MOLECULAR DIVERSITY AND RISK FACTORS OF AVIAN PARAMYXOVIRUS IN DOMESTIC POULTRY IN KENYA

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Molecular Diversity and Risk Factors of Avian Paramyxovirus in Domestic poultry in Kenya

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DECLARATION

This is my original work and has not been presented for a degree consideration/a ward in any other University.

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DEDICATION

This work is dedicated to my husband, Philip Ngere and our sons Thomas Junior and Matthew for their continued support throughout my academic journey. To my little angel Michelle who I unfortunately lost as I fought in this journey “You are not forgotten”.

In all things, "Christ is my anchor and Strength."

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ACRONYMS AND ABBREVIATIONS

µl	Microliter
µg	Micrograms
mg	Milligrams
ng	Nano grams
APMV	Avian paramyxovirus
BEB	Bayes Empherical Bayes
cDNA	Complimentary deoxyribonucleic acid
CDD	Conserved Domains Database
DEPC	Diethyl pyro carbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
ddNTP	Dideoxynucleoside triphosphate
GB	Gigabytes
GDP	Gross Domestic Product
HA	Hemagglutination Assay
HI	Hemagglutination inhibition
HIV	Human immunodeficiency virus
ICPI	Intracerebral pathogenicity index
IVPI	Intravenous pathogenicity index
Kb	Kilobytes
KNBS	Kenya National Bureau of Statistics
mAB	Monoclonal antibodies
MDT	Mean death time
MLD	Minimum lethal dose
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
OIE	World organization of Animal Health

PCR	Polymerase chain reaction
PDB	Protein Data Bank
PPMV	Pigeon paramyxovirus
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
rRT-PCR	Real time polymerase chain reaction
SPF	Specific pathogen free
USDA	United States Department of Agriculture

ABSTRACT

Avian paramyxoviruses (APMV) circulating in domestic poultry in Kenya were evaluated for their genetic characteristics and identification of risk factors. Through a cross-sectional study of 225 poultry farms and 21 live bird markets in five regions in Kenya. The study used the large polymerase protein (L) and Fusion (F) gene fragments as well as the whole genome to characterize the genetic diversity of APMV and multivariate regression to analyze the association between APMV occurrence and. management/trade factors. Based on this evaluation, the virus was significantly higher in poultry farms which introduced 'new' birds from markets or neighbors, kept mixed poultry species or those with chicken flock size (>30). Live bird markets located in cities or major towns and traders who purchased their stock from multiple sources also had significantly higher APMV infection. The average mean haplotype diversity L and F gene was 0.759 (±0.02) and 0.906 (±0.03) respectively. The average mean nucleotide diversity L and F gene was 0.018 (±0.001) and 0.021 (±0.001) respectively Phylogenetically, Kenyan APMV-1 together with Ugandan strains were classified in a novel sub-genotype of genotype V, Clade II. The genome of Kenyan strains was 18% (±1.7%) distant from that of commonly used vaccine strain *Lasota*. Unique signatures were identified in APMV-1 strains circulating in Kenya. The study also detected signature of selection on different lineages and amino acid sites of APMV-1 genes. Selective pressure was highest on the fusion protein gene (*dN/dS*=0.289*)* and phosphoprotein gene *(dN/dS*=0.272***)***. Three amino acid codons of fusion gene and one codon of the nucleoprotein gene under positive selection. In conclusion, the study showed that management and trade factors are associated with APMV occurrence in domestic poultry in Kenya and revealed presence of virulent closely related genetic variants of APMV-1 strains of a novel sub-genotype. Further, the study showed that natural selection affects the variation and diversity of lineages and proteins of global APMV-1 strains.

CHAPTER ONE

INTRODUCTION

1.0 Background information

This subsection provides information on the importance of poultry on livelihoods, poultry production systems and constraints. Background information on Newcastle disease and avian paramyxovirus in the global and Kenyan context is also summarized.

1.0.1 Definition of poultry

Poultry are domesticated birds kept by humans for their eggs, meat or their feathers. Poultry includes avian species of the Superorder *Galloanserae* (fowl), specifically of the order *Galliformes* that is chicken (*Gallus domesticus*), quails (*Coturnix japonica*) and turkeys (*Melleagris gallopavo*) as well as birds of the order *Anseriformes* or waterfowl such as domestic ducks (*Anas platyrhynchos*) and geese (*Anser anser*). Poultry also includes birds that provide meat such as pigeons (*Columba livia)* and birds considered game like pheasant (*Phasianus colchicus)* and Guinea fowl (*Numida meleagris*). Ninety percent (90%) of the world's poultry population are chicken (Bagnol, 2009). Domestication of poultry took place more than 2000 years ago (Vaisanen *et. al.*, 2005). Since then, poultry keeping has grown worldwide to be an important industry. It accounts for 30% of total meat consumed worldwide (Gilbert *et al.*, 2015) and is second most widely eaten meat type globally after pork (USDA, 2018). The largest poultry producers are China (28%), United States (12%), Brazil (7%) and Indonesia (6%) (Gilbert *et al.*, 2015).

In sub-Saharan Africa, poultry contributes considerably to the livelihoods of poor rural and urban populations (Copland & Alders, 2013). Poultry meat and eggs are a source of the much needed protein and essential minerals to Africa's malnourished rural populations (Bruyn *et al.*, 2015). It also provides a ready source of income and plays an important socio-cultural role (Wong *et al.*, 2017). Poultry also boosts the socio-

economic status of vulnerable groups such as widows, the elderly and people leaving with HIV (Gabanakgosi *et al.,* 2013; Moreki *et al.,* 2010). The poultry population in Africa is estimated at 3.8 billion chicken, 32 million ducks, 19 million turkeys and 8 million geese (FAOSTAT, 2016). It is estimated that Africa produces approximately 4.5 million metric tons of poultry meat and 3.2 million metric tons of eggs annually (FAOSTAT, 2016). The largest poultry producers in Africa are South Africa, Egypt, Morocco, Algeria and Nigeria (FAOSTAT, 2016). Eighty percent (80%) of the total poultry population in Africa are kept in the rural communities under the traditional smallholder extensive system (Alders & Pym, 2009).

