	Live bird market
MEGA	Molecular Evolutionary Genomic Analysis
NCBI	National Center for Biotechnology information
ND	Newcastle disease
NDV	Newcastle disease virus
OIE	Organisation for animal health
OP	Oropharyngeal swab
PCR	Polymerase chain reaction
POPART	Population analysis with reticulate trees
QGIS	Quantum Geographic information system
RNA	Ribonucleic acid
RT-qPCR	Quantitative reverse transcription-polymerase chain reaction
SAN	Specific antibody negative
SNP	Single nucleotide polymorphism

SSRNA	Single-stranded ribonucleic acid
USDA-ARS	United States Department of Agriculture-Agriculture Research Service
WOAH	World Organisation for Animal Health

ABSTRACT

Indigenous chicken farming is a source of livelihood for rural and urban families in Kenya. However, the farmers experience challenges such as infections from Newcastle disease. Vaccination of flocks against Newcastle disease virus (NDV) outbreaks is an approach for controlling the spread of Newcastle disease (ND). Nevertheless, vaccinated chickens' contract NDV. The study objective was to determine NDV in indigenous chickens (ICs) and the association of the disease with the weather (temperature, humidity, and wind speed) at the time of sample collection, production system, and the interaction of ICs with other species and make inference on the genetic diversity of the NDV matrix and fusion genes. A total of 1,585 samples were collected between 2017 and 2019 from NDV-vaccinated and unvaccinated ICs. The samples collected were from ICs in eight live bird markets in Nairobi (N=372) and ICs from 68 households (N=1210) within the Bomet, Baringo, Kilifi, Nakuru, Kakamega, and Machakos counties in Kenya and clinical samples (N=3). The collected samples were oropharyngeal and cloaca swabs from ICs in markets and households and samples (long bone and internal organs (liver, intestine, caecal tonsils, kidney and heart tissues)) from clinically sick ICs. NDV matrix genes were detected using quantitative reverse transcription-polymerase chain reaction (RT-qPCR), and amplicons of matrix and fusion genes were sequenced using a capillary sequencer. Chi-square and correlation tests were used to test the association between the prevalence of Newcastle disease in IC and the associated factors. The genetic diversity was analysed by haplotype analysis (TCS network) and phylogeny. The NDV prevalence in the Nairobi live bird markets was 65.3% and 72.4% in ICs from the household. Among the vaccinated ICs, the prevalence of NDV was 77%. There were significant relationships between the prevalence of NDV and vaccination history ($p = 0.039$), of the ICs, the type of production system for ICs ($p < 0.010$) and the interaction between ICs and other birds ($p = 0.037$). There was no correlation between the prevalence of NDV and the prevailing weather condition. Haplotype analysis revealed non-synonymous mutation of sequences with low nucleotide densities and high haplotype density of the matrix gene and high nucleotide diversity and haplotype diversity of the fusion gene. The phylogeny revealed similarity for the matrix gene with previous isolates and heterogeneity of the fusion gene. The study also revealed the non-relatedness of the circulating NDV strains with the NDV La Sota strain used in NDV vaccine development. The presence of matrix and fusion genes in samples from vaccinated flocks indicated the presence of both virulent and low-virulence strains of NDV. These findings highlight the occurrence of NDV among ICs and suggest the type of production system, viral shedding post-vaccination and interspecies transmission as the drivers of Newcastle disease outbreaks. Therefore, associated factors to the prevalence of NDV and the circulating strains should be considered during the development of vaccines for effective control of ND outbreaks in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Poultry farming accounts for about 30% of agricultural activities contributing to Kenya's gross domestic product(GDP) (Nyaga, 2007). The distribution of households rearing chicken in Kenya is approximately 3.3 million with 92.5% rearing indigenous Chicken (IC) (Kenya National Bureau of Statistics, 2019). Indigenous chicken is an important component of the rural and the urban household as a source of food, income, nutrition, insurance against emergencies and has the potential to reduce poverty. Rearing of IC is also linked to different ceremonial roles among communities in Kenya (Owour et al, 2013; Magothe et al., 2012).

Indigenous chicken farming is characterised by several challenges including slow growth and maturity rates, poor feeding, high feed cost and high chick mortality due to poultry disease. Newcastle disease (ND) is one of the most important infectious diseases of poultry globally and a big constraint in poultry production in rural areas (Alders, 1999; Dortmans et al., 2011). Newcastle disease virus (NDV), is the causative agent for the ND (Aldous & Alexander, 2001), a single-stranded RNA virus of the genus *Avulavirus* (Aldous et al., 2003). NDV is also called *Avian ortho-avulavirus 1* (AOAV-1), formerly known as *Avian avulavirus1* (AAvV-1) or *Avian paramyxovirus 1* (APMV-1) (ICTV, 2019; King et al., 2018). AOAV-1 virions are pleomorphic in shape, and consist of single-stranded, non-segmented, negative-sense RNA genomes (Miller et al., 2010). The RNA genome contains six major genes encoding the structural proteins which include nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and the RNA-dependent RNA (large) polymerase (Miller et al., 2010; Rabalski et al., 2014).

Newcastle disease is a highly contagious poultry disease affecting all birds globally, regionally and locally. The endemicity of the virus has been attributed to several factors; weather, agro-ecological zoning, disposal of infected carcasses, vaccination processes, interspecies farming and restocking of birds from markets (Njagi et al., 2010; Otim et al., 2007). Prevalence of ND by sero-surveillance in parts of Kenya has

been reported to be higher in dry hot zones (17.8%) than in cool wet zones (9.9%) and cold seasons (Njagi et al., 2010). In Kenya, different local communities have adopted names for ND depending on the prevailing weather condition when a suspected outbreak is reported. During the sample collection, the following names were registered as the local name for ND, “muyekha” in the western region “Kidere” at the coastal lines, “Kihuruto” at the central area, “Amalda” in the south rift, “Chepkinuch” in the north rift and “Mavui” in eastern Kenya.

The World Organisation for Animal Health (WOAH) lists ND as one of the notifiable poultry diseases and recommends vaccination as a control measure (OIE, 2019). In Kenya, the vaccine used is a live attenuated viral vaccine prepared from the "F" strain, and the La Sota strain, 1-2 thermostable ND and B strain vaccine. However, there have been reported cases of vaccination failures among the bird population (Miller et al., 2013). This has been attributed to several factors including virus shedding in vaccinated challenged birds, environmental contamination after the previous outbreaks, improper administration, and vaccine neutralization due to passive immunity from maternal antibodies (Ahmed, 2015; Miller et al., 2013). Other species of birds are reared alongside chickens, such as ducks, which are known to harbour strains of APMV-1 and therefore can transmit the virus to non-infected birds (Alders, 1999) and should be vaccinated alongside ICs.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) has been used to detect the different genes of the virus. Detection of the M gene has been done by the use of RT-qPCR for clinical samples by the use of primers from the conserved matrix gene (Cattoli et al., 2009; Wise et al., 2004). This has been used to detect the lentogenic (low virulence), Mesogenic (moderate virulence) and velogenic (highly virulent) strains of *AOAV-1*(Al-Habeeb et al,. 2013; Dortmans et al., 2011; Kant et al., 1997). It is used as a screening test to detect the presence of *AOAV-1*. Detection of the fusion gene by use of RT-qPCR is done to determine the virulence of the disease (OIE, 2019).

1.2 Statement of the problem

Newcastle disease has a negative economic impact on poultry farming and food security in Kenya. The disease results in morbidity in chickens and eventually mortality of up to 100% when poultry is infected with the virulent strain (velogenic vNDV) of the virus (OIE, 2019; Andriamanivo et al., 2012). The occurrence and prevalence of Newcastle disease are attributed to climatic changes in zones in Kenya. Dry hot zones have shown a higher prevalence (17.8%) compared to the cool wet zone (9.9%) (Njagi et al., 2010). Indigenous chickens under different production systems are exposed to predisposing factors to Newcastle disease and mortality of the chicken. The type of production system also influences the ability to monitor signs of illness and vaccination history of the chicken (Owour et al., 2013). Infection with NDV can occur despite the poultry being vaccinated (Miller et al., 2013). Vaccine failures have been attributed to inadequate application (Dortmans et al., 2011). The use of vaccines different from the challenge genotype virus has resulted in viral shedding and subsequent infection of other birds with lowered immunity (Miller et al., 2013). The evolution of NDV genotypes occurs continuously at different geographical locations. The possibility of evolved genotypes may have an impact on the diagnosis of the disease due to failures and the inability to identify new strains (Miller et al., 2010). The inability to identify new strains will in turn have an impact on vaccines produced and its' ability to provide protection against the challenging virus.

1.3 Justification

There is a need to understand the disease dynamics of Newcastle disease and establish proper disease diagnosis and control tools based on an understanding of circulating strains. Detection of variants of Newcastle disease virus in poultry populations, in important poultry locations across Kenya and poultry value chain (live bird markets), provides an opportunity to understand the ND drivers. Determining the presence of the disease at varied geographical location gives information on the endemicity of the disease in Kenya (Miguel et al., 2013) and therefore lead to the development of appropriate mitigation measures to curb the spread of the disease in poultry farms across Kenya. In Africa as a whole, there is limited data on circulating genotypes (de Almeida et al., 2013; Miguel et al., 2013), as well as minimal information on the occurrence of the disease. In Kenya, minimum research has been done to indicate if

the virus circulating is maintained by wild birds (Swayne & King, 2003) or if the strains have evolved depending on location as was observed in Uganda (Otim et al., 2004). This information on the endemicity of the virus and circulating genotypes, can be achieved by molecular characterisation of the circulating strains and identifying new genetic strains contributing to the spread of viruses in Kenya

The genotype information will be useful in identifying proper laboratory diagnostic tools and can be used in the development of a heterologous vaccine to include the circulating genotype in Kenya. This is because the use of matched vaccines to field isolates has the potential to provide superior protection against transmission by reducing the magnitude of viral shedding (Miller et al., 2013).

Studies on the prevalence of NDV and the genetic diversity of the virus have been carried out by many scholars. In Kenya, studies have been done on the genetic diversity of NDV based on the fusion (F) and the L gene. This study will provide information on the genetic diversity of the matrix(M) gene and the fusion (F) gene from samples collected from ICs from the Nairobi live bird market, households in key indigenous chicken rearing areas and clinical samples. The study also provides information on the association of the Newcastle disease prevalence in ICs found in households with the type of production system, vaccination history, interaction with other bird species and the prevailing weather condition. The results of this study shall provide insight on appropriate management practices for IC, appropriate timing of vaccination and the development of vaccines genotypically similar to circulating strains. This will improve control of NDV, improve the resilience of IC to NDV and thereafter increase the productivity of IC and an improvement on the country's food security.

1.4 Research questions

1. What is the prevalence of Newcastle disease in indigenous chicken found in six counties in agroecological ecological zones III, IV, V and Nairobi markets?
2. What is the association between the prevalence of Newcastle disease and the production system, interspecies interaction, vaccination history and prevailing weather conditions?

3. What is the relationship between local circulating NDV strains and globally identified strains?

1.5 Objectives

1.5.1 General objective

To characterise Newcastle disease by determining the prevalence of Newcastle disease virus in indigenous chicken from Nairobi markets and households in six Kenya counties, determining the associated factors to ND prevalence and determining the genetic relationship among NDV strains circulating in Kenya and globally.

1.5.2 Specific objectives

1. To determine the prevalence of Newcastle disease in Nairobi markets and six counties lying in agroecological ecological zones III, IV, V.
2. To determine the associated factors (production system, interspecies interaction and prevailing weather condition) to the prevalence of Newcastle disease in ICs in the study counties.
3. To determine the genetic relationship between the circulating NDV strain and globally identified strains.

1.6 Scope of the study

The scope of the study is to collect samples from vaccinated and unvaccinated indigenous chickens from households, markets and clinical samples. The scope of analysis is to detect the Newcastle disease virus, analyse the association of prevalence of the Newcastle disease with the production system, vaccination history, interspecies interaction and prevailing weather and investigate the relationships between circulating strains of NDV.

CHAPTER TWO

LITERATURE REVIEW

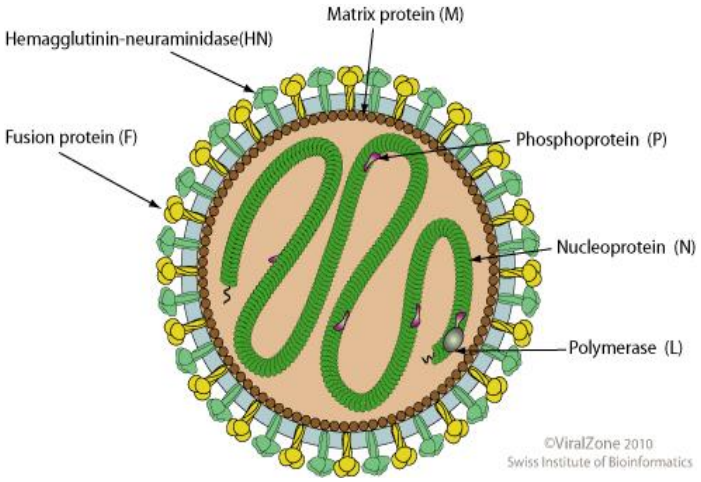
2.1 Newcastle disease virus

2.1.1 Classification of the Newcastle disease virus

Newcastle disease virus (NDV), also known as *Avian orthoavulavirus 1* (AOV-1) (Previously known as avian paramyxovirus type 1 (APMV-1)). The virus is classified in the species *Avian orthoavulavirus 1*; genus *Orthoavulavirus*; sub-family *Avulavirinae;* family *Paramyxoviridae*, order *Mononegavirales;* class *Monjiviricetes;* Subphylum *Haploviricotina; Phylum Negarnaviricota;* kingdom *Orthornavirae;* realm *Riboviria.* (ICTV, 2019; King et al., 2018; OIE, 2019). NDV strains are classified into three pathotypes; velogenic, mesogenic and lentogenic (Alexander et al., 2001). Velogenic strains are virulent strains and are further classified as viscerotropic velogenic associated with haemorrhagic intestinal lesions and neurotropic velogenic associated with respiratory and neurologic signs. Mesogenic strains are moderately pathogenic and lentogenic strains are lowly pathogenic (Alexander et al., 2001).

2.1.2 Structure of Newcastle disease virus

The NDV virions are pleomorphic in shape, and consist of single-stranded, non-segmented, negative-sense RNA genomes (Miller et al., 2010). The virus has three different genome lengths of 15,186, 15,192 or 15,198 and six genes producing six structural proteins (**Figure 2.1**). The structural proteins are nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and RNA-dependent RNA (large) polymerase (L) (Miller et al., 2010; Rabalski et al., 2014).



Source: Viral zone 2010

Figure 2.1: Structure of Newcastle disease virus

The matrix (M) protein is associated with the inner surface of the membrane. Three proteins, nucleocapsid (NP), phosphoprotein (P), and large (L) protein, constitute the viral transcriptase complex (Molouki et al., 2011). Fusion(F) and hemagglutinin-neuraminidase (HN) are the NDV surface protein. HN is involved in virus attachment and release whereas F, a class I transmembrane protein, mediates the fusion of the viral envelope with cellular membranes (Heiden et al., 2014) and is the determinant of virulence of NDV.

2.2 Infection by Newcastle disease virus

During fusion of the virus to the host cellular membrane, the F structural conformation converts into a large irreversible conformational change which promotes the merger of the viral and cellular bilayers opening a pore to deliver the viral genome into the cytoplasm (Swanson et al., 2010; Swanson et al., 2011). The F gene is synthesized as precursor protein F0 and further cleaved into F1 and F2 by cellular protease. This cleavage occurs at a specific amino acid motif, polybasic for Mesogenic and velogenic viruses and is necessary for infectivity of the virus (Paldurai et al., 2014). The polybasic amino acid motif 112(K/R)-R-(Q/K)-(R/K)-R116 at the carboxy terminus of F2 and phenylalanine at the amino terminus of the F1 subunit, position aa 117 are substrates for ubiquitously existing furin-like proteases detected in a wide range of cells and tissues, resulting in systemic infections (Heiden et al., 2014). The F protein of lentogenic viruses is characterized by leucine at position 117 and a monobasic aa

motif at the carboxy terminus of F2 112(G/E)-(K/R)-Q-(G/E) R116 resulting in a virus digested only by trypsin-like enzymes restricted to the respiratory and intestinal tract which limits virus replication (Heiden et al. 2014).