In Kenya, the poultry population is estimated at 49 million, out of which 98% are chicken while ducks, geese, turkeys, guinea fowls, quails and pigeons constitute 2% (KNBS, 2017). Like the rest of Africa, 80% of the poultry population is concentrated in the rural areas under the smallholder production system with minimal resource inputs (Kingori *et al.,* 2010). Poultry plays an important role in the livelihoods of Kenya's rural dwellers and contributes 50% of the total egg production and 80% of the total poultry meat consumed in Kenya (KNBS, 2017). The poultry sub-sector also creates employment especially for women and the youth (Ndirangu *et al.,* 2009). Poultry production is especially attractive in Kenya, as it requires low startup capital and low maintenance cost. Moreover, in Kenya where landlessness is increasing due to high population growth (The World Bank, 2016), many households are embracing poultry keeping as an investment of choice (Ajani *et al.,* 2007; Ngugi & Nyariki, 2005).

1.0.2 Poultry management

Almost 80% of poultry production in Africa is found in rural and peri-urban areas where birds are raised in small numbers in traditional smallholder extensive or semi-intensive systems. These systems are characterized by low inputs low output management with no or minimal feed supplementation and rudimentary housing (Melesse, 2014). Under this system, chicks are hatched by natural incubation and are raised by mother hens until they are naturally weaned. They are therefore exposed to

predators and harsh environmental conditions and diseases. Weaned birds on the other hand, search for their own food and usually obtain this from the scavengeable feed resource base that consists of kitchen wastes, insects, fruit seeds and other feed in the environment (Kryger *et al.*, 2010). Previous work reported that scavengeable feedstuffs can provide a bird with 9-27g/bird/day of the required nutrients (Sonaiya, 2002). This is insufficient to provide required nutrients (Hayat, *et al.*, 2016) and optimize production. The production parameters are generally low. Hens mature at 6 months at a live body weight between 1.5 to 1.7 kg and produce 40 to 60 eggs per annum (Alders & Pym, 2009; Khalafalla, *et al.*, 2002). Chick mortality ranges between 40-80% (Tirunesh & Gerima, 2016; Lwesya *et al.*, 2004).

In Kenya, small-scale poultry production accounts for 80% of poultry production (KNBS, 2017). Majority of the small-scale poultry producers are poor and prefer to keep the poultry in low input backyard or extensive systems with little or no feed supplementation (Kingori *et al.*, 2010; Ochieng *et al.*, 2010). In this system, local or '*kienyeji'* poultry are preferred to the improved or exotic breeds because of their ability to produce even with low input, cultural preference and disease tolerance (Magothe *et al.*, 2012). Poultry flock size per household in Kenya is reported to range from 5-25 birds (Olwande *et al.*, 2010). Poultry are kept under extensive scavenging system with little grain supplementation. In this system, poultry from various households interact freely among themselves and with wild birds as they scavenge (Kingori *et al.*, 2010). Most households source their breeding stocks from the neighbors while other source from local markets (Mwobobia *et al.*, 2016; Kaingu *et al.*, 2010). Poultry health management and sanitation is not practiced on a regular basis, where practiced herbal products are used in treatment of poultry diseases (Okitoi *et al.*, 2007). Like the rest of Africa, poultry performance in Kenya remains generally poor and is characterized by low feed intake, slow growth rates, small body size, low chick survival rates and high mortality, low egg and meat production (Atela *et al.*, 2016; Olwande *et al.*, 2010).

1.0.3 Challenges encountered in poultry production

Despite its various advantages, the maximum potential of the poultry sector in Africa to contribute to food and income security can only be realized if the constraints to production are addressed. Top among these constraints include: diseases, predators, inadequate nutrition, housing genetic improvement, marketing and access to veterinary services (Wong *et al.,* 2017). Diseases are the most common cause of high mortality in the poultry sector in Africa (FAO, 2014).

The common diseases are Newcastle disease (ND), fowl pox, Gumboro, helminthiasis and ecto-parasites (Sadiq & Mohammed, 2017; Olwande *et al.,* 2016; Tirunesh & Gerima, 2016; Alfred *et al.,* 2012). Previous studies from many African countries report ND as the most economically important disease of poultry in Africa. In Uganda, Semakula *et al.,* (2015) reported that ND was the highest cause of chicken mortality followed by salmonellosis, coccidiosis, fowl cholera and fowl pox in that order. In Ethiopia, ND was reported to be the most devastating disease in smallholder poultry production (Meskerem, 2017). The same also authors reported that helminthes, ecto-parasites, fowl pox and coccidiosis were important constraints to smallholder poultry production in Ethiopia.

Newcastle disease (ND) was reported to be the major health constraint to poultry production in Rwanda followed by ecto-parasites and helminths (Mazimpaka *et al.,* 2018). In Kenya, ecto-parasites and helminths have been reported as important constraints for rural poultry production systems (Sabuni *et al.,* 2011; Kaingu *et al.,* 2010; Mungube *et al.,* 2008). However, ND is by far the most devastating cause of mortality in poultry in Kenya (Olwande *et al.,* 2016).

Newcastle disease affects many domesticated poultry and wild bird species (Dimitrov *et al.,* 2016a). The disease is classified under list A of epizootics and notifiable diseases of the World Organization of Animal Health (OIE) (OIE, 2012). In developing countries, ND is endemic and occurs as annual outbreaks resulting in high mortality of

up to 100% in susceptible poultry (Ashraf, & Shah, 2014). Other avian paramyxoviruses cause disease of varying severity in different poultry species (Alexander & Senne, 2008).

1.0.4 Avian Paramyxovirus (APMV)

1.0.4.1 Classification

Avian paramyxoviruses belong to genus *Avulavirus* of the family *Paramyxoviridae* in the order *Mononegavirales* (Lamb & Parks, 2007). The genera *Avulavirus* consists of distinct avian paramyxovirus (APMV) serotypes that only infect avian species (Gogoi *et al.,* 2015).

Like other *Paramyxoviridae*, the structure of APMV consist of a lipid bilayer envelope that originates from the host's cell membrane. In a review by Gogoi *et al.* (2015), the APMV virion consists of a helical ribonucleoprotein, the nucleocapsid surrounded by a pleomorphic envelope which is mostly spherical in shape and ranges in diameter from 100 to 300 nm. APMV has six non-overlapping genes in the order; 3'-NP-P-M-F-HN-L-5' that encode six major polypeptides (nucleocapsid protein, phosphoprotein, matrix, fusion, hemagglutinin-neuraminidase and the large protein respectively) (Lamb *et al.*, 2006).

There are thirteen serotypes of avian paramyxovirus (APMV-1 to APMV-13) capable of infecting avian species (Miller *et al.,* 2010). APMV-1, which is the most diverse serotype (Dimitrov *et al.,* 2016a), is presently classified into two clades; Class I and Class II. Avian paramyxovirus serotype 1 strains of Class I are mostly non-pathogenic and have been isolated from waterfowl and shore birds in various parts of the world (Kim *et al.,* 2008; Czegledi *et al.,* 2006). Avian paramyxovirus of Class II are mainly pathogenic strains isolated from domestic poultry and wild birds. They are responsible for the most devastating outbreaks causing huge economic losses in poultry globally (Kim *et al.,* 2008). The class II strains are highly diverse and the current classification

groups the strain of class II APMV-1 into eighteen genotypes I-XVIII (Courtney *et al.*, 2013; Almeida *et al.*, 2013; Diel *et al.*, 2012a).

1.0.4.2 Transmission

Information on the transmission of other serotypes of avian paramyxovirus other than APMV-1 is limited. However, it's suspected that all APMVs have similar mechanisms of transmission since the viral replication mechanisms are similar (Khattar et al., 2013; Coffee et al., 2010). The major means of transmission of avian paramyxoviruses remains the fecal-oral route and direct contact between healthy birds and bodily discharges of infected birds (Alexander, 2009; Alexander, 2003). APMV-1 is shed during incubation and the clinical stage of disease but seems to be limited during the convalescence period (Kahn & Line, 2010).

1.0.4.3 Diagnosis

APMV is readily cultivated in specific-pathogen-free (SPF) embryonated chicken eggs. Chicken embryo kidney (CEK) cells or chicken embryo fibroblast (CEF) cells can also be used to propagate the virus. Virus isolation is done from swab and tissue samples collected in viral transport media (OIE, 2012). Hemagglutination inhibition test using specific antiserum and controls is the OIE recommended serological test for avian paramyxoviruses (OIE, 2016). Other serological tests such as virus neutralization, plaque neutralization, agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) available. However, serological assays are associated with multiple shortcomings including the inability to classify some strains based on the already classified APMV and cross-reactivity between serotypes (Terregino *et al.*, 2013; Cattoli *et al.*, 2011).

Molecular techniques have made diagnosis of APMV much more rapid and accurate (Cattoli *et al.*, 2011). Rapid diagnostic tests such as real-time reverse transcription polymerase chain reaction (rRT-PCR) and sequencing have greatly reduced time

needed for diagnosis and implementation of control measures (Gopinath *et al.*, 2011). RT-PCR assays provide quick amplification and are essential diagnostic tools for virus detection. APMV have negative sense RNA that requires reverse transcription into complimentary DNA (cDNA) prior to RT-PCR amplification. Reverse transcription of single-stranded RNA using an RNA-dependent DNA polymerase enzyme results in single-stranded cDNA (King, 2010).

Nucleotide Sequencing has enabled a detailed analysis of APMV strains. Sequencing techniques originated with use of di-deoxynucleoside triphosphate (dd-NTP) mediated chain termination and chemical degradation methods (Maxam & Gilbert, 1977; Sanger *et al.,* 1977). Sequencing techniques have rapidly improved since that time and next generation or automated sequencing techniques have been developed globally (Kürekçi & Dinçer, 2014; Ansorge, 2009). Due to their cost effectiveness, next generation sequencing techniques are now routinely used in characterization of APMV (Thampaisarn et al., 2017; Goraichuk et al., 2016).

1.1 Statement of the Problem

The agricultural sector in Kenya targets to increase its contribution to the GDP by Kshs 80-90 billion. The sector also plans to achieve a reduction in the number of people living below absolute poverty line and food insecurity by 25-30 per cent by the year 2030 (GoK, 2007). In addition, the agricultural sector is expected to achieve a 7 to 10 per cent annual average growth rate (GoK, 2007).

The agricultural sector must be transformed from the current subsistence orientation to a more commercial activity. According to the Agriculture Sector Development plan 2010-2020, special focus will be on agricultural or livestock commodities that impact on the livelihood of majority of Kenyans especially vulnerable groups (GoK, 2010). Poultry is one such commodity; it is important in the livelihoods of over 80% of Kenyans; especially poor women, widows and orphans of HIV/AIDS. Moreover,

poultry production has been identified as a useful development tool with benefits that meet the aims of the Sustainable Development Goals (GoK, 2010).

The poultry sector has potential to improve incomes and food security in the rural. However, poultry disease, especially rampant Newcastle disease (ND) outbreaks, hamper efforts to improve poultry productivity and profitability (Bagnol *et al.*, 2013). Losses from ND in Kenya could result in a loss of Ksh 3.8 billion per annum (Thurlow, 2011). With persistence ND outbreaks, the goal of transforming the poultry sector to a vibrant competitive sector that contributes to the sustainable development of the country will be difficult to achieve.