2.3 Genetic diversity of Newcastle disease virus

The NDV strains are classified into two classes I and II viruses according to the genome size of the F and L genes (de Almeida et al., 2013). Class I virus has nine genotypes (1-9) and Class II viruses have ten genotypes (I-X) identified (Miller et al., 2010; Kapczynski et al., 2013). Class I viruses are typically isolated from wild birds and all reported strains are of low virulence except for one strain, chicken/Ireland/1990 (Dimitrov et al., 2016; Kapczynski et al., 2013). Class II viruses are responsible for fatal infections in poultry and they have been responsible for major outbreaks worldwide (Dimitrov et al., 2016; Miller et al., 2010). Isolates of class II, genotype X are of low virulence and most often found in wild birds, but some have been isolated from some poultry species (Diel, et al., 2012). Genotype I contained mostly avirulent strains whereas genotypes II, III, and IV were involved in the first panzootic started in 1920 and vanished around 1950 (de Almeida et al., 2013). Genotypes V, VIa, and VIII were responsible for the second panzootic between the 1960s and the 1970s. Sub-genotypes VIb, VIc, and VId caused the third panzootic emerging from pigeons during the 1980s, and sub-genotypes VIIa, VIIb, VIIc and, VIc appeared in the 1980s and the 1990s in the Far East, Europe, and South Africa (de Almeida et al., 2013). Genotype VII has been the predominant genotype circulating throughout the world, particularly in Asia (Roohani et al., 2015) and Africa, and it was recently reported in South America. South African, European, American, and Asian isolates were previously typed as genotypes VIb, VIIb, and VIII (de Almeida et al., 2013; Otim et al., 2004). In West Africa, new sub-genotypes VIIf, VIIg, and VIIh were described (de Almeida et al., 2013). In Mali, a new sub-genotype, VIIi from phylogenetic analyses of partial F coding sequence of NDV isolates from apparently healthy poultry, was proposed. In Madagascar, other original strains were also isolated, sequenced, and ascribed to a new genotype, genotype XI (de Almeida et al., 2013). Isolates of genotype VII and XIII were isolated from Tanzania and genotype XVIII from Ghana (da Silva et al., 2020). In Uganda, strains from an undetermined genotype but close to genotype VI were also isolated (Otim et al., 2004). Isolates of genotype VII and XIII have been isolated from

Tanzania and Genotype XVIII from Ghana. Isolates of genotype V have also been isolated from Uganda, Tanzania and Kenya (Byarugaba et al., 2014; da Silva et al., 2020; Kariithi et al., 2021; Ogali et al., 2018).

2.4 Newcastle disease virus lineages

Newcastle disease viruses have been subsequently re-classified broadly into six lineages (1–6), which correspond to the existing groups (I–IX), and these lineages are divided into 13 sub-lineages. Lineages are grouped geographically while others circulate worldwide (Aldous et al., 2003). Avirulent viruses are grouped in lineages 1 and 6 and virulent viruses are grouped in lineages 3, 4 and 5; lineage 2 contains both virulent and avirulent viruses. The Isolates obtained from South Africa and the Middle East region group together in lineage 5 (genotype VII) and those from the Far East and Western Europe group in lineage 3d (genotype VIII) and 5b (VIIb) (Tan et al., 2008) (Roohani et al., 2015). The distinct cluster of lineages and genotypes cover distinct clusters but are based on the same genomic information (de Almeida et al., 2013).

2.5 Newcastle disease

This is a very important disease of poultry caused by NDV. It is reported to cause mortality in birds of up to 90 per cent. The disease is listed in the World Organisation for Animal Health (WOAH) Terrestrial Animal Health Code and must be reported to the WOAH (OIE Terrestrial Animal Health Code) (OIE, 2019). Clinical manifestation of ND in chickens varies significantly among isolates. The systems affected include; the digestive, respiratory, reproductive, blood circulatory system and nervous systems. Infection with virulent viruses causes three well-defined clinical forms: Viscerotropic velogenic, which is characterized by acute lethal infections, usually with haemorrhagic lesions in the intestines of dead birds; Neurotropic velogenic, which is characterized by high mortality following respiratory and neurological disease, but where gut lesions are usually absent; Mesogenic, which causes respiratory and neurological signs, but low mortality (Miller et al., 2010) and lentogenic viruses which result in low mortality and loss of egg production and are mainly used in the production of NDV vaccines such as La Sota, B1 and F vaccines (Ayala et al., 2016). No clinical sign can be regarded as ND specific because the signs are dependent on the species of bird, the age

of the host, the immune status of the host and environmental conditions. (Ayala et al., 2016). No clinical sign can be regarded as ND specific because the signs are dependent on the species of bird, the age of the host, the immune status of the host and environmental conditions. Chicken infected with virulent ND virus strains may die without showing any signs of illness. The most observable symptoms of NDV in chickens include lethargy, inappetence, respiratory signs such as mild rales and snick accompanied by severe respiratory distress and gasping, swelling of the head and neck; greenish diarrhoea; marked decrease in egg production (OIE, 2019). Sometimes deformed eggs may be produced; Nervous signs of tremor, torticollis, convulsions and paralysis of wings and legs will not be seen until the disease is advanced. Torticollis is generally seen in chickens only when ND is at an advanced stage (Alders, 1999).

2.6 Transmission of Newcastle disease

Newcastle disease can be transmitted by inhalation or ingestion, as birds shed these viruses in both faeces and respiratory secretions. Domestic birds are thought to excrete NDV for 1-2 weeks. NDV may persist in undispersed chicken faeces for more than six months or 8 weeks in hot dry tropical areas at 40^0C but under village conditions, the virus is unlikely to survive outside a host for more than one month (Awan et al., 1994). NDV is present in all parts of the carcass and can persist for some time at cold temperatures. The virus can survive on chicken skin for up to 160days at a temperature of 1-2ºC, this in bone marrow for nearly 200 days (Francis, 2016). Wild birds have been considered reservoir hosts such as the waterfowl, pigeons and parrots when in contact with domestic birds (Ayala et al.,2020). Other non-avian host animals such as dogs and rats act as transient reservoir hosts when they consume infected birds and excrete the virus within 72hrs in their faeces to the environment (Awan et al., 1994). The virus infection has an incubation period, of 4 to 5 days (range 2 to 15 days) but varies with the strain of virus (Miller *et al*., 2010). The incubation period for NDV infections in poultry ranges from 2 to 15 days and is commonly 2-6 days in chickens infected with velogenic isolates (Alders, 1999).

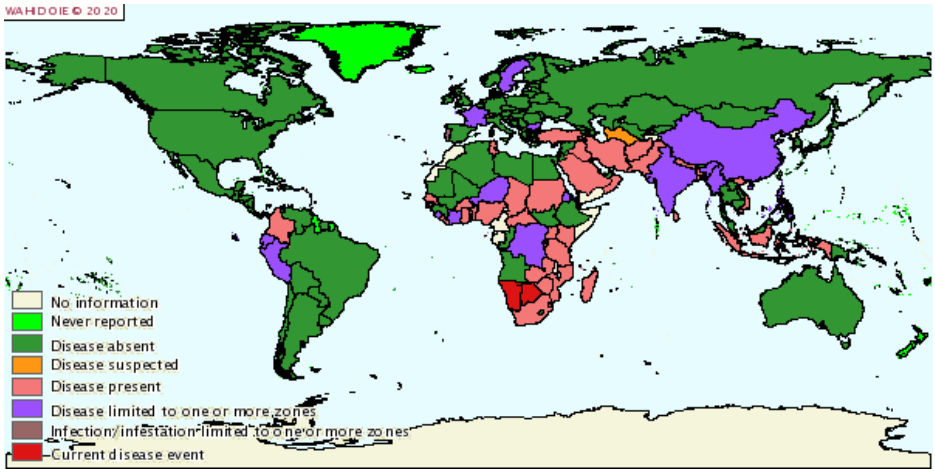
2.7 Newcastle disease host range

The species susceptible to the ND infection are chicken, turkey, pigeons, ducks, geese, peafowl, guinea fowl, pheasants, quails, canaries, psittacine and ratites (Health, 2014; Seal et al., 1998). Domestic Chicken (*Gallus gallus domesticus*) are more susceptible to the velogenic strain with a 100% mortality and morbidity rate (Alders, 1999). Turkeys develop less severe signs than chickens, and the susceptibility of other gallinaceous game birds (pheasants, partridges, peacocks, quail and guinea fowl) is variable. Infections are usually inapparent in ducks and geese; however, some isolates have caused outbreaks among geese in China since the 1990s. Normally ducks are resistant to the disease but on occasions, ducklings may be affected (Tan *et al.*, 2008).

2.8 Global and regional distribution of Newcastle disease virus

The information on Newcastle disease occurrence from specific countries in the world is submitted annually to the WOAH (**Figure 2.2**), indicating NDV is distributed globally. Velogenic NDV viruses are endemic among poultry in parts of Asia, Africa and the Middle East, and some countries in Central and South America. The virus has been recovered from both wild and domestic birds. Non-velogenic strains have been isolated from both domestic and wild birds sampled in North America and Eurasia (Dimitrov et al., 2016). Newcastle disease (exotic NDV) is not present in domestic birds in North America and USA, but it has been isolated in wild birds (Brown & Bevins, 2017). The disease is also not present in Europe and the European food safety authority has in place containments to prevent and eradicate the spread (Nielsen et al., 2021)

Newcastle disease is an important disease-causing huge economic loss in the case of the ND outbreak. In Bangladesh (Khatun et al., 2018), it was estimated that the country incurred losses equivalent to US$ 288.49 per annum. In Nigeria (Sadiq & Mohammed, 2017), the estimated losses due to ND were cumulatively equivalent to approximately US$ 30,000. In Kenya, an outbreak of ND has been attributed to economic losses (Ipara et al., 2021).



Source: World Animal Health Information Database (WAHID Interface

Figure 2.2: Global prevalence of Newcastle disease in 2020

2.9 Prevalence of Newcastle disease in Kenya

Newcastle disease affects chickens, other domestic poultry and wild birds. The disease causes nonlife-threatening conjunctivitis developing within 24hrs of NDV exposure. However, there is no evidence of human-to-human transmission (OIE, 2019). In Kenya, studies have indicated Newcastle disease prevalence of 39.5% in households (Kariithi et al., 2021) and 11% in live bird markets (Ogali et al, 2018). A study on the seroprevalence of the Newcastle disease in Kenya indicated the prevalence of NDV was higher (17.8%) in the Mbeere district (Lower midland 5) as compared to 9.9 % in Embu district (Lower highland 1 (LH1. The lower midlands are the dry hot zones and the lower highlands are the cool wet zones. (Njagi et al., 2010). Generally, indigenous poultry has been shown to play a role in the endemicity of the virus in Kenya as they act as reservoirs for the virulent strains (Lichoti, 2000).

2.10 Laboratory diagnosis of Newcastle disease

Laboratory diagnosis of ND is done by isolating NDV from live or recently dead birds. An oropharyngeal, choanal cleft and cloacal swabs are usually taken from live birds. The cloacal swab should be covered with faeces (USDA, 2016). Tracheal or oropharyngeal swabs are generally the preferred samples, as false-negative results are less likely (Alexander et al., 2001). The recommended swabs to be used are rayon and polyester swabs (USDA, 2016). Commonly collected tissues at necropsy include the

spleen, lung, intestines (particularly the cecal tonsil), intestinal contents, long bone, liver, kidneys, heart and brain. The WOAH also recommends collecting oronasal swabs from the carcass (OIE, 2019). Viral isolation is done by inoculation of the virus in the allantoic membrane of nine-day-old embryonated eggs from specific pathogen-free (SPF) chicken or specific antibody-negative (SAN) chicken to multiply the viral particles. Serological tests that detect NDV include haemagglutination agglutination (HA), haemagglutination inhibition (HI) and Enzyme-linked immunosorbent assay (ELISA). HI is used for detecting antibodies against Newcastle disease virus and ELISA to assess post-vaccination antibody titres. The serological tests are used in diagnostic laboratories but are not of value in surveillance and diagnosis because of the varied vaccines used in the field of poultry (OIE, 2019). Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) and conventional reverse transcription-polymerase chain reaction (RT-PCR) are the molecular tests used to determine the circulating strains (lentogenic, mesogenic and velogenic) of the NDV by analysis of matrix gene of the virus and the virulence of the strain by analysis of the fusion gene of the NDV using specific primers. The test is sensitive and results are obtained within 3hours. The test has a high potential for contamination of samples and contamination of the laboratory. Gene sequencing and phylogenetic studies are used to map the disease evolutionary relationships (OIE, 2019).

2.11 Challenges in laboratory diagnosis of Newcastle disease

The challenges of diagnosis of ND in Africa are mainly due to minimum research as well as limited data on the circulating genotypes (de Almeida et al., 2013). Globally, there is an emergence of new virulent genotypes as well as changes in the genomic sequence of NDV resulting in genetic diversity of the virus (Miller et al., 2010). Laboratory diagnosis of ND in Kenya is by use of conventional PCR and RT-qPCR involves the use of primers specific for genotypes identified in other regions outside Kenya. This increases the possibilities of diagnostic failures and results in unidentified infections (Miller et al., 2010) within Kenya and possibly the East Africa trade block.

2.12 Prevention of Newcastle disease

Newcastle disease has no treatment and is only prevented by the use of a vaccine (OIE, 2019) The vaccines are derived from the virus strain with low virulence (Lentogenic virus) (**Table 2.1**). The use of lentogenic strain is based on an immunological concept that NDV strains are antigenically similar and can offer cross-protection against a challenge (Miller et al., 2010). Virus shedding occurs after vaccination by vaccines formulated with antigens heterologous to the challenge virus. To reduce shedding, the use of vaccines formulated with antigen homologous to the challenge virus has been used (Miller et al., 2010).

Table 2.1: Eight strains of Newcastle disease virus are used in live vaccines

(Grimes 2002)

Strain	Description
F	Lentogenic. Usually used in young chickens but suitable for use as a vaccine in chickens of all ages.
B1	Lentogenic. Slightly more virulent than F, used as a vaccine in chickens of all ages.
La Sota	Lentogenic. Often causes post-vaccination respiratory signs, used as a booster vaccine in flocks vaccinated with F or B1.
V4	Avirulent. Used in chickens of all ages.
V4-HR	Avirulent. Heat Resistant V4, thermostable, used in chickens of all ages.
I-2	Avirulent. Thermostable, used in chickens of all ages.
Mukteswar	Mesogenic. An invasive strain, used as a booster vaccine. Can cause adverse reactions (respiratory distress, loss of weight or drop in egg production and even death) if used in partially immune chickens. Usually administered by injection.
Komarov	Mesogenic. Less pathogenic than Mukteswar, used as a booster vaccine. Usually administered by injection.

2.13 Management of Newcastle disease

Management of the ND is by practising biosecurity measures and containment, especially during NDV outbreaks. The containment procedures include burning of dead carcasses, and cleaning and sanitation of the house by use of disinfectants (Ayala

et al., 2020). Sodium hypochlorite, glutaraldehyde, phenolic disinfectants and oxidizing agents are effective disinfectants against NDV(Owour et al., 2013).

2.14 Factors associated with the prevalence of Newcastle disease

2.14.1 Type of poultry production systems

The type of production system farmers choose for their ICs is determined by the economic situation of households (Ipara et al., 2021). However, the structure of the poultry production systems determines the effectiveness of the control of ND in terms of vaccination application and monitoring of disease signs and symptoms (Kemboi, 2014; Otiang et al., 2021; Owour et al., 2013). Indigenous chicken in Kenya is reared either under the semi-free range or the free-range production systems (Okello et al., 2010). In the semi-free range production system, housing is available for the IC at night, their feed supplemented and allowed to forage in a controlled space. In the free-range system, the chicken scavenges for food and water may be housed at night but are mostly left to perch outside (Apopo et al., 2019). The free-range system is preferred by small-scale farmers because it is cheap and not labour intensive (Campbell et al., 2018; Owour et al., 2013). An effective disease control practice in poultry should factor in the type of poultry production system (Marangon & Busani, 2007)

2.14.2 Indigenous chicken vaccination history

Vaccination of chicken against the Newcastle disease virus is one of the ways to control the Newcastle disease (OIE, 2019). However, unlike the commercial breed of chicken, the indigenous chickens are not routinely vaccinated against poultry disease because of the expected returns of investments in commercial breeds (Okello et al., 2010) and the perception that indigenous chickens are hardy and not susceptible to disease (Nyaiyo & Maangi, 2016). Vaccination ensures ND is controlled and the flock size for indigenous chickens increases (Campbell et al., 2018). Most IC farmers, purchase their breeding stock from the markets and the poultry value chain involves the sale of vaccinated spent layers in live bird markets (Okello et al., 2010). In the market setting, indigenous chicken is kept in the same environment with spent layers that are routinely vaccinated against NDV, increasing the possibility of viral shedding

and transmission from the vaccinated flock to the unvaccinated. In such cases, the purchased exposed indigenous chicken may transmit the acquired virus by virus shedding when introduced to a new flock (Miller et al., 2013). This could lead to disease among vaccinated IC due to the persistency of the virus in the exposed birds (Rehmani et al., 2015). Knowledge of the vaccination history of the chicken in the vicinity is equally important because it allows for uniform vaccination of IC in households, improved vaccine efficacy level and control of ND (Otiang et al., 2021).

2.14.3 Integration of indigenous chicken with other bird species

Integration of ICs with other poultry species is a common practice in households. Most common domestic poultry reared alongside ICs include ducks, turkeys and geese (Kariithi et al., 2021). Wild birds such as psittacine (Parrots) and Columbidae (Pigeons and doves) are also reared alongside ICs (Brown & Bevins, 2017). The wild birds such as grain-eating sparrows, weaver birds and robins integrate with the chicken, and they share food sources and water points (Ogali et al., 2018). Other poultry species and wild birds (feral and migratory) are susceptible to NDV (Otim et al., 2006) and they play a role in the maintenance of the disease in the environment and interspecies transmission (Ayala et al., 2020; Rahman et al., 2018; Snoeck et al., 2013). Passive vaccination of other poultry and wild birds takes place where vaccination of IC is done through drinking water and may result in the dispersion of the virus by shedding to different locations, and to different species, particularly migratory birds (Ayala et al., 2020)

2.14.4 Influence of prevailing weather conditions

The occurrence, distribution and prevalence of the disease are affected by weather change which in turn can influence the farming practices and the natural environment (Gale et al., 2009). NDV transmission could be influenced by sudden weather changes, therefore the intensity of outbreaks of disease correlates with alternations of different weather patterns (Apopo et al., 2019). Stress due to harsh weather conditions increases the vulnerability of the ICs to infection and disease transmissibility of ND (Balachandran et al., 2014; Miguel et al., 2013). Temperature, relative humidity and rainfall have shown correlation with the outbreak of Newcastle disease (Nyaiyo & Maangi, 2016). Directional wind flow is known to disperse NDV viral particles

transmitting the virus to ICs and therefore, the duration of strong winds and dry weather is considered an ideal condition for active vaccination of poultry against ND (Dimitrov et al., 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study description

This study was done to determine the prevalence of NDV in the poultry value chain that is markets and households. The inclusion of clinical samples in the study was to identify the strain in vaccinated ICs and the genetic relationship with global strains.