Vaccination, biosecurity and monitoring of avian paramyxovirus in the poultry sector have been used successfully in ND control in developed countries (Bagnol *et al.*, 2013). However, majority of poultry farmers in Kenya lack the resources to control ND in their flock (Alders *et al.*, 2007). This calls for the use of effective tailor made control measures relevant for these farmers. Development of such control measures requires adequate knowledge on Newcastle disease occurrence dynamics and nature of its causative agents. Such knowledge is largely lacking in Kenya's smallholder poultry.

Firstly, there is need to understand factors that contribute to APMV infection in the smallholder system so that appropriate mitigation strategies can be put in place for effective control of APMV. The type of poultry production system in Kenya allows APMV strains to exist as heterogeneous populations (Awan *et al.*, 1994).

Until this study was carried out, the genetic characteristics of APMV viruses circulating in the poultry in Kenya was poorly understood. This study utilized the genes associated with host invasion (F gene) and multiplication (L gene) and ultimately the whole genome to understand the genetic diversity of APMV and characterize them into a novel APMV-1 clade II sub-genotype Vd. Further, the study utilized the whole genome of APMV-1 strains from the world including those from poultry in Kenya to

identify unique signatures specific to novel sub-genotype Vd and identify selective pressures influencing the diversity of APMV-1 proteins and lineages.

1.2 Justification

The poultry sector in Kenya has traditionally been under-rated as a vehicle for sustainable rural development due to constraints that ND imposes on the development of this sector. Newcastle disease control will increase production of smallholder poultry in Kenya and open up further opportunities for improvement. In Kenya, there are national, community and individual efforts towards the control of Newcastle disease. The findings of this study will help policy makers, national and county diseases control teams and farmers to understand the circulating and disease causing avian paramyxoviruses. In turn they will be in a better position to, formulate affordable and quick APMV control strategies that are readily incorporated into farmer management systems.

Possible solutions to prevailing ND outbreaks in Kenya will be suggested, which once implemented; the farmers' welfare will increase. From this study, policy recommendations will be made pertaining poultry disease control in rural communities and these will play a vital role in guiding policy makers in the implementation of ND control strategies. Analyzing the genetic diversity of APMV strains in Kenya will provide a theoretical foundation for research on development of possible ND surveillance and control tools using the local strains and add to efforts in development of genotype-matched vaccines suitable for use in Africa's smallholder poultry sector.

1.3 Objectives of the study

The main objective was to assess the molecular diversity of avian paramyxoviruses in domestic poultry as well as investigate the management and trade practices that enhance the occurrence of the virus in domestic poultry in Kenya.

1.3.1 Specific objectives

1. To investigate the management and trade factors that influence APMV occurrence in domestic poultry in Kenya.
2. To determine the molecular diversity of Fusion (F) and large polymerase (L) protein gene of avian paramyxoviruses in domestic poultry in Kenya.
3. To determine the genetic variation in the genome of APMV strains from poultry in Kenya compared with vaccine strains.
4. To identify the signatures of selection on the genes encoding functional proteins of global APMV-1 strains.

1.4 Null Hypotheses

1.4.1 Main hypothesis

Avian paramyxovirus in circulating in domestic poultry in Kenya are homogenous strains that show no distinguishable genetic variation whose occurrence has no association with poultry management or trade factors.

1.4.2 Specific hypothesis

1. Poultry management and trade factors have no association with the occurrence of APMV in domestic poultry in Kenya.
2. The fusion and large RNA polymerase protein gene of APMV strains circulating in poultry in Kenya are homogenous and show no distinguishable genetic variation.
3. The genome of APMV strains circulating in domestic poultry in Kenya, not genetically distinguishable from other global APMV-1 strains and vaccine strains.
4. Natural selection has no influence on the diversity of lineages and proteins of global APMV-1 strains

CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

This Chapter explains theories on which guided the study and from which the study framework as drawn. The Chapter also provides the conceptual framework for the study. Previous studies conducted on avian paramyxovirus, their biological and genetic characteristics, risk factors and epidemiological dynamics are also discussed in the chapter.

2.1 Theoretical Review

This sub-section defines an overview of theories of disease causality, genetic variation and evolution in viruses and explains theories occurrence, genetic diversity and evolutionary mechanisms of avian paramyxovirus.

2.1.1 Definition of Newcastle disease

Newcastle disease occurs when a bird is infected with virulent strains of avian paramyxovirus serotype 1 (OIE, 2016). Three pathotypes of APMV-1 virus have been described based on their virulence: lentogenic, mesogenic and velogenic.

Alizon *et al.,* (2009) define virulence as the ability of a pathogen to induce the host's loss of fitness most often resulting in mortality or the degree of damage that the pathogen causes to the host where sub-lethal measures of virulence such as organ damage, weight loss among others have been used.

Virulence of APMV-1 pathotypes is determined through an *in vivo* test, the intracerebral pathogenicity index (ICPI) in day old chicks (OIE, 2016). The ICPI score is assigned from 0.0 to 2.0. APMV-1 strains with ICPI score of 0.7 are considered lentogenic while a score of more than 1.5 are considered velogenic. APMV-1 strains with ICPI scores between 0.7 and 1.5 are considered mesogenic (OIE, 2016).

Virulence can also be determined using molecular techniques whereby the amino acid residues at the cleavage site of the F protein is a virulence indicator (OIE, 2016). Lentogenic strains of APMV-1 are low virulent strains that contain monobasic or dibasic amino acid residues (^{112}G/E-K/R-Q-G/E-R^{116}) at the F protein cleavage site and Leucine (L) at residue 117. Their amino acid motif at the F gene cleavage is makes them insensitive to intracellular proteases and can only be cleaved by extracellular proteases (Ganar *et al.,* 2014). These have been used as primary ND vaccines and constitute strains such as *LaSota* strain.

Velogenic strains are highly virulent viruses with a polybasic amino acids containing lysine (K) and arginine (R) (^{112}R-R-Q-R/K-R^{116}) at the F gene cleavage site (Dortmans *et al.,* 2011). This amino acid motif renders them cleavable by furin-like proteases, which are widely available in most host cells. Therefore, the viral F protein invades a wide range of host tissues causing fatal systemic infections (Samal *et al.,* 2011).

Mesogenic strains are viruses of mild virulence that may result in less severe disease with neurological signs such as head tremor, torticollis and paralysis with lower mortality of up to 50% in younger birds (Ashraf, & Shah, 2014). These have been used as secondary live vaccines and constitute strains such as *Mukteswar* strain.

2.1.2 Role of viral components in disease occurrence

The genome of all avian paramyxoviruses encode six major structural proteins namely: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and Large RNA polymerase (L) (Samal, 2008; Lamb & Parks, 2007).

The outer surface of the envelope bilayer has spikes which are composed of two glycoproteins namely: Fusion (F) and hemagglutinin-neuraminidase (HN) proteins (Ren *et al.,* 2012). The HN and F both play essential roles in the initiation of infection (Kim *et al.,* 2011). The F protein mediates viral replication and pathogenicity (Samal *et al.,* 2013).

The inner part of the envelop bilayer is coated with the Matrix (M) protein. The M protein is thought to control viral RNA synthesis and is thus important in viral morphogenesis. It plays a key role in virus assembly and budding (Takimoto & Portner, 2004). Three other proteins, the nucleoprotein (N), the phosphoprotein (P) and large polymerase (L) form the ribonucleocapsid core.

Nucleocapsid (NP) protein forms the helical nucleocapsid of the virus while L and P proteins are associated with the transcriptive process and function as RNA dependent RNA polymerase (Cox & Plemper, 2017; Hamaguchi *et al.*,1983). The phosphoprotein (P) gene encodes the structural phosphoprotein, which serves as a polymerase co-factor thereby facilitates RNA synthesis and is essential in viral transcription and replication.

The large polymerase (L) gene synthesizes viral mRNA and assists in replication of genomic RNA. Since it is the last gene to be transcribed, the L protein plays an important role in 5´capping, methylation and poly-A polymerase activity on newly formed mRNA (Lamb & Parks, 2007; Lamb *et al.*, 2006). It is thought that the L protein has a potent role in the virulence of APMV-1 by increasing the rate of viral RNA synthesis during replication (Rout & Samal, 2008).

2.1.3 Association of avian paramyxovirus occurrence and risk factors

In an intensive poultry production system, introduction of the virulent APMV-1 is enough to result in Newcastle disease (East *et al.*, 2006). However, this is not the case in the extensive poultry production system where the occurrence of disease results from a combination of unique factors (Awan *et al.*, 1994). Management factors including live bird trade and movement constitute a higher proportion of the unique factors that influence APMV in extensive poultry production system (Awan *et al.*, 1994).

The occurrence of Newcastle disease is associated with various factors among them host factors and environmental factors (Chaka *et al.*, 2013; Njagi *et al.*, 2010*; Otim et*

al., 2007). These factors influence the introduction and maintenance of pathogens in a poultry flock and therefore indirectly dictate the disease outcome (Otim *et al.*, 2007).

The multi-causal theory postulated by Hill (1965) states that health outcomes are affected by multiple underlying social and environmental factors. To draw a conclusion of causality of certain factors, there must first be an association between the factor and the disease. Further investigation would then define the strength and consistency of such an association to draw conclusion on its causality (Hill, 1965).

A measure of association quantifies the relationship between the disease and other factors such as characteristics of the disease agent (virulence), inherent (age, sex) and biological characteristics (immune status) of the host, socioeconomic characteristics (poverty, education). Measures of association compare the probability of disease occurrence in one group exposed to a potential factor with another group not exposed to this factor (Thun & Jemal, 2003).

2.1.4 APMV control interventions and their influence in reducing APMV risks

The most important factors in preventing APMV introduction into a flock are the conditions under which poultry are reared. Biosecurity measures are practices put in place to reduce introduction of APMV into a poultry flock (Rimi *et al.*, 2017; Okeno *et al.*, 2012). In commercial enterprises, APMV-1 has been successfully controlled through vaccination and strict implementation of biosecurity. In smallholder poultry production systems biosecurity has been difficult to implement but may result in drastic reduction of disease occurrence if implemented (Conan, *et al.*, 2012; Msoffe *et al.*, 2010).

In countries in which APMV is endemic, regular vaccination is required so as to reduce disease outbreaks and losses (Copland & Alders, 2013). However, despite extensive use of vaccine, some countries still report ND outbreaks (Samuel *et al.*, 2013; Mohamed et al., 2011). Current vaccines boost the immune system of poultry and thus reduce infection. However, once challenged with a virulent virus, vaccinates, shed the

virus and could be a source of infection to susceptible birds (Grund et al., 2014; Afonso & Miller, 2013). Previous research indicates that use of current vaccines on non-vaccine related strains may change the evolution of APMVs by allowing co-infection, recombination and introduction of unique signatures on viral glycoproteins (Chong et al., 2010).

2.1.5 Mechanisms underlying the genetic diversity of APMV

Genetic diversity of APMV is a well demonstrated in the large genetic differences between APMV serotypes and within serotypes (Jeong et al., 2018; Lee et al., 2017). For APMV-1, which is the most diverse serotype, wide genetic differences have been revealed between strains in different regions of the world (Diel et al. 2012). Novel genetic variants of APMV-1 are still being discovered (Dimitrov et al., 2016b). Some of the variants are associated with increased virulence and host range (Guo et al., 2014); Gould et al., 2001).