The live bird markets (LBM) sampling site was in Nairobi County. Sample collection was carried out in Nairobi and data on sources of indigenous poultry in Kenya was obtained. The LBM markets in Nairobi County were Burma market (Burma stadium, and Burma Maziwa), Kangemi, Kariokor, Kariobangi North, Kawangware, Kasarani and Mutindwa located in different sub-counties. Nairobi live bird markets were selected to identify the potential source of Indigenous chicken in the country and the possibility of NDV transmission in the poultry value chain. The selection of Nairobi live bird markets is because Nairobi county is the capital county of Kenya with the highest number of poultry traders (McCarron et al., 2015).

Field sample collection was carried out from households located within Kakamega, Bomet Nakuru, and Baringo counties within Agro-ecological zone (AEZ) III and counties Machakos, Kilifi within Agro-ecological zone (AEZ) IV, and Baringo within (AEZ) V. The sampled sub-counties included Bomet east, Chepalungu and Sotik in Bomet county, Lurambi, Shinyalu, Ikolomani, Matungu and Lumakanda in Kakamega county, Tenges, Sacho, Riwo, Karbatonjo, Eldama Ravine, Mogotio, Bogoria and Lunoi in Baringo county, Subukia, Molo, Sachangwan and Sirikwa in Nakuru county, Mwala, Kathiani, Kangundo, Yatta and Machakos central in Machakos county and Kilifi North in Kilifi County. Sample collection in the household was carried out to assess the living conditions of the chicken and relate to the presence of the ND.

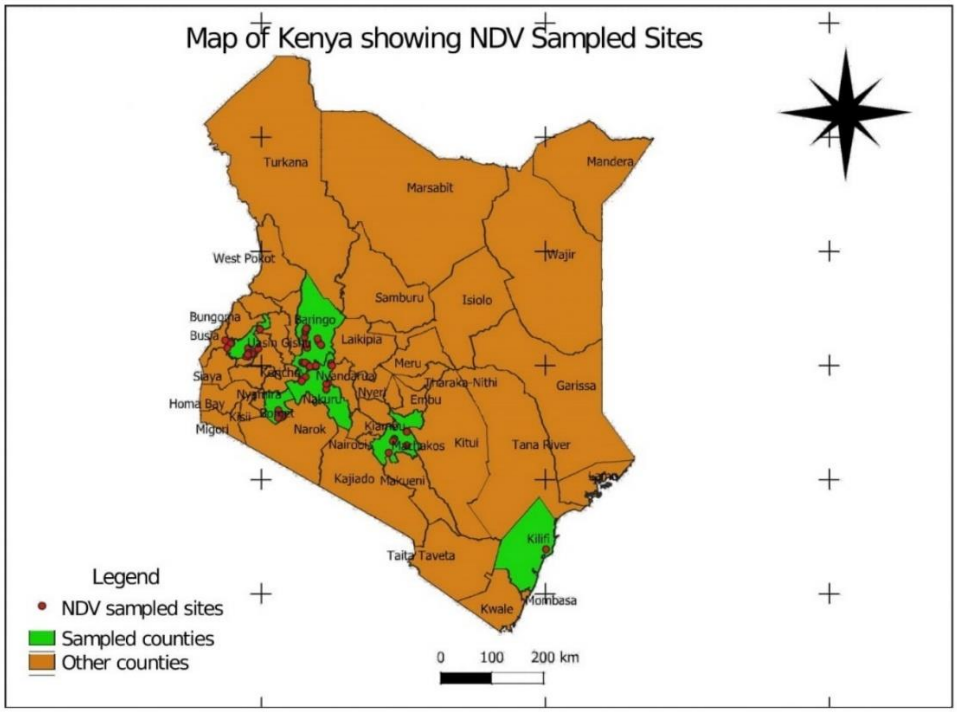
The sample source for the clinical samples was the Central Veterinary Laboratory (CVL), a government veterinary diagnostic/research lab located in Nairobi County, Westlands sub-county within the Directorate of Veterinary Services headquarters. CVL is the referral laboratory for veterinary disease and therefore receives animal samples from all over the country.

3.1.1 Description of study sites

The live bird markets are located in the Nairobi sub-counties of Embakasi west, Westlands, Kasarani, Starehe, Dagorretti North, and Makadara.

Household samples were collected in counties clustered in three agroecological zones (AEZ), III, IV and V. The areas were Kakamega, Bomet Nakuru, and Kilifi within AEZ III, Machakos within AEZ IV and Baringo within AEZ V. AEZ III-are areas with elevations between 900-1800 m with annual rainfall between 950 and 1500 mm. This zone is the most significant for agricultural cultivation and several legume fodders are found here in crop-livestock systems. It is also the most populated. It occurs in the vast parts of Western (Kakamega) and a good proportion of Central Rift Valley (Nakuru, Bomet) and a small strip in the Coast province that is Malindi, Kilifi and Taita Taveta. AEZ IV are areas with an elevation of 900-1800mm with a lower rainfall of about 500-900mm. These are Naivasha, parts of Laikipia and Machakos county. AEZ V includes areas at lower elevations with annual rainfall of 300-600. This includes parts of northern Baringo, Tana River and lower Makueni (Otolo & Wakhungu, 2013). The coordinate's readings were collected using a GPS reader and the study site map (**Figure 3.1**) was developed using QGIS-3®.

Sample preparation and analysis were done at Kenya Agricultural and livestock research organisation (KALRO) Biotechnology Laboratory and the Central Veterinary Laboratory (CVL), molecular and virology section. KALRO Biotechnology Laboratory is a government animal research laboratory located in Nairobi city along Waiyaki way, Kenya. Primer design and sequencing were done at contracted laboratories (Macrogen, Seoul, Korea and Inqaba™, Pretoria, South Africa).



Developed using QGIS-3®

Figure 3.1: Sample collection sites for NDV in Kenya.

3.2 Research study design

The cross-sectional study design was used to assess the association of the NDV in indigenous chickens to factors such as vaccination, production system, interaction with other species and prevailing weather conditions and the genetic diversity of the isolates. Data for the study were collected on different dates in January 2017, May 2017, June 2017, September 2017, March 2018 and 2019.

The research design has been used previously to describe the relationship between poultry management practices and the presence of NDV by observation of multiple variables in poultry farms and LBM and the genetic analysis of the isolates (Ogali et al., 2018).

3.3 Study approval

The study approval was issued by the Kenya Agricultural and Livestock Research Organisation (KALRO) (Appendix I). The poultry handling procedures used in the study were done under the KALRO institutional animal care and use committee (IACUC) recommendations. The respective country governments, through the county veterinary officer, granted permission to collect samples at the markets and interact with the farmers. The farmers and traders gave verbal consent to collect samples from their flock and their information and responses were confidential.

3.4 Sample size determination

The overall sample size was estimated, and then prorated based on the population of birds in the different regions (KNBS, 2010). However, a slightly higher proportion was allocated to Baringo as compared to proportion of IC in Baringo during the KPHC 2009. To obtain the minimum sample size, prevalence of NDV was estimated as 11% in poultry found in backyard poultry, based on the results obtained by (Ogali et al., 2018), assuming a small reduction in the prevalence. Sample size was then calculated based on the formula (Charan & Biswas, 2013).

$$n = \frac{Z^2 \text{ x } (\text{ p}(1 - \text{p})}{d^2}$$

Where, n= sample size, Z= z statistics for a level of confidence, p is the expected proportion of the population and d =margin of error. Taking Z score at 95% confidence interval (CI) = 1.96, and d (margin of error) = 0.05, then minimum sample size per zone is 151. For each bird, two samples were to be taken, (oropharyngeal swabs and cloacal swabs). This is what now gives the total for the regions as 1210 samples. This number was then prorated to the regions as follows: Baringo 350 (29%), Bomet 160 (13%), Kakamega 338 (27%), Kilifi 70 (6%), Machakos 140 (12%) and Nakuru 152 (13%).

For the live birds' markets in Nairobi, a total of 372 IC's was sampled from 32 poultry traders. Each market had an allocated number of poultry traders sampled. The sample size for the market was determined by a random selection of 10% of the total number of birds in every cage per trader and dependent on the county source of IC. These samples were thus obtained from a previous study looking at determining the potential

sources of ICs in the Nairobi live bird's market and surveillance of poultry diseases in Nairobi markets (Kariithi et al., 2021, 2020). Finally, the clinical sample was limited to the number of ICs vaccinated and clinically sick submitted to the CVL.

3.5 Sampling criteria

The stratified random sampling technique was used to ensure the likelihood of the population of IC in markets and households being selected for the study. The characteristics of Kenya's indigenous chicken described by (Akondo, 2012) were used to identify indigenous chickens in markets and households from other chicken breeds.

A total of 1585 samples were collected from the chickens in this study. Samples collected from Nairobi markets IC were n=372 (oropharyngeal swabs (n=186) and cloacal swabs (n=186). Samples collected from household IC were 1,210 (oropharyngeal swabs (605) and cloacal swabs (605)). Clinical samples collected from IC submitted to the CVL were long bone (n= 1) and intestinal contents (n=2).

The selection of traders in Nairobi live bird markets was based on the type of chicken flock in their stock. Traders with indigenous chicken were selected for the study. The inclusion of other breeds in the flock did not exempt the traders from sampling, however, samples were only collected from indigenous chickens. Traders gave information on the county source of the chicken. The chickens were randomly sampled based on the county source of chicken from each trader.

The household sampling sites within the sub-counties were selected under the guidance of the regional veterinary government officers. The distance from one household to the next was over 1 km. This was to ensure that indigenous chickens in a given region were from unrelated households and were not interacting. The chicken was baited using available feed and captured randomly. The sampled birds were marked on the beak to avoid repeated sample collection on the same chicken. An average of ten chickens were sampled in every homestead. The homestead had other species of poultry such as duck, turkey and geese, wild birds such as guinea fowl, parrots, pigeons and doves as well as evidence of small birds such as weaver birds and robin birds interacting with the indigenous chicken. The sample collection was done in the homesteads regardless of the type of production system and vaccination history. Vaccination in this context means that the farmer, through their intervention or that of

an animal service provider, had administered the NDV vaccine within three months, inclusive of the sample collection date. Vaccination of ICs was performed less than three months before sampling. La Sota was the most administered vaccine, mainly through drinking water (oral route).

Clinical samples were collected from IC with presenting NDV clinical symptoms submitted to the Central Veterinary Laboratory (CVL) by IC farmers who had vaccinated their flock against NDV in 2019.

The samples selected for sequencing were selected as representative of samples for markets, households and clinical samples.

3.6 Sample collection

3.6.1 Sample collection from indigenous chicken in live bird market (LBM)

The data was collected using the market sampling questionnaire (**Appendix II**). The data collected included the name of the market, trader details, sample type, age of the IC, number of IC in stock, source of the ICs, (county of origin, duration of stay at the market), the health status of birds (if sick, number of sick birds).

The samples were collected from 32 traders selling ICs (**Table 3.1**). The ICs were randomly selected from the cages based on their source of purchase. The IC was physically checked for any signs suggestive of ND infection such as nasal and ocular discharge, diarrhoea, respiratory distress or nervous signs. Disposable plastic-Shafter flocked sterile swabs (polyester or rayon) (Puritan Medical, Guilford, ME), were used to collect oropharyngeal swabs (OP), choanal and cloacal swabs (CL) (Killian, 2012). The samples were collected in duplicates. The oropharyngeal and choanal swabs were collected by gently swabbing the oropharyngeal area and oral cleft (**Figures 3.3 and 3.4).** The cloacal swabs were collected by inserting the sterile swab into the cloaca and swabbing in circular motions applying gentle pressure on the mucosal surface (Whitworth et al., 2007). The swabs were immediately placed into appropriately labelled cryovials containing 1ml of viral transport media (brain-heart-infusion broth; Difco, NZ) supplemented with antibiotics and antifungals according to standard procedures. Samples were then immediately placed in a cool box and later placed in liquid nitrogen and transported to the Kenya Agricultural and Livestock Research

Organisation (KALRO) Biotechnology laboratories where they were stored at -80^{o}C awaiting processing(OIE, 2019).

Table 3.1: Number of samples collected from indigenous chicken from the traders in Nairobi live bird markets

Market	Market location (Subcounty)	Number of Poultry traders per market	Number of samples collected from indigenous chicken (%)
Burma Maziwa	Makadara	10	72(19.4%)
Burma Stadium	Makadara	5	34(9.14%)
Kangemi	Westlands	6	70 (19%)
Kariakor	Starehe	3	24(7%)
Kariobangi North	Embakasi West	3	48(13%)
Kasarani	Kasarani	2	54(15%)
Kawangware	Dagoretti North	2	50(13.4%)
Mutindwa	Embakasi West	1	20 (5.4%)
Total		**32**	**372**

3.6.2 Sample collection from indigenous chicken in households

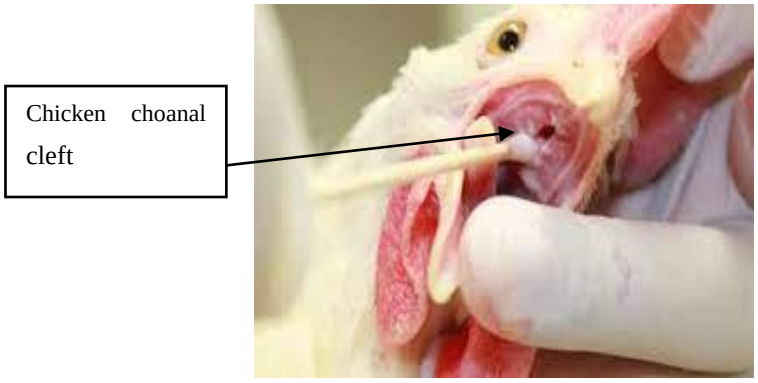
The sample collection in households was done in villages/wards in different counties (**Table 3.2**). The production systems in homesteads were either the free-range chicken or semi-free-range production systems. Samples were collected from farms where the IC had either interacted with other mixed species of poultry or not. Data collection was done using the Central Veterinary Laboratory (CVL) form (**Appendix III**). The data collected from the household included the farmers' details, farm details (location and county including GPS readings), sample type, animal history (age, clinical signs, source of bird, vaccination history, and treatment), flock history (number of animals in flock, number affected by disease, number dead due to disease and the duration of outbreak). Samples were collected from live domestic indigenous chickens of both sexes and all ages from 3 weeks old. The samples were collected from both healthy

birds and birds with signs suggestive of ND infection. The samples were collected from both farms that had not vaccinated their IC and those that had vaccinated theirs against NDV (**Figure 3.2**)**.** Disposable plastic-Shafter flocked sterile swabs (polyester or rayon) (Puritan Medical, Guilford, ME), were used to collect oropharyngeal swabs (OP), and cloacal swabs (CL) (Killian, 2012). The samples were collected in duplicates. The oropharyngeal swab was collected by gently swabbing the choanal cleft and oropharyngeal area (**Figure 3.3**). The cloacal swab was collected by inserting the sterile swab into the cloaca (**Figure 3.4**) and swabbing in circular motions applying gentle pressure on the mucosal surface (Whitworth et al., 2007). The swabs were immediately placed into appropriately labelled cryovials containing 1ml of viral transport media (brain-heart-infusion broth; Difco, NZ) supplemented with antibiotics and antifungals according to standard procedures. Samples were immediately placed in a cool box and on placed in liquid nitrogen and transported to the KALRO Biotechnology laboratories where they were stored at -80^{o}C awaiting processing (Stear, 2005).

Table 3.2: Number of samples collected from indigenous chicken in different households in the counties

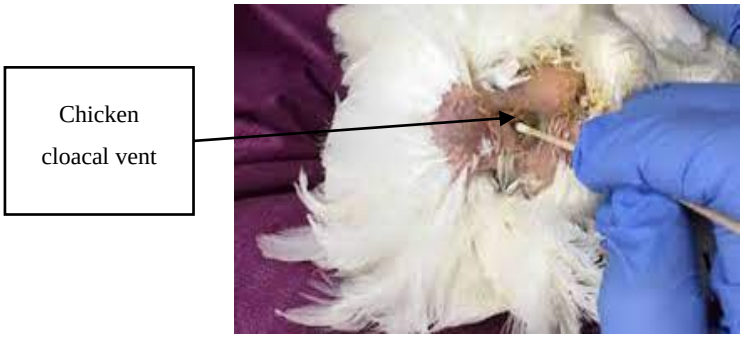
County	**Number of indigenous chicken farmers**	**Number of samples collected from indigenous chicken (%)**
Baringo	21	350 (29%)
Bomet	5	160 (13.2%)
Kakamega	20	338 (28%)
Kilifi	1	70 (6%)
Machakos	10	140 (12%)
Nakuru	11	152 (13%)
Total	**68**	**1210**

Figure 3.2: Collection of samples from ICs in homesteads



Picture courtesy (Hy-Line, 2016)

Figure 3.3: Oropharyngeal swab sample collected from the choanal cleft



Picture courtesy (Hy-Line, 2016)

Figure 3.4: Cloacal swab sample collected from the cloacal vent

3.6.3 Clinical samples from a suspected case of Newcastle disease in indigenous chicken

The clinical samples in this study were collected at necropsy by a veterinary pathologist at the Central Veterinary Laboratory in 2019. The samples were from vaccinated ICs. The samples collected are shown in **Table 3.3.**

Table 3.3: Clinical samples submitted to the Central veterinary laboratories in 2019

Sample Identification	Sample submitted for necropsy	Sample submitted for laboratory diagnosis
S-95	IC carcass	Internal organs (liver, intestine, caecal tonsils, kidney and heart tissues)
S-602	IC carcass	Internal organs (liver, intestine, caecal tonsils, kidney and heart tissues)
S-378	IC carcass	Long bone

3.7 Sample preparation and analysis

3.7.1 Sample preparation

Samples from live birds were pooled with consideration of the sampling route and same premises. The pooling of oropharyngeal (OP) swab from samples collected from ICs in the same premise (cage for markets) is because the NDV is transmitted through the respiratory route and the pooling of samples from the cloaca(CL) is because NDV is transmitted through the enteric route (Killian, 2012). Samples for the markets were pooled depending on the trader, source and cage of the birds.