RNA virus populations, including APMV are heterogeneous in nature with high genetic diversity. This is often attributed to their large population size in an individual, short generation times and error-prone replication of these viruses (Domingo & Holland, 1997). Garcia-Arenal *et al.,* (2001) defined genetic diversity as the probability that two genetic variants randomly chosen from a population are different. Genetic variants of viruses may present at the sequence level as changes in single bases (single-nucleotide polymorphism, SNP) up to rearrangements of the genome architecture (Sanjuan *et al.,* 2010).

The diversity at the molecular or sequence level is what enables organisms of different species to survive and adapt to changes in the environment. (Sanjuan *et al.,* 2010).Variant forms of a genetic sequence are alleles, and the position at which they occur is locus. For viruses, this includes changes in host-invasion mechanism and virulence among others. Such genetic variation in viruses may arise through two main

mechanisms mutation and genetic exchange, which are intrinsic of the virus genome and its replication and expression systems (Bull *et al.,* 2007).

Mutation is a result of errors during the replication of a genome due to mis-incorporation of nucleotides in the daughter strand that do not correspond to the template (Garcia-Arenal *et al.,* 2003). High mutation rates in RNA viruses are due to lack of proofreading activity of RNA polymerases (Holland *et al.,* 1982). Mutation is therefore the most common cause of genetic variation of RNA viruses. Genetic exchange occurs when genetic information from different genetic variants within the same host cell is switched between them to form a new variant (Simon-Loriere & Holmes, 2011).

Evolution is the change with time of the genetic structure of a population that is dependent on the fitness of an individual or genetic variant (Maynard, 1989). In an ideal population of infinite size, an estimate of the fitness of a variant is the frequency at equilibrium with which this variant is present in the progeny. The evolutionary processes that affect the frequency of genetic variants and structure of virus populations include: natural selection, genetic drift, and gene flow (Moya *et al.,* 2000; Bull *et al.,* 2007).

Natural selection is a directional process by which variants that are fittest will increase their frequency in the population (positive or adaptive selection), whereas less fit variants will decrease their frequency (negative or purifying selection) (Schneider & Roossinck, 2001). On the other hand, genetic drift occurs when the population is not large enough for each variant to have progeny, resulting in stochastic changes in allele frequencies so that variants pass to the next generation by chance (random effects), not by their respective fitness (Garcia-Arenal et al., 2001). The effects of these evolutionary forces are affected by the virus biology (host type and range), the ecological environment, and population parameters (population size and history of population bottlenecks).

2.2 Conceptual Framework

To determine the association between APMV occurrence and poultry management/ trade factors, a conceptual framework was derived from literature. The conceptual framework illustrates the potential factors that were considered in the study to explain the occurrence of avian paramyxovirus in domestic poultry in Kenya. The dependent variable was the APMV PCR test result. Avian paramyxovirus positivity was determined at individual and farm (flock)/trader level. A farm/trader who had at least one bird that tested APMV positive was considered to have a positive flock or stock of poultry (**Figure 2.1**).

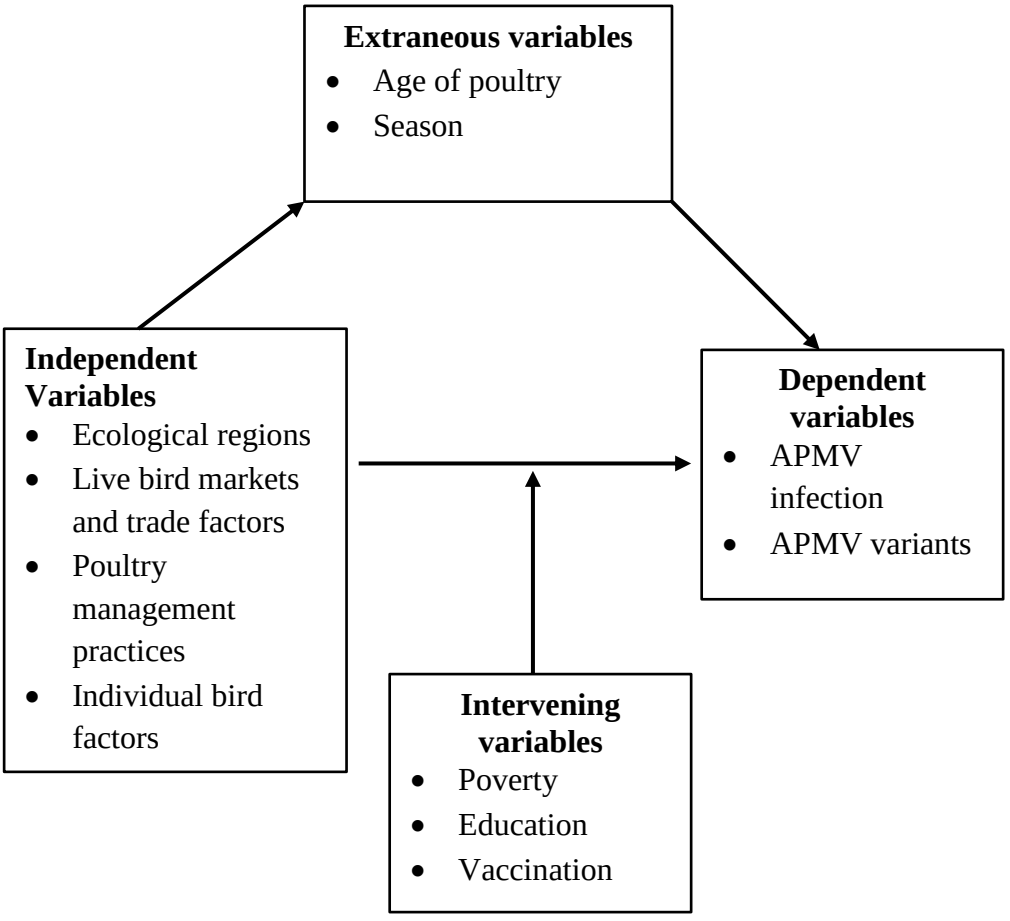

Figure 2.1: Conceptual framework of the study on risk factors of APMV

The independent variables explaining avian paramyxovirus occurrence at farm level comprised the individual bird characteristics (species, sex and breed), poultry management factors including flock size, restocking method, feeding system, confinement and waste/carcass disposal methods and ecological region.