Samples from the same region, same household, and type (oropharyngeal (OP) or cloaca (CL) were pooled in labelled cryovials. The samples were given new identification traceable to the original samples, the farmer and trader. Pooling was performed by centrifuging the cryovials containing the swabs at 1000 × g for approximately 10 min in a refrigerated centrifuge (4 °C). Centrifugation ensured the release of the virus into the viral transport medium by dislodging the particles from

the swab. After gently shaking the vial, 100 µL of each sample was transferred to a sterile cryovial to make a pooled sample. The pooled samples were marked to know the originality of each sample that can be traced to the location and farmer.

The long bone was crushed and the marrow contents were transferred into isotonic phosphate-buffered saline (PBS), Ph 7.0-7.4 containing antibiotic. The internal organs (liver, intestine, caecal tonsils, kidney and heart tissues) were pooled into antibiotic isotonic phosphate-buffered saline(PBS), Ph 7.0-7.4 containing antibiotic (OIE, 2019).

3.7.2 Reliability of laboratory techniques

Reliability of the laboratory techniques was ensured by carrying out all the tests in duplicates and including the negative and positive controls. Inoculation of the samples was done in four eggs embryonated eggs including positive control (La Sota strain isolate of NDV) and negative control (sample negative for NDV by Haemagglutination inhibition test). Extraction of the RNA was done using the conventional phenol-chloroform extraction procedure. The residual salt was removed by incorporating additional ethanol washes.

3.7.3 Validity of laboratory techniques

The quality of the embryonated eggs was examined by holding a strong light below the egg in a dark room to determine if the eggs were viable (candling method). A healthy embryo responded to the light by moving and had well-defined blood vessels.

The validity of the PCR test was ensured by the procurement of quality materials and reagents from recommended companies. The purity of the extracted RNA was determined by the use of a NanoDrop™ 2000/2000c spectrophotometer. After initializing the system using blank water 2 µl, the sample of equal volume 2 µl was loaded to the spectrophotometer. A read of the A260/A280 ratio was recorded. RNA sample that had an A260/A280 ratio of >1.8 was considered pure.

The amplified cDNA for the fusion gene quality was assessed by the use of 1% agarose gel dissolved in TAE buffer and loading dye, stained by the use of EZ visual dye and separated by electrophoresis at 100volts 2 amp for 1 hour. Well-defined bands that showed illumination were considered for sequencing.

The primers used in the study were subjected to BLASTN query to establish their identity with a wide range of NDV.

3.7.4 Isolation of Newcastle disease virus

Virus isolation was through the allantoic route of nine-day-old embryonated specific antibody negative (SAN) eggs (**Figure 3.5**). The eggs were obtained on day eight from Kenchic® hatcheries-Nairobi. They were allowed to settle at 36 °C ± 1 °C. The pooled samples were vortexed for 15s to mix the sample. The inoculation site of the viable eggs was marked using a sterile pencil during candling, the site was disinfected using an iodine solution. A volume of 0.2 mL of the pooled sample was inoculated into the allantoic cavity of each of the five labelled embryonated SAN eggs. The eggs were incubated at 36 °C ± 1 °C and 60%–65% humidity for five days. Embryo development and appearance were monitored daily using an egg candler. The dead embryos were stored at 4 °C for further harvesting. The allantoic fluid was harvested aseptically using 2 mL syringes, transferred to sterile vials, and stored at −80 °C (Stear, 2005).

Figure 3.5: Viral isolation of NDV by use of nine-day-old SAN embryonated chicken eggs

3.7.5 Molecular detection and characterization

3.7.5.1 RNA extraction and amplification of the matrix gene using real-time RT-qPCR

Total RNA was extracted from the allantoic fluid using TRIzol^{TM} LS reagent (Invitrogen, Carlsbad, California, USA). The aseptically harvested allantoic fluid was homogenised with phenol reagent at a ratio of 1:3, incubated at room temperature and chloroform added to the homogenate at a ratio of 1:5 to confine the total RNA in a

clear aqueous phase-separated from protein and cell debris by centrifugation. The RNA was recovered by precipitation using isopropanol, washed with ethanol and reconstituted RNAse free water.

The primer sequence (**Table 3.4**) was obtained from a study by (Wise et al., 2004) and the primer information was confirmed by the use of the basic local alignment search tool (BLASTN) from the National Center for Biotechnology Information (NCBI). The primers were synthesized by contracted laboratories (Macrogen, Seoul, Korea and Inqaba™, Pretoria, South Africa).

The extracted RNA was amplified by a one-step RT-qPCR machine (QuantStudio™ 5 System) using the formulated PCR master mix. The La Sota vaccine strain was used as a positive control. A known negative clinical sample (sample negative for NDV by Haemagglutination inhibition test) was used as the negative control. The primer master mix for the PCR run was prepared to include primers 0.5 µl (forward and reverse) and probe 0.5 µl, 2x RT buffer 12.5 µl, enzyme mix 1 µl, nuclease-free water 2.5 µl and a sample volume of 8 µl The cycling conditions for real-time RT-qPCR were 40 cycles of denaturation at 94 °C for 10 s, annealing at 56 °C for 30 s, and extension at 72 °C for 10 s (Wise et al., 2004).

Table 3.4: Primer sequence for the isolation of matrix gene using RT-qPCR

Primer name	**Primer sequence**	**Fragment size (bp)**	**Reference**
M+4100 (Forward Primer)	5’_-AGTGATGTGCTCGGACCTTC-3’	121bp	(Wise et al., 2004)
M+4169 (Matrix Probe)	5’[FAM]TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]-3’	121bp	
M-4220 (Reverse Primer)	5’CCTGAGGAGAGGCATTTGCTA-3’	121bp	

3.7.5.2 Complementary DNA preparation

Eight samples from Nairobi LBM IC (n=2), household IC (n=3) and clinical samples (n=3) were selected for sequencing (**Appendix V**). The samples from markets and households were positive for the matrix gene (Ct values< 30). The clinical samples had pathological findings determined by a veterinary pathologist that was informative of ND. The LBM, household and clinical samples were passaged in nine-day-old embryonated SAN eggs obtained from Kenchic®. Total RNA was extracted from the allantoic fluid using TRIzol^{TM} LS (Invitrogen, Carlsbad, California, USA). The aseptically harvested allantoic fluid was homogenised with phenol reagent at a ratio of 1:3, incubated at room temperature and chloroform added to the homogenate at a ratio of 1:5 to confine the total RNA in a clear aqueous phase-separated from protein and cell debris by centrifugation. The RNA was recovered by precipitation using isopropanol, washed with ethanol and reconstituted RNAse free water. The extracted RNA was converted to cDNA by first-strand cDNA synthesis using a Thermo-Scientific^{TM} revert-aid first-strand cDNA synthesis kit (Waltham, Massachusetts, USA) according to the manufacturer's instruction.

3.7.5.3 Amplification of cDNA targeting the matrix gene

The cDNA was sent to a contracted laboratory where they were amplified using one pair of matrix gene universal primers targeting 232 bp (Putri et al., 2017) (**Table 3.5**). The PCR conditions for amplification of the NDV matrix gene were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. The amplicon quality was assessed using agarose gel electrophoresis and purified using a Millipore plate MSNU030 (Millipore SAS, Molsheim, France).

Table 3.5:Matrix gene primer sequence for amplification of cDNA and subsequent sequencing of the matrix gene

Primer	Primer sequence	Fragment size (bp)	Reference
MATRIX MF	5′-TCGAGTCTGTACAATCTTGC-3′	232	Putri et al., 2017
MATRIX MR	5′-GTCCGAGCACATCACTGAGC-3′	232	

3.7.5.4 Amplification of cDNA targeting the fusion gene

The cDNA was amplified using five paired primers of different sequences of NDV fusion gene from studies by (Byarugaba et al., 2014; Ogali et al., 2018) (**Table 3.6**) using the formulated master mix consisting of 10x PCR buffer 5 µl,10 mM dNTP 1 µl, $MgCl_2$ 3 µl, forward and reverse primers 3.0 µl, Taq polymerase 0.5 µl, nuclease-free water 35.5 µl and samples 2 µl The PCR conditions for amplification of the NDV fusion gene were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and initial extension at 72 °C, and final extension at 72 °C for 45 s.

The amplicons of the clinical samples were assessed using 1% agarose gel electrophoresis in TAE buffer and a 100bp molecular marker. The staining dye used was EZ visual. The quality assessment was done before shipment for sequencing.

Table 3.6:Fusion gene primer sequences for amplification of cDNA and subsequent sequencing of the fusion gene

Primer Name	Primer Sequence	Fragment size (bp)	Reference
NDVF1F1-FORWARD	5'-GCAAGATGGGCYCCAAACC-3'	550	Byarugaba et al., 2014
NDVF1R2–REVERSE	5'-CATCTTCCCAACTGCCACT-3'	550	
NDVF2F2–FORWARD	5'-GACCACTTTACTCACTCCTC- 3'	642	
NDVF2R2–REVERSE	5'-GTAGGTGGCACGCATATTATT-3'	642	
NDVF3F2–FORWARD	5'-CGACTCACAGACTCAACTC-3'	685	
NDVF3R2–REVERSE	5'-TATARGTAATRAGRGCRGATG-3'	685	
NDVF4F2–FORWARD	5'-GCAAGATRACAACATGTAGRTG-3'	709	
NDVF4R2–REVERSE	5'-CTTGGCTAACYGCRCGGTCCAT-3'	709	
NDVF5F–FORWARD	5'-ATGGGCYCCAGACYCTTCTAC-3'	535	
NDVF5R–REVERSE	5'-CTGCCACTGCTAGTTGTGATAATCC-3'	535	(Ogali et al., 2018)

3.7.5.5 Sequencing of Amplified cDNA

The cleaned amplicons were sequenced using Sanger sequencing. Forward and reverse sequence reads were obtained for each amplicon. Before sequencing, the PCR products were cleaned using the ExoSAP-IT^{TM} (Waltham, USA), which consists of exonuclease and alkaline phosphatase at a temperature of 37 °C for 15 min. Fragments were sequenced using the BrilliantDye™ terminator cycle sequencing kit V3.1, BRD3-

100/1000 (Nimagen, Nijmegen, Netherlands) according to the manufacturer's instructions. The labelled products were then cleaned with the ZR-96 DNA sequencing clean-up kit (Zymo Research, Irvine, California, USA). The cleaned products were injected into a Genetic Analyser with a 50 cm array, using POP7 (Applied Biosystems ABI 3500XL). Sequence chromatogram analysis was performed using FinchTV analysis software (Geospiza, Inc.).

3.8 Data management and analysis

3.8.1 Test of association

Data entry for the poultry management in households was tabulated in Microsoft excel® 2016. These were associated with the RT-qPCR matrix gene results. Sample with Ct values <30 was positive for NDV. The Statistical Package for Social Sciences software (SPSS® 2019) was used for data analysis. The *Chi*-square and Fisher's exact tests were used to test the independence of the variables with a statistical significance of $p \leq 0.05$. The analysis was performed to examine the association between the prevalence of NDV in indigenous chicken and other variables (vaccination history of the ICs, interaction with other domestic poultry and the production system). A correlation test was performed to test the relationship between the weather conditions (temperature, relative humidity and wind speed) and the prevalence of ND at a statistical significance of $p \leq 0.05$. The weather data was obtained from Kenya meteorological department (**Appendix VI(a)**) and historical weather data from https://www.visualcrossing.com/weather-api (**Appendix VI(b)**).

3.8.2 Analysis of sequence results for the matrix gene and fusion gene

The five raw M gene paired reads and seven raw F gene paired reads were trimmed and assembled using *Geneious Prime version* 2020.2 software (https://www.geneious.com/) by mapping to the reference sequence MN685356.1, which was retrieved from the National Centre for Biotechnology Information (NCBI). Consensus sequences were retrieved from the generated contigs and multiple sequence alignment was performed using the Muscle algorithm within the Mega X version 10.1.6 software.

3.8.3 Haplotype and Phylogenetic analysis

Genetic indices were computed using DNA Sequence Polymorphism (DNA SP) version 6.0 software (http://www.ub.edu/dnasp/downloadTv6.html) and the TCS network was computed using Population Analysis with Reticulate Trees (PopArt) version 1.7(http://popart.otago.ac.nz/) (Leigh & Bryant, 2015). The matrix and fusion gene phylogeny was inferred using the maximum likelihood method by applying neighbor-joining and BioNJ algorithms on nucleotide pairwise distances, which were estimated using the maximum composite likelihood approach with a bootstrap of 1000 repeats (Kumar et al., 2018).

CHAPTER FOUR

RESULTS

4.1 Newcastle disease virus detection in Nairobi live bird markets

The NDV matrix gene detection rate in eight Nairobi live bird markets was 243/372 (65.3%, 95% CI = 60.5-70.2) and the result was statistically significant p <0.05. There was a variation in the detection rates of NDV in sampled Nairobi LBM (**Table 4.1**).

Table 0.1: Number and proportion of NDV matrix (M) gene positive samples from Nairobi live bird market

Nairobi Live bird market	**Number of samples**	**Number of positives**	**Proportion (%) positive (95% CI)**
Burma Maziwa	72	42	58.3 (47-70)
Burma Stadium)	34	28	82.4 (70-95.2)
Kangemi	70	33	47.14 (35.4-59)
Kariobangi North	48	24	50 (36.14-64)
Kariokor	24	18	75 (58-92)
Kasarani	54	43	80 (69-90.4)
Kawangware	50	45	90 (82-98.3)
Mutindwa	20	10	50 (28.1-72)
Total	**372**	**243**	**65.3 (60.5-70.2)**

4.2 Newcastle disease virus detection in households

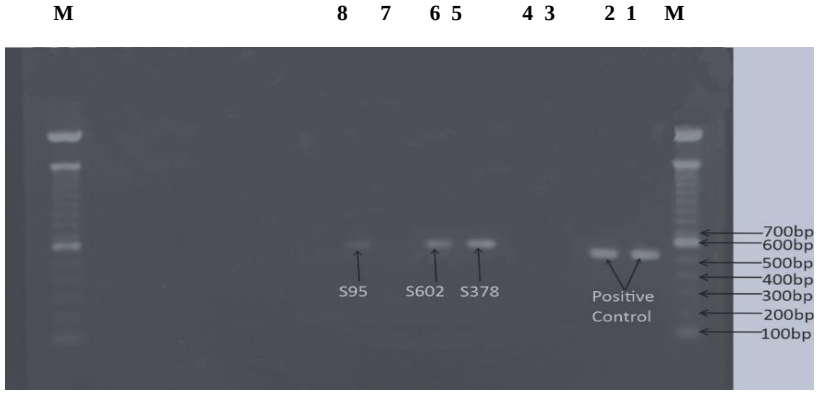
The NDV matrix gene detection rate in the six Kenya counties was 876/1210 (72.4%**,** 95% CI= 66.3-78**)** and was statistically significant at p <0.05. There was variation in the detection rates of NDV in the sampled household in the counties (**Table 4.2**).

Table 0.2: Number and proportion of NDV matrix (M) gene positive samples from households

AEZ	County	Number of samples	Number of positives	Proportion (%) positive samples (95% CI)
AEZ V	Baringo	350	233	67 (62-72)
AEZ III	Bomet	160	125	78.1 (72–85)
AEZ III	Kakamega	338	225	67 (62–72)
AEZ III	Kilifi	70	55	79 (57.1-100)
AEZ IV	Machakos	140	116	83 (77–89)
AEZ III	Nakuru	152	122	80.2 (73.9–87)
	Total	**1210**	**876**	**72.4(66.3-78)**

4.3 Newcastle disease virus in clinical samples

Amplification of the fusion gene showed positive samples from the clinical samples with the expected band size of 600bp (**Figure 4.1**). All the samples were selected and the S95 sample though the band was not very clear, was considered based on the presenting clinical signs.



M-DNA marker -100bp. Lane 1 and 2 positive controls (La Sota vaccine). Line 3 and 4 Negative control. Line 5,6 and 8

Figure 0.1: Images of amplified F gene fragments on agarose gel.

4.4 Factors associated with the prevalence of Newcastle disease virus in household indigenous chicken

4.4.1 Prevalence of Newcastle disease virus and vaccinated indigenous chicken

Among the households (n = 68), only 17/68 (25%, 95% CI = 14.7–35.3) had vaccinated their ICs Samples were collected from one household in Kilifi that had an active case with signs and symptoms of suspected NDV despite having vaccinated their flocks (Plate 4.1) Symptoms were observed by the farmer two days after the vaccination. The birds were positive for the Newcastle disease virus. One household in Kakamega had ICs with symptoms similar to those of fowl-pox disease and had vaccinated their IC, and had chickens that were positive for Newcastle disease.

Plate 0.1: Indigenous chicken with clinical signs of NDV after vaccination

The vaccination history per county indicated that all the counties had a higher number of unvaccinated ICs (434/605; 72%, 95% CI = 68.3–75.5) than vaccinated ICs (171/605; 28.3%, 95%= 24.5–31.7) and none of the households in Bomet County had vaccinated their ICs (**Figure 4.2**)

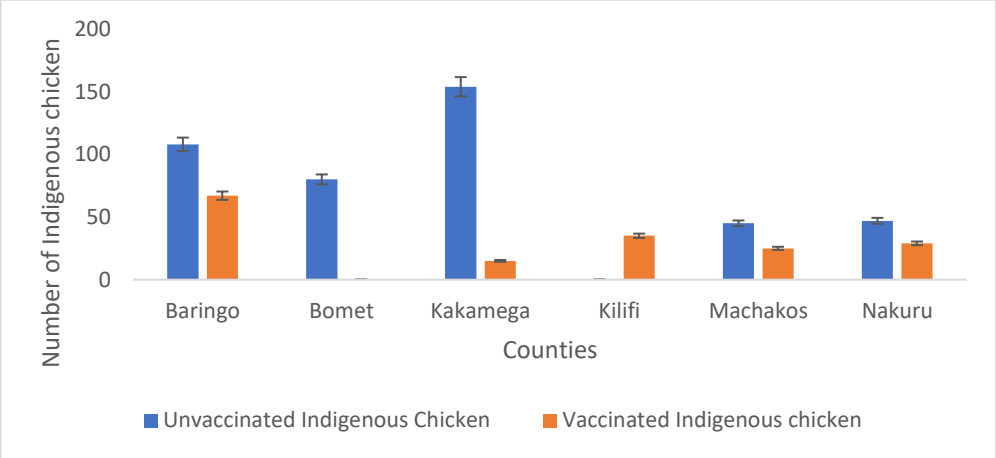


Figure 0.2: Number of vaccinated and unvaccinated indigenous chickens in households in the counties

The prevalence of NDV in unvaccinated ICs was 613/868 (71%, 95%CI= 68-74) and the prevalence of NDV in vaccinated ICs was 262/342 (77%, 95% CI = 69–72.1-88.1). There was a variation of matrix gene positive samples in unvaccinated and vaccinated ICs across the counties (**Table 4.3** and **Table 4.4**).

The was a statistically significant (p= 0.039) association between the prevalence of NDV and the vaccination history of the IC (**Table 4.5**)

Table 0.3: Number and proportion of NDV matrix (M) gene-positive samples from unvaccinated indigenous chicken in the counties

County	**Number of samples from unvaccinated flocks**	**Number of positive samples**	**Proportion (%) positive samples (95% CI)**
Baringo	215	151	70.2(64.1–76.3)
Bomet	160	125	78.1(72-85)
Kakamega	309	196	63.4 (58-69)
Kilifi	0	0	0
Machakos	90	67	74.4 (65.4-84)
Nakuru	94	74	79 (70.4–87)
Total	**868**	**613**	**71 (68-74)**

Table 0.4: **Number and proportion of NDV matrix (M) gene-positive samples from vaccinated indigenous chicken in the counties**

County	**Number of samples from vaccinated flocks**	**Number of positive samples**	**Proportion (%) positive samples (95% CI)**
Baringo	135	81	60(52–68.2)
Bomet	0	0	0
Kakamega	29	29	100 (100)
Kilifi	70	55	79 (69-88.2)
Machakos	50	49	98 (94–100)
Nakuru	58	48	83 (73.03–93)
Total	**342**	**262**	**77 (72.1–81.1)**

Table 0.5: Association between prevalence of NDV and vaccination history of indigenous chicken

		Vaccination history		
		Unvaccinated	Vaccinated	**Total**
PCR-M results (CT VALUE< 30)	Detected	613	262	875
	Not detected	255	80	335
Total		868	342	1210
***Chi*-square and fishers exact test significance *p*=0.039**				

4.4.2 Prevalence of Newcastle disease virus and the type of production systems

The sampled households had either a free-range or a semi-free-range production system. The majority of the ICs were kept in the free-range system 430/605 (71.1%, 95% CI = 68–75). Nakuru county household kept their ICs under the semi-free-range system. Machakos, Bomet and Kilifi counties had their chicken kept under the free-range system. Baringo county and Kakamega county had their ICs in both semi-free

range and free-range systems (**Figure 4.3**). A majority of the households had an average of 10 ICs of all ages and sexes. There were more vaccinated ICs in the semi-free-range system 59/175 (34%, 95 CI= 27-40.1) than in the free-range system 112/430 (26.04% 95%CI 22-30.2).

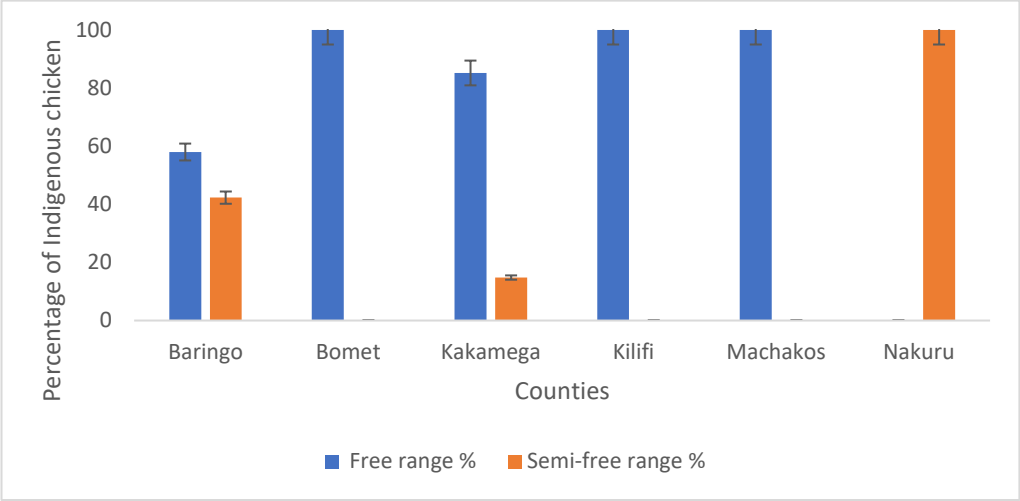


Figure 0.3: The percentage (%) of indigenous chicken kept under different poultry production systems in the counties

The prevalence rate of NDV in the free-range production system was 611/870 (70.2, 95% CI = 67.2-73.3) and in the semi-free range was 264/340 (78, 95% CI = 73.2-82.1). There was a variation of NDV matrix gene positive samples in the production system across the counties (**Table 4.6 and Table 4.7**)

There was a significant relationship *(p=0.010*) between the prevalence of Newcastle disease virus and the type of production system (**Table 4.8**)

Table 0.6: Number and proportion of NDV matrix(M) gene positive samples from indigenous chicken kept under the free-range production systems in the counties

County	Number of samples from Free-range chicken	Number of positive	Proportion (%) positive samples (95% CI)
Baringo	212	130	61.3 (55-68)
Bomet	160	125	78.1(72-85)
Kakamega	288	185	64.2(59-70)
Kilifi	70	55	78.5(69-88)
Machakos	140	116	83(77-89.1)
Nakuru	0	0	0
Total	**870**	**611**	**70.2(67.2-73.3)**

Table 0.7: Number and proportion of NDV matrix(M) gene positive samples from indigenous chicken kept under the semi-free range production systems in the counties

County	Number of samples from Semi-Free-range chicken	Number of positive	Proportion (%) positive samples (95% CI)
Baringo	138	102	74(67-81.2)
Bomet	0	0	0
Kakamega	50	40	80(69-91.1)
Kilifi	0	0	0
Machakos	0	0	0
Nakuru	152	122	80.3(74-87)
Total	**340**	**264**	**78(73.2-82.1)**

Table 0.8: Association between the prevalence of NDV and the production system for the indigenous chicken

		Production system		
		Free-range	Semi free-range	**Total**
PCR-M results (CT VALUE< 30)	Detected	611	264	875
	Not detected	259	76	335
Total		870	340	1210
Chi-square and Fisher exact test Significance *p*=0.010				

4.4.3 Prevalence of Newcastle disease virus and interspecies interaction

The sampled households had their ICs interacting with other poultry species and wild birds. However, the proportion of ICs that had no interaction with other species was relatively higher 332/605 (55%, 95% CI=51-59) compared to the ones that had interaction 273/605(45.1%, 95% CI= 41.2-49.1) (**Figure 4.4**).

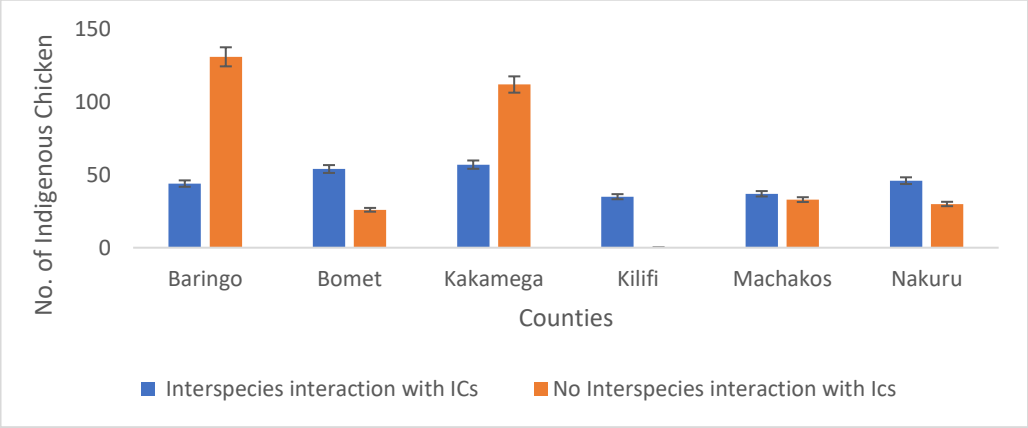


Figure 0.4: Number of indigenous chickens that had Interaction with other bird species in the counties

The prevalence rate of NDV in ICs that had interspecies interaction was 380/503(76%, 95% CI= 71.7–79.3). There was a variation of matrix gene positive samples in ICs that had interspecies interaction across the counties (**Table 4.9**).

There was a significant (p = 0.037) relationship between the interaction of ICs with other birds and the presence of NDV (**Table 4.10**).

Table 0.9: Number and proportion of NDV matrix (M) gene positive samples from indigenous chickens interacting with other birds in the counties

County	The number of samples from IC interacted with other bird species	Number of positive samples	The proportion (%) of positive NDV (95% CI)
Baringo	87	47	54(44-65)
Bomet	108	90	83.3(76.3-90.4)
Kilifi	70	55	79(67-88.2)
Kakamega	104	75	72.1(64-81)
Machakos	74	63	85.1 (77-93.2)
Nakuru	60	50	83.3 (74-93)
Total	**503**	**380**	**76 (71.7–79.3)**

Table 0.10: Association between the prevalence of NDV and the interaction of the indigenous chicken and other bird species

		Interaction with other poultry		
		N	Y	Total
PCR-M results (CT VALUE< 30)	Detected	495	380	875
	Not detected	212	123	335
Total		707	503	1210
***Chi*-square and fishers exact test significance p=0.037**				

Key N=No interaction Y=Interaction

4.4.4 Correlation between the prevalence of Newcastle disease virus in indigenous chickens and the prevailing weather condition

There was no correlation between the prevalence of NDV and the prevailing weather condition of temperature, humidity and wind speed in Baringo, Bomet, Kakamega, Nakuru and Machakos. Kilifi county data was not computed because there was no variation in the weather conditions. After all, samples were collected on the same day.

4.5 Genetic diversity of Newcastle disease virus

4.5.1 Haplotype analysis of the Newcastle disease virus matrix gene

A total of six mutated sites were observed in the five analyzed sequences, with each sequence harbouring at least one mutation. Two singleton variable sites (single nucleotide polymorphism) were observed at loci 62 and 77, whereas the remaining sites with mutations (loci 41, 66, 149, and 203) were informative for parsimony (occurred in more than one sequence). Parsimony informative sites 41, 66, and 203, where the nucleotide base guanine was substituted by adenine, adenine by guanine, and guanine by adenine, respectively, were observed in sequences of isolates (2C2, KF2C, and KF30), and mutation at locus 149 occurred only in sequences of isolates KF2C and KF30 (**Figure 4.5**)

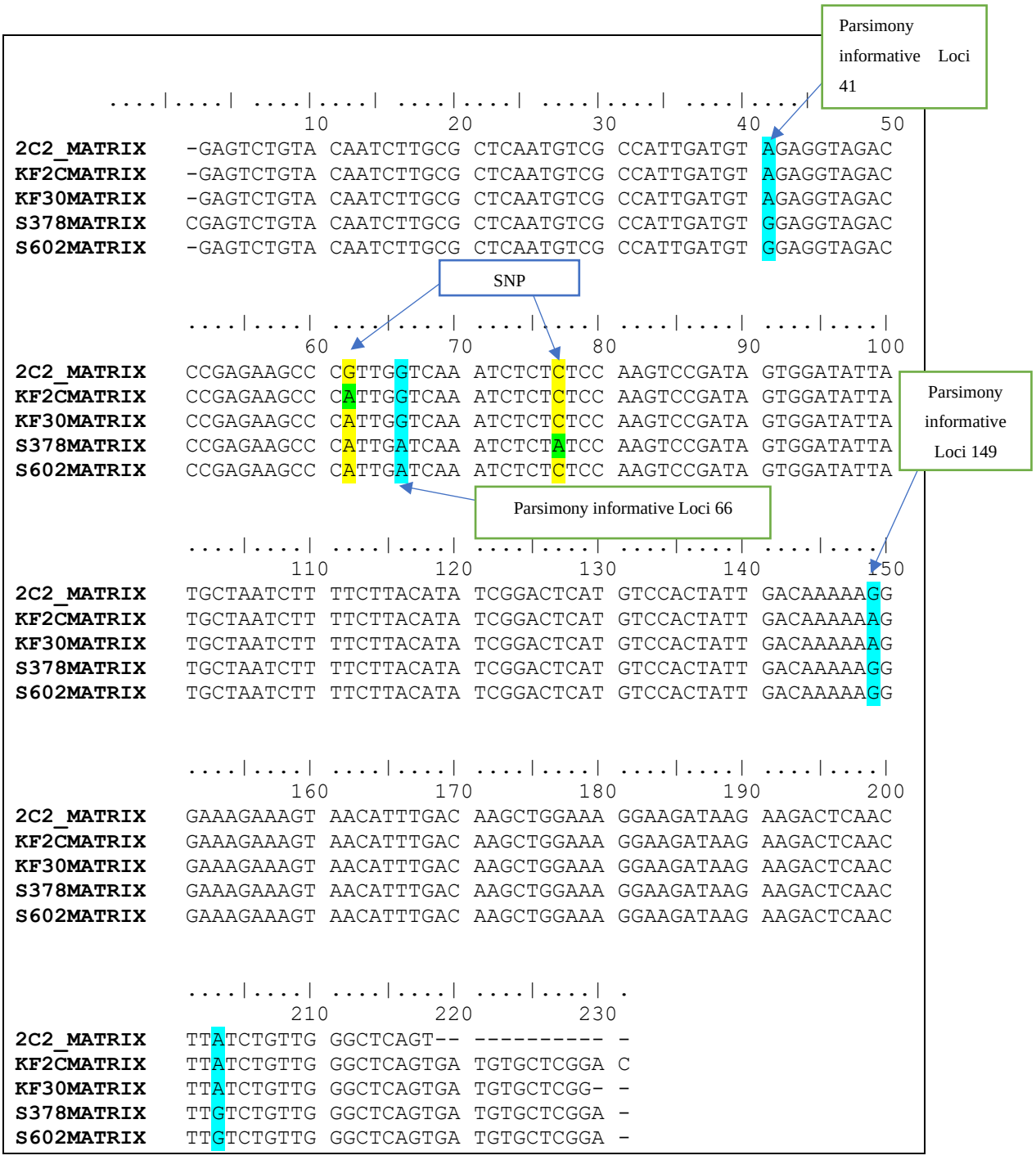


Figure 0.5: Alignment of the NDV matrix gene showing single nucleotide polymorphism (SNP) and parsimony-informative site

The observed mutations across all sites (165.03) analyzed were non-synonymous with Pi (a), Jukes & Cantor model equaling 0.01972. Despite the presence of mutated sites, the matrix gene from the five isolates had high sequence conservation (C) with a C

value of 0.974 out of 229 net sites analyzed. The five sequences exhibited low nucleotide diversity, with π = 0.01475 and a high haplotype diversity (Hd) value of 0.900 (**Table 4.11**). Four haplotypes based on matrix genes were found to be circulating within Kenya (**Figure 4.6**).