At the live bird markets, independent variables included individual bird characteristics (species, breed and sex), market characteristics such as location, frequency of operation (daily or weekly), volume of trade, and presence of enclosures/ fixed bird-holding structures and presence of bird slaughter slabs. Trader related factors include: frequency and distance of trade, method of sourcing poultry stock, carcass/waste disposal, frequency of cleaning bird holding cages and bird transport methods.

Host of the host as well as season were considered extraneous variables and were controlled for in the study. For instance, the study targeted poultry similar age (8-10 weeks) to control for age effect. To address seasonal effects, samples were collected within the same season throughout the study. Flock immunity, education and socioeconomic status of the farmers are considered significant intervention/protective factors against risks of ND outbreak. The study also controlled for intervention variables by targeting only non-vaccinated flocks and farmers of similar socioeconomic status.

The bulk of the emergent variant APMV-1 strains have been isolated from the African continent (de Almeida *et al.*, 2013). It is thought that the poultry production system could favor co-existence of heterogeneous strains of APMV-1 which may enhance mutation and emergence of newer genotypes (Snoeck *et al.*, 2013a).

Genetic variants of APMV strains in Kenya were determined by considering the proportion of sequence variants or alleles at any particular loci. Poultry management, rrade factors, host factors and ecological location were considered to potential factors in influencing genetic variation in domestic poultry in Kenya (**Figure 2.2**). The study

therefore, compared genetic variation in individual birds, poultry farms, live bird markets and ecological zones.

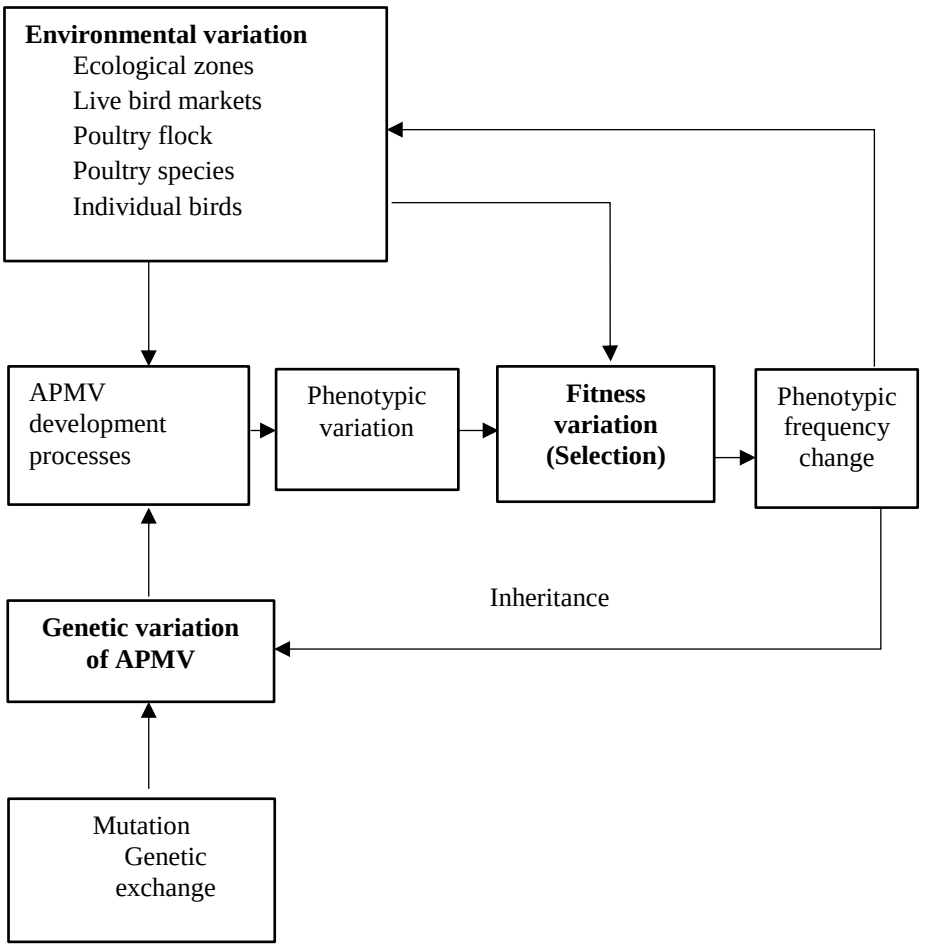

Figure 2.2 : Conceptual framework of the study on APMV diversity

2.3 Empirical Review

Previous research has examined the association between various factors in the extensive production system and the occurrence of avian paramyxovirus. Other studies have also shown evidence of genetic diversity in the Fusion and large polymerase protein gene of avian paramyxovirus strains. Studies characterizing the genome of avian paramyxovirus strains have provided evidence of existence of genetic variation shaped by environmental factors and evolutionary forces. This empirical review

section discusses the evidence in relation to aspects of avian paramyxovirus investigated in this study.

2.3.1 Factors associated with occurrence of APMV

Previous studies have shown evidence that factors such as management, host factors, ecology influence the occurrence of avian paramyxovirus. This section discusses empirical evidence of association between various factors and avian paramyxovirus occurrence.

2.3.1.1 Effect of season and Agro-ecological zones

Many studies have reported higher seasonal incidence and virulence of APMV. In Thailand, Ratanasethakul, (1989) reported cases of APMV-1 infection throughout the year with peaks in the dry season. In Bangladesh, Asadulla, (1992), reported higher incidence in winter. In Vietnam and Nepal, higher incidence of APMV-1 infection was reported in the winter and summer respectively (Mishra, 1992; Nguyen, 1992). Evidence links higher APMV-1 incidence with the dry season (Chaka *et al.,* 2014; Otim *et al.,* 2007). However, increased diseases incidence in the dry season in many African countries has been attributed to increased human movement, live bird trade and exchange among flock owners (Otim *et al.,* 2007).