Table 0.11: Matrix gene segregation site, number of mutations, nucleotide diversity, and haplotype density

No. of Sequences	Segregating sites (S)	Total No. of Mutations (Eta)	Nucleotide Diversity (π)	π (per site) from Eta	Haplotypes (h)	Haplotype (gene) diversity (Hd)	Standard Deviation of Hd
5	6	6	0.01	0.21	4	0.90	0.16

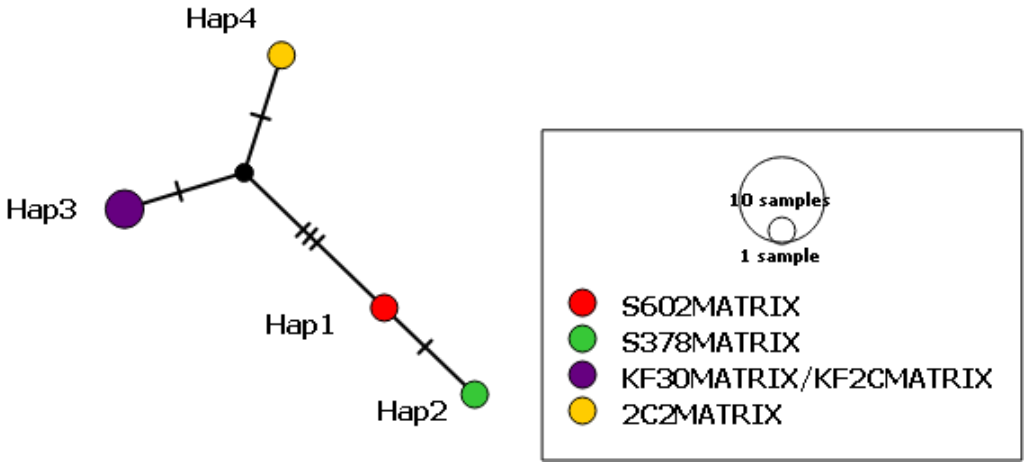


Figure 0.6: TCS haplotype network estimating the four haplotypes as well as genealogies of NDV genotypes based on the matrix gene.

The hatch marks represent the number of mutations that led to the emergence of a specific haplotype or genotype. The haplotypes are represented by the following colours: haplotype 1 (red), haplotype 2 (green), haplotype 3 (purple), and haplotype 4 (yellow). The size of the circles is proportional to the number of samples within the specific haplotype. Haplotype 3 was observed in two samples, thereby explaining the large size of the circle.

4.5.2 Phylogenetic analysis of the matrix gene

Forty-four (44) GenBank sequences were selected from a Basic local alignment search tool (BLASTN) query result that identified a percentage identity of >80% and an expected value (e-value) of 0.0 with the matrix gene sequences. GenBank retrieved sequences of NDV isolates from Kenya, Tanzania and wild birds. The La Sota strain sequence was queried independently. The retrieved sequences and five matrix gene sequences (KF2C(MZ062109), KF3O(MZ062110), 2C2(MZ062108), S378(MZ062111), and S602(MZ062112)) from the study were used to generate the phylogenetic tree (**Figure 4.7**). The bootstrap values >75% are presented next to the node

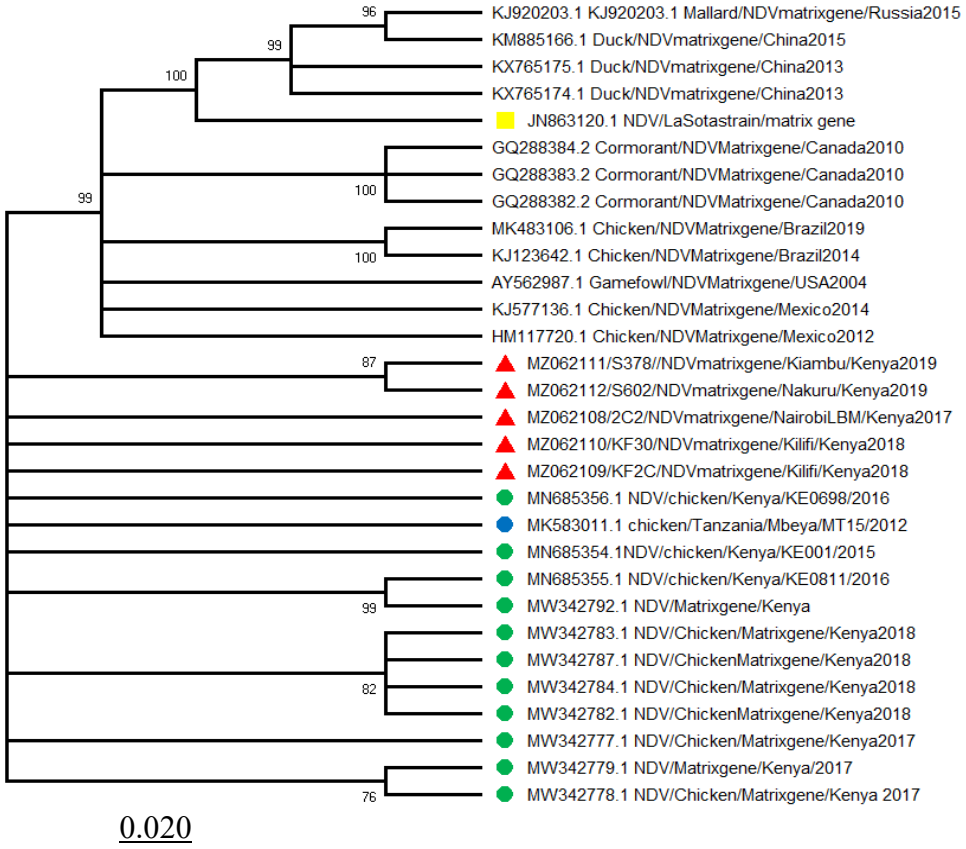


Figure 0.7: Phylogenetic tree inferring evolutionary relatedness of NDV matrix gene and previously isolated NDV.

The phylogenetic revealed seven clusters of the matrix gene and relatedness between the matrix gene sequences (Highlighted in red) and previously isolated sequences in Kenya (green) and Tanzania (blue). The La Sota strain (JN863120-yellow)) sequences

and sequences obtained from ducks and Mallard in China were outgroups in the phylogeny tree with no relatedness with the matrix gene sequences from Kenya.

Isolates KF2C(MZ062109) and KF30(MZ062110) from ICs in Kilifi were to be a recent genotype thought to have emerged as a result of mutations within the matrix gene of isolate 2C2(MZ062108) isolated from Nairobi market

4.5.3 Haplotype analysis of the Newcastle disease virus F gene

Seven analysed sequences revealed 106 mutations across 103 segregating sites that showed differences (polymorphisms) between related genes in a sequence alignment on the 535 base pairs of the seven aligned sequences. Of the 103 sites with mutations, 102 sites were parsimony informative, as the mutation was observed in more than one sequence (**Appendix VII**)

A high nucleotide diversity (π) of 0.24639 was observed across the seven analyzed NDV sequences (**Table 4.12**). Five haplotypes (**Figure 4.8**) with a high haplotype density (Hd) of 0.905 were identified among the seven analyzed sequences. Haplotypes one and five (H01 and H05) each had two samples, whereas the rest of the haplotypes (H02, H03, and H04) had a single sequence.

Table 0.12: **Genetic diversity indices of seven NDV isolates from Kenya.**

No. of Sequences	**Segregating sites (S)**	**Total no. of Mutations (Eta)**	**Nucleotide Diversity (π)**	**π (per site) from Eta**	**Haplotypes (h)**	**Haplotype (gene) diversity (Hd)**	**Standard Deviation of Hd**
7	103	106	0.25	0.22	5	0.91	0.10

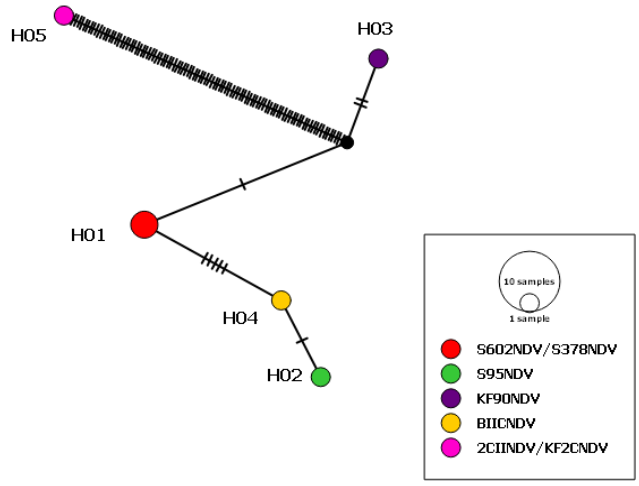


Figure 0.8: TCS network estimating haplotypes as well as genealogies of the NDV genotypes from the seven analyzed samples.

The hatch marks represent the number of mutations that led to the emergence of a specific haplotype or genotype. The haplotypes are represented by the following colours: haplotype 1 (red), haplotype 2 (green), haplotype 3 (purple), haplotype 4 (yellow), and haplotype 5 (pink). The size of the circles is proportional to the number of samples within the specific haplotype.

4.5.4 Phylogenetic analysis of the fusion gene

Sixty-four (64) nucleotide sequences were selected from a Basic local alignment search tool (BLASTN) query result that identified a percentage identity of >80% and an expected value (e-value) of 0.0 with the matrix gene sequences. La Sota strain sequence was queried independently with the seven sequences MZ062101(2CIINDV), MZ062102(BIICNDV), MZ062103(KF2CNDV), MZ062104(KF90NDV), MZ062105(S95NDV), MZ062106(S378NDV), and MZ062107(S602NDV), from this study to compute the tree. (**Figure 4.9**). The bootstrap values >75% are presented and shown at the nodes.

Isolated strain MZ062104(KF9O) had the same lineage as an isolate from Uganda HG937580.1 categorised as genotype V. Isolate MZ062102(BIICNDV), MZ062105(S95NDV), (MZ062107 (S602NDVand MZ062106(S378NDV) share the same lineage as isolates previously identified in Kenya and categorised as genotype

V. Isolate 2CIINDV(MZ062101) and KF2CNDV(MZ062103) were outgroups as well as an isolate from Uganda HG937575.1. There was no relatedness with isolates from Tanzania. There was no relatedness between the fusion gene isolates and the La Sota strain fusion gene. However, the La Sota strain had the same lineage as isolates from wild birds from Mexico and Argentina.

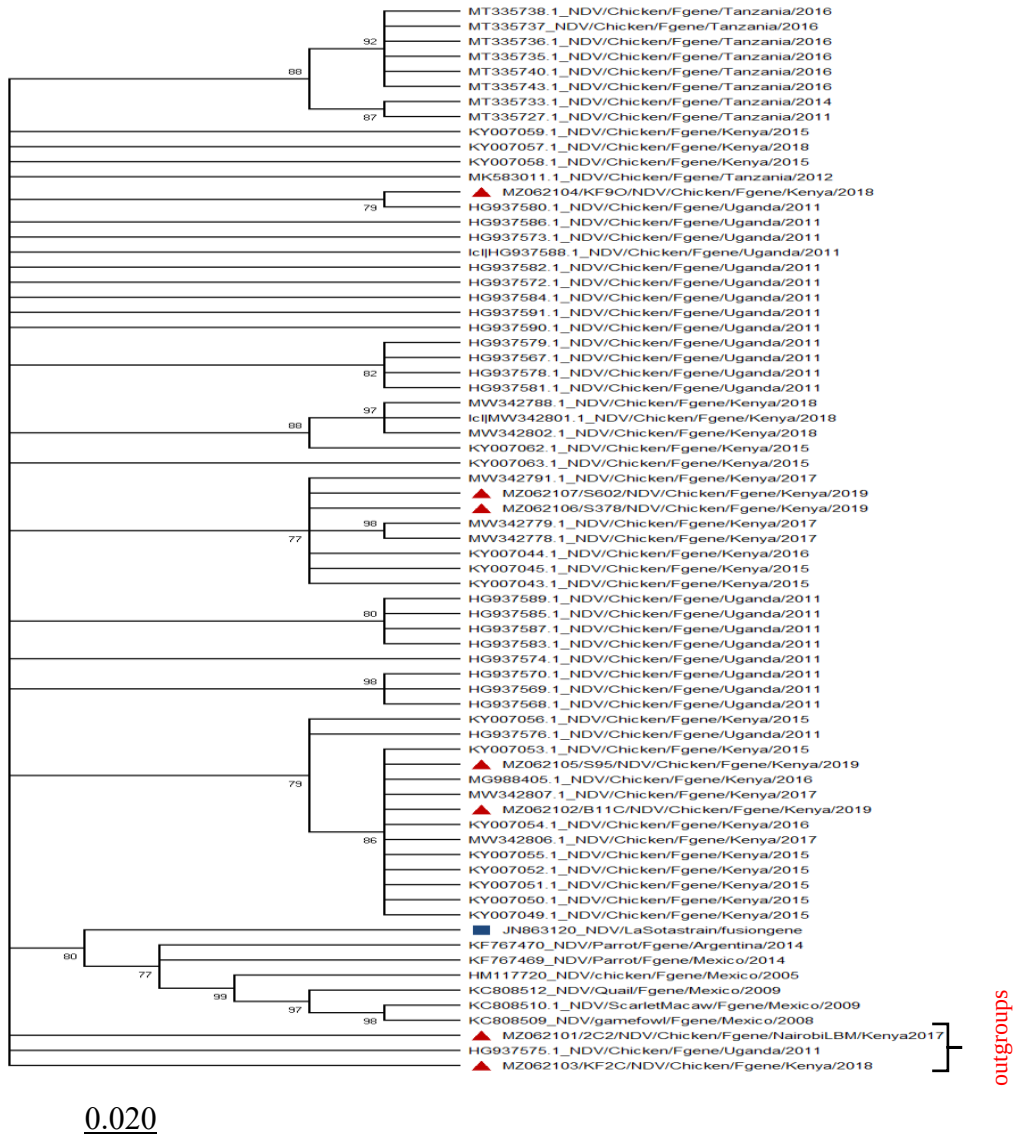


Figure 0.9: Phylogenetic tree inferring evolutionary relatedness of the NDV fusion gene and previously isolated NDV.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

This was the first molecular characterisation study that revealed the prevalence of NDV circulating in both vaccinated and unvaccinated indigenous chickens in Kenya.

This study's findings revealed the occurrence of NDV virulent, mesogenic and lentogenic strains in Nairobi live bird markets and households in key indigenous chicken counties.

The study findings revealed that the type of production system, vaccination history of ICs and interspecies interactions contribute to the presence of ND in households.

The study established that the matrix gene of the circulating strain of NDV had a similar lineage to previously identified strains in Kenya. The fusion gene of the circulating strain of NDV, however, was genetically diverse and two outgroups were identified. The strains isolated were distant from the La Sota vaccine strain.

5.1.1 Determination of prevalence of Newcastle disease virus

5.1.1.1 Newcastle disease virus in Nairobi live bird markets

This study's findings revealed the presence of the Newcastle disease virus in the Nairobi live bird markets. By targeting the matrix gene, this study identified all variants circulating in the market and suggests that ND is in circulation in live bird markets. The detection of NDV targeting the matrix gene was used to detect NDV from markets in Ethiopia (Mulisa et al., 2014). Previous studies have isolated NDV in different LBMs in Kenya (Ogali et al., 2018) however, this study's prevalence rate was higher because, unlike the previous studies where the target was the virulent gene (F gene), this study targeted all the variants in circulation. This study, therefore, iterates that live birds' markets (LBM) are an important distribution point for ICs in urban centres in Kenya with Nairobi live bird market as the main drop point for most chickens in the country as was demonstrated in a study in Kenya (McCarron et al., 2015; Ogali et al., 2018; Magothe et al., 2012). This study suggests that LBM is an epidemiological point for the distribution of poultry disease as was demonstrated in Indonesia (Kurscheid et al., 2017) and Vietnam (Fournie et al., 2013).

5.1.1.2 Newcastle disease virus in households

This study's findings revealed the prevalence of ND in households and suggest the dispersal of NDV in households in different agroecological regions in Kenya. The distribution of NDV is not limited to specific agroecological zones in Kenya and specific months of the year. ND in Kenya was previously identified in chickens by seroprevalence studies in different agroecological zones (Njagi et al., 2010) and virulent strains isolated from households (Kariithi et al., 2021; Ogali et al., 2018). This study, therefore, iterates that NDV is endemic in the country.

This study's findings revealed that all variants of NDV were circulating in vaccinated ICs by detection of the matrix gene. The detection of the NDV from the oropharyngeal and cloacal samples of vaccinated ICs can be attributed to viral shedding through the respiratory tract and the intestinal tract. The detection of the virus in the vaccinated flock was also reported in West Java Indonesia where NDV was isolated from vaccinated commercial chickens on farms (Putri et al., 2017). This study, however, did not estimate if the amount of virus shed would be enough to infect other unvaccinated and vaccinated birds. However, other studies have revealed that vaccination with the homologous strain of the circulating virus vaccine exhibited a low amount of virus shed (Miller et al., 2013).

5.1.2 Factors associated with Newcastle disease virus occurrence in households

5.1.2.1 Vaccination history

This study's findings revealed that there were fewer households that had vaccinated their ICs across different counties in Kenya. The study also revealed that the average number of ICs per homestead was ten (10). This study, therefore, suggests that the low uptake of NDV vaccination programmes is a result of low flock numbers of ICs. This IC population in Kenya's households was also revealed to be below ten (10) in a longitudinal study of indigenous chickens in Kenya (Otiang et al., 2021). Similar findings on the low uptake of vaccination for ICs were reported in Tanzania (Campbell et al., 2018) where large flock sizes were associated with high vaccine intake.

This study revealed that some flocks had other infections despite being vaccinated and they were positive for NDV. The presence of other diseases may interfere with the effectiveness of the NDV vaccine. Similar findings were reported on the importance of vaccinating healthy birds for an effective antibody response and effective administration of the ND vaccine on healthy birds (Kemboi, 2014; Otiang et al., 2021; Sharif, 2020). Therefore, this study suggests that the vaccination of unhealthy ICs may impact the effectiveness of the vaccine.

This study revealed that the vaccination history of the ICs had an association with the prevalence of NDV in ICs in households. The association suggests that vaccination is important in the control of NDV. This study's findings support a study on the impact of routine vaccination of ICs (Otiang et al., 2021) that suggests that vaccine efficacy is affected by variation in vaccination history which in turn affects the flock immunity. Therefore, this study suggests that routine vaccination of ICs in Kenya is important for effective vaccination and control of ND in indigenous chickens.

5.1.2.2 Production system

This study's findings revealed that the most preferred system for ICs production is the free-range system across the counties. This was also reported as the most preferred system particularly in rural households (Owour et al., 2012) due to the affordability and reduced labour in management. This study's findings revealed that there were more vaccinated ICs in the semi-free-range system compared to the free-range system and suggest that it is because of the association of the semi-free range production system with productive farming. These findings support a study that also revealed ICs in semi-free range systems as the most vaccinated because of the commercial nature of the system in Kenya and Tanzania (Lindahl et al., 2019)

This study's findings revealed that the type of production system had an association with the prevalence of Newcastle disease. The type of production system is indicative of the type of management system used in poultry farming and determines how effectively poultry disease are controlled. Therefore, effective control and management of ND in ICs is dependent on the type of production system. Similar studies on the effect of management practices have also suggested the importance of production systems in the effective management of ND (Ogali et al., 2018; Sharif,

2020). Therefore, this study suggests that the type of production system for the ICs should be considered for effective management, and control of the spread of Newcastle disease.

5.1.2.3 Interspecies interaction

This study's findings revealed that ICs had interaction with other poultry species and wild birds and there was an association between NDV prevalence and interspecies interaction. These birds interacted with the ICs in the feeding and watering points. Considering the mode of transmission of ND and the host range of ND, the possibility of interspecies transmission is expected. Other studies have accounted that chicken and other bird species are in constant interaction (Ayala et al., 2020) and they have indicated the possibility of interspecies transmission particularly isolates from chickens to wild birds (Ayala et al., 2020; Garcia et al., 2013) and the potential role of wild birds in maintaining the virus in the environment (Rahman et al., 2018). Therefore, this study suggests the possibility of interspecies transmission of ND from ICs to other species and other species to chickens. However, this study did not isolate NDV from the other bird species. Therefore, there is a need for further investigation on the role of other poultry species and wild birds in the transmission of NDV in Kenya.

5.1.2.4 Prevailing weather conditions

This study's findings did not provide any evidence of the association between the disease prevalence and prevailing weather conditions. The collection of samples was during one season across the years in relatively warm months. Possibly, if the samples were collected in two different seasons from the same region, as was done in other studies, the results would reflect a different situation. However, during sample collection, the farmers attributed the presence of the disease to the dry period before the wet season characterised by strong winds. Previous studies have shown the effect of weather on the transmission of ND in Kenya (Kemboi, 2014; Nyaiyo & Maangi, 2016). Therefore, further longitudinal study on the correlation between weather and the prevalence of ND should be undertaken.

5.1.3 Genetic diversity of Newcastle disease Virus

5.1.3.1 Matrix gene

This study's findings revealed that there was no genetic diversity of the matrix gene between the study sequences and previously isolated sequences in Kenya. However, the non-synonymous mutations on the sequences indicate divergent sequences circulating in Kenya. Similar studies on the analysis of the matrix gene have revealed the impact of divergent sequences on the matrix gene in the molecular detection of NDV (Cattoli et al., 2009; Wise et al., 2004). Therefore, the study suggests that the emergence of new haplotypes of the conserved matrix gene should be considered in the development of primers to avoid detection challenges and possibilities of false-negative results.

An analysis of the relatedness between the isolated matrix gene sequences and the La Sota strain NDV vaccine virus strain revealed that there was no shared lineage among the sequences. This points out to dissimilarity between the conserved gene of the circulating strain and the most commonly used vaccine.

5.1.3.2 Fusion gene

The fusion gene is the determinant of virulence in the NDV. The detection of virulent strains suggests that virulent strains of NDV are in circulation in ICs in Kenya's markets and households. These findings support studies that identified the virulent strain of ND in live bird markets and households in Kenya (Kariithi et al., 2021; Ogali et al., 2020). Virulent strains have been isolated in households within East Africa (da Silva et al., 2020), West Africa (Samuel et al., 2013), Asia (Rabiei et al., 2020) and South America (Garcia et al., 2013; Fernandes et al., 2014). Other studies in Madagascar (Andriamanivo et al., 2012), West Africa (Samuel et al., 2013) and East Africa (Byarugaba et al., 2014) have identified virulent NDV in markets.

This study's findings revealed high genetic diversity of the NDV fusion gene circulating in ICs in Kenya with a high number of mutations. The study findings revealed the evolution of new genotypes of the NDV fusion gene due to mutations. The presence of two outgroups is an indication of different types of genotypes in

circulation and may pose diagnostic challenges. Other studies have identified that NDV genotypes are evolving at different geographical locations and may result in diagnostic challenges of NDV(Miller et al., 2010). The two out-groups had a close identity to wild birds isolated on different continents and may result from the interspecies interaction. Therefore, this study supports other studies on the possibility of interspecies transmission of NDV (Ayala et al., 2016, 2020).

This study's findings revealed the similarity between Kenya strains and previously isolated strains in Uganda indicating the possibility of transboundary transmission of NDV. Other studies have revealed that transboundary transmission of NDV occurs through the ICs value chain particularly, in markets and during cross-cultural practices (Byarugaba et al., 2014; Ogali et al., 2020). This study, however, did not analyse samples from the Kenya borders and therefore the similarity of the sequences could be a result of the interaction of ICs in the market. This study revealed clustering of the isolated strain with previously identified strains in Kenya and Uganda of genotype V. Therefore, this is a good indication that the strain circulating in Kenya is genotype V. Genotype V is considered to be the most virulent strain in circulation globally and one of the causes of major outbreaks of NDV(de Almeida et al., 2013). However, the two out groups cannot be classified different because they did not meet the threshold of >60 % to be identified as different genotypes (Diel et al., 2012; Dimitrov et al., 2016).

The study revealed that virulent strains were present in vaccinated IC and that there was no relatedness between the virulent strain and the La Sota vaccine. The difference in genotype between the vaccine and the circulating strain does not deter the vaccine's ability to protect against disease but the non-relatedness may result in ineffective control of circulating Newcastle disease. Other studies have shown non-relatedness between the La Sota vaccine strain and the circulating strain in the countries (Bello et al., 2018; Tan et al., 2008) and suggest the possibility of vaccine failures due to administration of non-related vaccine resulting in reduced control of NDV. Previous studies have outlined the importance of using a heterologous vaccine consisting of a genotypic similar vaccine in the control of Newcastle and reducing virus shedding (Bello et al., 2018; Miller et al., 2010; Roohani et al., 2015). The study, therefore,

suggests the use of a vaccine that consists of genotype from the circulating strain in Kenya could result in a significant reduction in virus replication and shedding.

5.2 CONCLUSION

This study reveals that there is the dispersal of NDV in ICs within Nairobi markets and households in different agroecological zones in Kenya. Therefore, from the prevalence results, a conclusion can be made to support previous studies that NDV is endemic in the country despite efforts to control the disease by the use of vaccination. The distribution and maintenance of NDV in households are attributed to the different factors that are associated with the disease's presence. Therefore, it is important to consider the ICs vaccination history, type of production system and interspecies interaction as factors in the control of the spread and maintenance of NDV in the IC flock. Prevailing weather condition, however, was not impactful on the prevalence of NDV in Kenya households and further investigations recommended.

The relatedness between the circulating strains in the study sequences and previously isolated sequences in Kenya and Uganda indicates that the circulating strain is similar and can be inferred to be of genotype V. Two strains considered as outgroups is an indication of mutation of circulating strains. The phylogeny study clearly outlines that the circulating matrix and fusion gene strain in Kenya and the commonly used vaccine in Kenya (La Sota) are phylogenetically distant.

This study, therefore, concludes that continuous surveillance of circulating strains of NDV among ICs is important to understand the disease dynamics in the country and if there are any evolving genotypes. This will improve laboratory detection and control of ND by vaccination, use of a vaccine that can reduce viral shedding and completely eradicate ND among chickens in Kenya and improve Kenya's food security.

5.3 RECOMMENDATIONS

5.3.1Recommendations for improvement

From the study findings, the following are recommended for the control of NDV in Kenya

1) The national government and county government carry out annual surveillance for NDV, to detect any evolving genotypes annually under different weather conditions.
2) The competent authorities on animal production and animal health should factor in the unique conditions of ICs farming methods in control of ND in Kenya.
3) An intergovernmental partnership developed towards funding for the development of a vaccine that is compatible with circulating strains of NDV and produces a heterologous vaccine

5.1.2 Recommendation for further studies

To gain more information on circulating strains of NDV the study recommends the following:

1) Further isolation of NDV circulating among wild birds and other domestic birds to determine the strains circulating in the country by detection and characterisation.
2) A longitudinal study on the correlation between prevailing weather conditions and the prevalence of ND.
3) Studies to evaluate the effectiveness of vaccines produced in Kenya in comparison to vaccine strains used worldwide by analysis of the transmission characteristics of Newcastle disease virus field strain after different vaccination scheme

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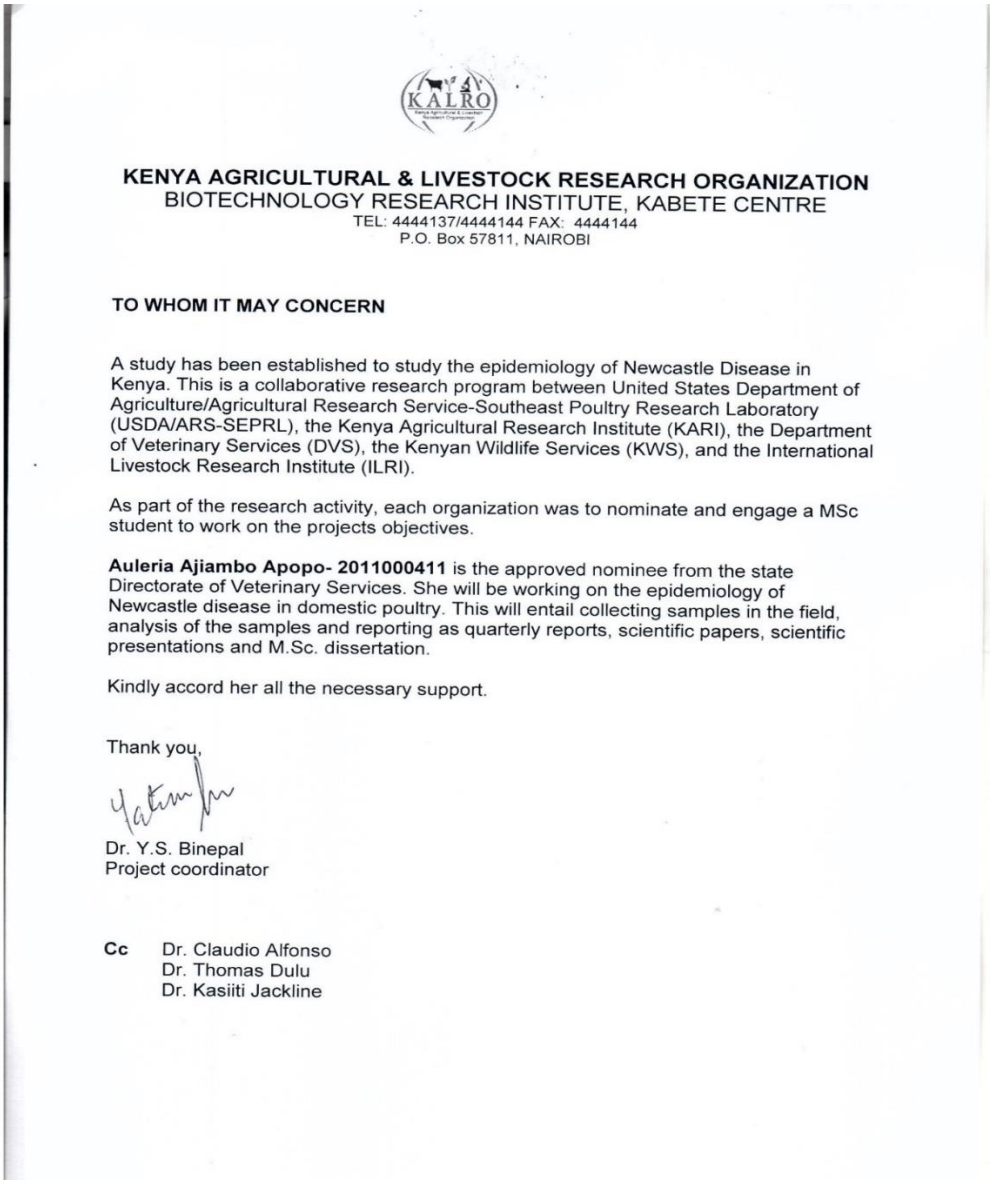
APPENDICES

Appendix I: Study approval document



KENYA AGRICULTURAL & LIVESTOCK RESEARCH ORGANIZATION
BIOTECHNOLOGY RESEARCH INSTITUTE, KABETE CENTRE
TEL: 4444137/4444144 FAX: 4444144
P.O. Box 57811, NAIROBI

TO WHOM IT MAY CONCERN

A study has been established to study the epidemiology of Newcastle Disease in Kenya. This is a collaborative research program between United States Department of Agriculture/Agricultural Research Service-Southeast Poultry Research Laboratory (USDA/ARS-SEPRL), the Kenya Agricultural Research Institute (KARI), the Department of Veterinary Services (DVS), the Kenyan Wildlife Services (KWS), and the International Livestock Research Institute (ILRI).

As part of the research activity, each organization was to nominate and engage a MSc student to work on the projects objectives.

Auleria Ajiambo Apopo- 2011000411 is the approved nominee from the state Directorate of Veterinary Services. She will be working on the epidemiology of Newcastle disease in domestic poultry. This will entail collecting samples in the field, analysis of the samples and reporting as quarterly reports, scientific papers, scientific presentations and M.Sc. dissertation.

Kindly accord her all the necessary support.

Thank you,

Dr. Y.S. Binepal
Project coordinator

Cc Dr. Claudio Alfonso
Dr. Thomas Dulu
Dr. Kasiiti Jackline

Appendix II: Sample collection form for market samples

County of sampling		Date	
Market of sampling			
Name of Trader			
Trader contact			
Species			
Samples collected			
Age of animals			
Breed			
Number in stock currently			
Number bought from the supplier			
The market where birds sourced from			
County of origin of birds			
Date of delivery of birds			
Any sick birds			
Birds bought for organ sampling			
Any other observations			

Appendix III: Sample collection form for field samples

MINISTRY OF AGRICULTURE, LIVESTOCK AND FISHERIES
STATE DEPARTMENT OF LIVESTOCK
CENTRAL VETERINARY LABORATORIES

Reg. No.: CVL/PATH/REC/FORM001 **Effective date:** 11/10/2013
Edition: 002 **Page** 1 **Authorized by:**

LABORATORY REPORT FORM

FOR LABORATORY USE ONLY
Specimen No: _____ Date Received: _____ M.R. No.: _____
Veterinarian: _____ Reception officer: _____

Client details
Submitted by: _____ Owner's Name: _____
Address: _____ Address: _____
_____ _____
Telephone: _____ Telephone: _____

Farm details
Location of farm: _____ County: _____

Specimen details
Species: _____ _____ Date collected: _____
Specimen submitted: _____ Date dispatched: _____
_____ Test(s) requested: _____

Animal History
Age of animal: _____ Sex: _____ Breed: _____
Clinical signs: _____
_____
Length of illness: _____ Temperature: _____
Hatchery/Source: _____ Feeds: _____
Vaccination _____ Treatment: _____

Herd History
No. of animals in herd/flock: _____ Affected: _____ Dead: _____
Clinical signs: _____
_____
Duration of outbreak: _____

Notes: _____
_____
_____

Appendix IV: County Source of Indigenous chicken for Nairobi markets

	Number of indigenous chicken and their source January 2017											
	Bomet	Kericho	Kiambu	Kitui	Machakos	Makueni	Nairobi	Narok	Nyandarua	Trans-Nzoia	Uasin Gishu	West Pokot
Nairobi Live Bird market												
Burma - Maziwa	12	0	0	12	21	24	0	0	0	6	6	0
Burma Stadium	6	0	0	4	4	4	0	0	0	16	0	0
Kangemi	30	0	10	0	3	0	10	20	0	0	0	0
Kariakor	0	0	0	12	0	0	0	0	12	0	0	0
Kariobangi-North	0	0	12	6	30	0	0	0	0	0	0	0
Kasarani	0	0	0	0	48	0	0	0	6	0	0	0
Kawangware	0	50	0	0	0	0	0	0	0	0	0	0
Mutindwa	0	0	0	0	0	0	0	0	0	0	0	20

Appendix V: Samples selected for sequencing targeting the matrix and fusion gene

Sample Name	Sample Source	Vaccination history	Original Sample Type
2CII	Nairobi markets	Unknown	Cloacal swab
B11C	Nairobi markets	Unknown	Cloacal swab
KF9O	Kilifi farm	Vaccinated against NDV	Oropharyngeal swab
KF2C	Kilifi farm	Vaccinated against NDV	Cloacal swab
KF3O	Kilifi farm	Vaccinated against NDV	Oropharyngeal swab
S-602	Nakuru farm	Vaccinated against NDV	Internal organs
S-378	Kiambu farm	Vaccinated against NDV	Long bone
S-95	Nakuru farm	Vaccinated against NDV	Internal organs

Appendix VI: Sources of weather data

a) Kenya Meteorological Department

REPUBLIC OF KENYA
MINISTRY OF ENVIRONMENT ANDFORESTRY
KENYA METEOROLOGICAL DEPARTMENT
Dagoretti Corner, Ngong Road, P. O. Box 30259, 00100 GPO, Nairobi, Kenya
Telephone: 254 (0) 20 3867880-7,**Fax:** 254 (0) 20 3876955/3877373/3867888,
e-mail: director@meteo.go.ke, info@meteo.go.ke **Website:** http://www.meteo.go.ke

Attention: **State Department of Veterinary** **09/04/2019**

Following are monthly values for the four parameters for requested stations. Rainfall values are in millimetres while temperature in degree centigrade RH in percentage while wind run in kilometres per day.

Station/Year/Month	Max temp.	Min temp.	Total rainfall	RH 9am	RH 3PM	Wind run in km/day
Kakamega May 2017	27.9	16.0	217.5	81%	65%	56.2km/day
Mtwapa March 2018	31.9	24.0	98.3	80%	69%	80.5km/day
Machakos September 2017	25.5	12.1	5.7	71%	43%	124.2km/day
Nakuru June 2017	26.6	11.1	36.7	73%	41%	104.3km/day

Christine Mahonga
For: Director KMD

b) Historical weather data for the different homestead location Source- https://www.visualcrossing.com/weather-api

Date of sample collection	Region	Temperature(0C)	Relative Humidity %	Wind-speed (Km/hr)
22/5/2017	Bomet	26.2	71	21.2
23/5/2017		26.4	68.5	24.8
24/5/2017	Kakamega	26.9	72	20.5
27/5/2017		27	82.9	16.4
29/5/2017		22	77.5	14.8
30/5/2017	Baringo	21	85.2	14.8
31/5/2017		23	83.5	18.4
1/6/2017		22	84.2	13
2/6/2017		25	20.5	73.5
3/6/2017		25	71.9	24.1
5/6/2017	Nakuru	26.2	77.2	16.6

Date of sample collection	Region	Temperature(0C)	Relative Humidity %	Wind-speed (Km/hr)
6/6/2017		25.7	72.6	12.6
7/6/2017		26.7	71.4	11.2
27/9/2017	Machakos	29.1	54.6	30.4
28/9/2017		27	62.4	28.4
29/9/2017		28.3	25.6	61.2
8/3/2018	Kilifi	31.5	78.7	26.2

Appendix VII: Kenya NDV fusion gene sequence alignment

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
2CIINDV         ---------- ---------- ---------- ---------- ----------
BIICNDV         ---------- ---------- ---------- ---------- ----------
KF2C_NDV        TGTATTCCCA AGAGCTGGGT CTGTGAGTCA TATAGTATAG GGTTGCCAGT
KF90NDV         ---------- ---------- ---------- ---------- ----------
S95NDV          ---------- ---------- ---------- ---------- ----------
S378NDV         ---------- ---------- ---------- ---------- ----------
S602NDV         ---------- ---------- ---------- ---------- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
2CIINDV         ---------- ---------- ---------- ---------- ----------
BIICNDV         ---------- ---------- ---------C CAAACCTTCT ACCAGAATCC
KF2C_NDV        GACCAAGCCA CTACTAATTA ACGAGCTGAG TTGATTGTTC CCTACACCTA
KF90NDV         ---------- ---------- ---------- -------TCT ACCAGAATCC
S95NDV          ---------- ---------- -----GGCTC CAAACCTTCT ACCAGAATCC
S378NDV         ---------- ---------- ---------- ------TTCT ACCAGAATCC
S602NDV         ---------- ---------- ---------- ------TTCT ACCAGAATCC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
2CIINDV         ---------- ---------- ---------- ---------- ----------
BIICNDV         CAGTACCCCT GACACTGATC ACCCAGATTA CGTTGACACT GAGCTGTGTC
KF2C_NDV        ATTTAGTCAA CAAGTAATCC ATATTGCCAC CAGCGAGATT ATAAAGTGCC
KF90NDV         CAGCACCCTT GACACTGATC ACCCAGATTA TGTTGGCACT GAGCTGTGTC
S95NDV          CAGTACCTCT GACACTGATC ACCCAGATAG CGTTGACACT GAGCCGTGTC
S378NDV         CAGTACCTCT GACACTGATC ACCCAGACCA TGTTGACACT GAGCTGTGTC
S602NDV         CAGTACCTCT GACACTGATC ACCCAGACCA TGTTGACACT GAGCTGTGTC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
2CIINDV         ---------- ---------- ---------- ---------- ----------
BIICNDV         TGCATGACGA GCTCTCTTGA TGGTAGACCT CTGGCAGCTG CAGGGATTGT
KF2C_NDV        TGGATGGTCA GCTGAGTTAG GGCAGGAGAA GTGATTTGTG GCCCGAACGC
KF90NDV         CGCATGACGA GCTCTCTTGA TGGTAGACCT CTTGCAGCTG CAGGGATTGT
S95NDV          TGCATGACGA GCTCTCTTGA TGGTAGACCT CTGGCAGCTG CAGGGATTGT
S378NDV         TGCATGACGA GCTCTCTTGA TGGTAGACCT CTGGCGGCTG CAGGGATTGT
S602NDV         TGCATGACGA GCTCTCTTGA TGGTAGACCT CTGGCGGCTG CAGGGATTGT


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
2CIINDV         ---------- ---------- ---------- ---------- ----------
BIICNDV         GATAACAGGA GATAAAGCAA TCAACATATA CACTTCATCC CAAACAGGGT
KF2C_NDV        TGTAGTCAG- --TTCAGTTA AGTACAAATT GAGCTCGACG CCAACCTGTT
KF90NDV         GATAACGGGA GATAAAGCAG TCAACATATA CACTTCATCT CAAACAGGGT
S95NDV          GATAACAGGA GATAAGGCAA TCAACATATA CACTTCATCC CAAACAGGGT
S378NDV         GATAACGGGA GATAAAGCAG TCAACATATA CACTTCATCT CAAACAGGGT
S602NDV         GATAACGGGA GATAAAGCAG TCAACATATA CACTTCATCT CAAACAGGGT
```

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
2CIINDV         ---------- ---------- ---------- ---------- ----------
BIICNDV         CGATCATAGT CAAGTTACTC CCAAATATGC CCAAGGATAA AGAGGCGTGC
KF2C_NDV        GTGCAATTTT TATGC-AGTC CAATTCTCGC GCTGTATTAT TAAACTGATC
KF90NDV         CGATCATAGT CAAGTTACTC CCAAATATGC CCAAGGATAA AGAGTCGTGT
S95NDV          CGATCATAGT CAAGTTACTC CCAAATATGC CCAAGGATAA AGAGGCGTGC
S378NDV         CGATCATAGT CAAGTTACTC CCAAATATAC CCAAGGATAA AGAGGCGTGT
S602NDV         CGATCATAGT CAAGTTACTC CCAAATATGC CCAAGGATAA AGAGGCGTGT


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
2CIINDV         ---------- ---------- ---------- ---------- -TGTGATAAT
BIICNDV         GCAAAGGCCC CACTGGAGGC ATACAATAGA ACACTGACCA CTTTACTCAC
KF2C_NDV        ATTAACAAAC TGCTGCATCT TCCCGACTGC CACCTGCTAG CTGTGATAAT
KF90NDV         GCAAAGGCTC CACTGGAGGC ATACAATAGA ACACTGACCA CTTTACTCAC
S95NDV          GCAAAGGCCC CACTGGAGGC ATACAATAGA ACACTGACCA CTTTACTCAC
S378NDV         GCAAGGGCCC CACTGGAGGC ATACAATAGA ACACTGACCA CTTTACTCAC
S602NDV         GCAAAGGCCC CACTGGAGGC ATACAATAGA ACACTGACCA CTTTACTCAC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
2CIINDV         CCGTC--AGT GACCTCGTGC ACAGCCTCAT TGGTCGCAGC AATGCTCTCC
BIICNDV         TCCTCTCGGC GACTCCATCC GCAGGAT-AC AGGAGTCCGC GA----CTAC
KF2C_NDV        CCGTC--AGT GACCTCGTGC ACAGCCTCAT TGGTCGCCGC AATGCTCTCC
KF90NDV         TCCTCTTGGC GACTCCATCC GCAGGAT-AC AAGAGTCCGC GA----CTAC
S95NDV          TCCTCTCGGC GACTCCATCC GCAGGAT-AC AGGAGTCCGC GA----CTAC
S378NDV         TCCTCTTGGC GACTCCATCC GCAGGAT-AC AGGAGTCCGC GA----CTAC
S602NDV         TCCTCTTGGC GACTCCATCC GCAGGAT-AC AGGAGTCCGC GA----ATAC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
2CIINDV         TTAAGTCGGA GGATGTTGGC AGCGTTTT-T GTTGGCTTGT ATCAGGGCCG
BIICNDV         ATCTGGGGGA AGGAGACAAA AACGTTTTGT AGGTGCCGTT ATCGGCAGCG
KF2C_NDV        TTAAGTCGGA GGATGTTGGC AGCGTTTT-T GTTGGCTTGT ATCAGGGCCG
KF90NDV         ATCTGGGGGA AGGAGACAGA AACGCTTTGT AGGTGCCGTT ATCGGCAGCG
S95NDV          ATCTGGGGGA AGGAGACAAA AACGTTTTGT AGGTGCTGTT ATCGGCAGCG
S378NDV         ATCTGGGGGA AGGAGACAGA AACGCTTTGT AGGTGCCGTT ATCGGCAGCG
S602NDV         ATCTGGGGGA AGGAGACAGA AACGCTTTGT AGGTGCCGTT ATCGGCAGCG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
2CIINDV         CGGCCGCTGT TACCTGTGCA GCTGTTGCAA CCCCAAGAGC TACGCTGCCG
BIICNDV         TAGCTCTTGG GGTTGCAACA GCTGCACAGG TAACAGCGGC CGCGGCCCTG
KF2C_NDV        CGGCCGCTGT TACCTGTGCA GCTGTTGCAA CCCCAAGAGC TACGCTGCCG
KF90NDV         TAGCTCTTGG GGTTGCAACA GCTGCACAGG TAACAGCGGC CGCGGCCCTG
S95NDV          TAGCTCTTGG GGTTGCAACA GCTGCACAGG TAACAGCGGC CGCGGCCCTG
S378NDV         TAGCTCTTGG GGTTGCAACA GCTGCACAGG TAACAGCGGC CGCGGCCCTG
S602NDV         TAGCTCTTGG GGTTGCAACA GCTGCACAGG TAACAGCGGC CGCGGCCCTG
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
2CIINDV         ATAACGGCAC CTACAAAGCG TTTCTGTCTC CTTCCCCCAG ATGTAG----
BIICNDV         ATACAAGCTA ACA-AAAACG CGGCCAACAT CCTCCGGCTT AAGGAGAGCA
KF2C_NDV        ATAACGGCAC CTACAAAGCG TTTCTGTCTC CTTCCCCCAG ATGTAG----
KF90NDV         ATACAAGCCA ACA-AAAACG CTGCCAACAT CCTCCGACTT AAGGAGAGCA
S95NDV          ATACAAGCTA ACA-AAAACG CGGCCAACAT CCTCCGGCTT AAGGAGAGCA
S378NDV         ATACAAGCCA ACA-AAAACG CTGCCAACAT CCTCCGACTT AAGGAGAGCA
S602NDV         ATACAAGCCA ACA-AAAACG CTGCCAACAT CCTCCGACTT AAGGAGAGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
2CIINDV         TCGCGGACTC CTGT-ATCCT GCGGATGGAG TCGCCAAGAG GAGTGAGTAA
BIICNDV         TTGCTGCAAC CAATGAGGCT GTGCACGAGG TCACT--GAC GGATTATCAC
KF2C_NDV        TCGCGGACTC CTGT-ATCCT GCGGATGGAG TCGCCAAGAG GAGTGAGTAA
KF90NDV         TTGCGGCGAC CAATGAGGCT GTGCWCGAGG TCACT--GAC GGATTGTCAC
S95NDV          TTGCTGCAAC CAATGAGGCT GTGCACGAGG TCACT--GAT GGATTATCAC
S378NDV         TTGCTGCAAC CAATGAGGCT GTGCACGAGG TCACT--GAC GGATTATCAC
S602NDV         TTGCTGCAAC CAATGAGGCT GTGCACGAGG TCACT--GAC GGATTATCAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
2CIINDV         AGTGGTCAGT GTTCTATTGT ATGCCTCCAG TGGGGCCTTT GCACACGACT
BIICNDV         AACTAGCAGT GG-CAGWTGG GAAGATGCAG CAGTTTGTTA ATGATCAGTT
KF2C_NDV        AGTGGTCAGT GTTCTATTGT ATGCCTCCAG TGGAGCCTTT GCACACGACT
KF90NDV         AACTAGCAGT GG-CAGTCGG GAAGATGCAG CAGTTTGTTA ATGATCAGTT
S95NDV          AACTAGCAGT GG-CAGTCGG GAAGATGCAG CAGTTTGTTA ATGATCAGTT
S378NDV         AACTAGCAGT GG-CAG---- ---------- ---------- ----------
S602NDV         ARCTAGCAGT GG-CAG---- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
2CIINDV         CTTTATCCTT GGGCATATTT GGGAGTAACT TGACTATGAT CGACCCTGTT
BIICNDV         TAATAATACG GCGCGAGAGT TGGAATG-CA TAAAAATTGC ACAACAGGTT
KF2C_NDV        CTTTATCCTT GGGCATATTT GGTAGTAACT TGACTATGAT CGACCCTGTT
KF90NDV         TAATAATACA GCGCGAGAGT TGGNCTG-CA TAAAAATTGC ACAACAGGTT
S95NDV          TAATAATACG GCGCGAGAGT TGGACTG-CA TAAAAATTAC ACAACAGGTT
S378NDV         ---------- ---------- ---------- ---------- ----------
S602NDV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        710        720        730        740        750
2CIINDV         TGAGATGAAG TGTATATGT- --TGACTGCT TTATCTCCCG TTATCACAAT
BIICNDV         GGTGTCGAAC TCAATTTGTA CCTAACTGAA TTGACTACAG TGTTCGGGCC
KF2C_NDV        TGAGATGAAG TGTATATGT- --TGACTGCT TTATCTCCCG TTATCACAAT
KF90NDV         GGTGTCGAAC TCAATTTGTA CTTAACTGAA TTGACTACAG TGTTCGGGCC
S95NDV          GGTGTTGAAC TCAATTTGTA CCTAACTGAA TTGACTACAG TGTTCGGGCC
S378NDV         ---------- ---------- ---------- ---------- ----------
S602NDV         ---------- ---------- ---------- ---------- ----------
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
2CIINDV         CCCTGCAGCC GCCAGAGGTC TA--CCATCA AGAGAGCTCG TCA-TGCGGA
BIICNDV         ACAAATCACT TCTCCTGCCC TAACTCAGCT GACTATCCAG GCACTGTATA
KF2C_NDV        CCCTGCAGCC GCCAGAGGTC TA--CCATCA AGAGAGCTCG TCA-TGCGGA
KF90NDV         ACAAATCACT TCTCCTGCCC TAACTCAGCT GACCATCCAG GCACTTTATA
S95NDV          ACAAATCACT TCTCCTGCCC TAACTCAGCT GACCATCCAG GCACTGTATA
S378NDV         ---------- ---------- ---------- ---------- ----------
S602NDV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
2CIINDV         CACAGCTCAG TGTCAACATA GTCTGGGTGA TCAGTGTCAG AGGTACTGGG
BIICNDV         ATCTAGCTGG TGGCAATATG GACTACTTGT TGACTAAATT AGGTGTAGGG
KF2C_NDV        CGCAGCTCAG TGCCAACATA GTCTGGGTGA TCAGTGTCAG AGGTACTGGG
KF90NDV         ATCTAGCTGG TGGCAATATG GATTACTTGT TGACTAAATT AGGTGTAGGG
S95NDV          ATCTAGCTGG TGGCAATATG GACTACTTGC TGACTAAATT AGGTGTAGGG
S378NDV         ---------- ---------- ---------- ---------- ----------
S602NDV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
2CIINDV         ATTCTGGTAG AA-------- ---------- ---------- ----------
BIICNDV         AACAATCAAC TCAGCTCGTT AATTGGTAGT GGCTTGGTCA CTGGCAACCC
KF2C_NDV        ATTCTGGTAG AAG------- ---------- ---------- ----------
KF90NDV         AACAATAAAC TCAGCTCGTT AATTAGTAGT GGCTTGGTCA CTGGCAACCC
S95NDV          AACAATCAAC TCAGCTCGTT AATTGGTAGT GGCTTGGTCA CTGGCAACCC
S378NDV         ---------- ---------- ---------- ---------- ----------
S602NDV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| .
                       910        920        930        940
2CIINDV         ---------- ---------- ---------- ---------- -
BIICNDV         TATACTATAT GACTCACAGA CCCAGC---- ---------- -
KF2C_NDV        ---------- ---------- ---------- ---------- -
KF90NDV         TATACTATAT GACTCACAGA CCCAGCTCTT GGGCATACAG G
S95NDV          TATACTATAT GACTCACAGA CCCAGCTCTT ---------- -
S378NDV         ---------- ---------- ---------- ---------- -
S602NDV         ---------- ---------- ---------- ---------- -
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Appendix VIII: Publication from this study

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Presence of Newcastle Disease Virus in Vaccinated Indigenous Chicken in Selected Regions in Kenya — A Cross-Sectional Study

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