In Kenya Njagi *et al.,* (2010a) found higher APMV-1 infection in the dry lower middle land zone of Eastern Kenya. Moreover, Njagi *et al.,* (2010a) associated higher infection with change in season from hot to cold weather conditions. Another study in Eastern Kenya reported a seasonal variation in APMV-1 infection with higher infection being experienced in the wet than dry season (Kemboi *et al.*, 2013).

2.3.1.2 Effect of poultry management practices

Many studies have linked management practices among smallholder farmers with increased occurrence of virulent and heterogeneous strains of APMV. In Australia East et al., (2006) found association between hygiene practices, control of human and wild

bird entry into the farm with occurrence of virulent APMV-1. Wild birds are thought to serve as reservoir hosts for APMV and their contact with poultry results in infection in poultry (Kahn & Line, 2010). In Switzerland, (Schelling *et al.*, 1999) found increased occurrence of virulent APMV-1 among poultry owners who introduced poultry or eggs into their farm and those that allowed contact between their poultry flock and poultry from other farms. In Bangladesh, Belgrad *et al.*, (2018) associated APMV-1 infection with frequency of cleaning and litter disposal methods.

Similarly, studies in Africa have found an association between APMV-1 and poultry management factors such as: source for flock restocking, flock size, cleaning frequency, water sources, and feeding methods (Chaka, et al., 2013a; Otim et al., 2007).

In Kenya, the occurrence of virulent APMV-1 has been associated with confinement of birds, mode of carcass disposal and introduction of new birds have been reported to be risk factors for ND. Presence of carrier birds, village population dynamics, keeping mixed poultry species and wild birds have also been associated with higher APMV occurrence (Njagi et al., 2010a) in Eastern Kenya. However, the scenario in other regions of Kenya is not well understood.

2.3.1.3 Effect of poultry markets and trade practices

Introduction of birds from LBMs poses an important source of APMV-1 introduction into a flock (Chaka, *et al.*, 2013a; Otim *et al.*, 2007). Informal live bird markets (LBM) that may enhance movement of infected birds and spread of APMV are rampant in Africa and may be important in the epidemiology of APMV (Eze *et al.*, 2015; Jibril *et al.*, 2014). Factors in LBMs that predispose to APMV-1 infection are not well understood. However, poor biosecurity in LBMs is thought to be a major factor that enhances transmission and spread of poultry diseases (McCarron *et al.*, 2015; Kirunda *et al.*, 2014a; Mulisa *et al.*, 2014).

Movement of birds such as migratory birds, have been thought to introduce APMV into new areas. The potential of racing and captive birds in introducing APMV-1 is highlighted by panzootics that have occurred since 1980s (Ashraf, & Shah, 2014). Trade in birds of recreation has also been implicated in introduction and spread of APMV-1 in European countries (Alexander, 2011). In developing countries, where the major method of trade is by live bird poultry markets. Spread of APMV through live bird trade is common (Barman *et al.,* 2016; Abolnik *et al.,* 2012). In Kenya, no study has attempted to associate trade factors and occurrence of virulent APMV.

2.3.1.4 Effect of host factors

Previous studies have shown that avian paramyxovirus occurrence is influence by host factors such as: age structure and immunity, concurrent infections and breed (Martin, 1992). Young birds are more susceptible to APMV-1 infection. The multi-aged structure in smallholder poultry shows varying age susceptibility to APMV-1. This suggests the persistence of APMV-1 infection in smallholder poultry throughout the year. Flock immunity plays an important role in APMV-1 infection. Concurrent infections from bacteria, viruses and parasites impair the immunity of poultry (Awan *et al.,* 1994). This increases the occurrence of APMV-1 in a flock. APMV-1 susceptibility varies in different breeds and species of poultry. In Kenya, Njagi *et al.,* (2010a) found higher APMV infection in cocks and younger birds.

2.3.2 Genetic diversity in APMV fusion and large polymerase protein gene

Molecular diversity of APMV strains has been based on the F protein gene which displays the greatest variation than the other genes and is thus important to study the diversity in closely related APMV populations (Diel *et al.,* 2012a). The L protein gene is also thought to have sufficient genetic variation (Fuller *et al.,* 2010) and has also been used in genetic characterization of APMV strains.

Studies from across the world have established that APMV-1 is the most diverse serotype of APMV (Afonso & Miller, 2013; Miller *et al.,* 2010). Genetically, strains

of APMV-1 have been classified into two clades: Class I and II. Classes I are mainly avirulent viruses that have been recovered primarily from waterfowl and shorebirds (Kim *et al.,* 2007 ; Seal *et al.,* 2005).

Class I strains are characterized by low genetic diversity and are classified into only one genotype with three sub-genotypes (Ia, Ib and Ic). On the other hand, Class II strains consist of both avirulent and virulent viruses which have been isolated from domestic fowl and some wild birds (Lee *et al.,* 2009; Aldous *et al.,* 2003). Class II strains of APMV-1 are more diverse genetically. A previous study by (Aldous *et al.,* 2003b) classified class II strains into six (1-6) lineages based on the fusion gene.

A more recent classification system by Diel *et al.,* (2012a) has used the Fusion gene to re-classified the class II strains into fifteen genotypes. At present, novel APMV-1 strains have been recovered across the world and have been classified into three other genotypes (XVI to XVIII) (Almeida *et al.,* 2013). The genotypes classified by Diel *et al.,* (2012a) correspond well with the earlier lineage classification by Aldous *et al* (2003) where Lineage 1, 4 and 5 were re-classified to genotype I, II, VI and VII respectively. The genotypes III, IV, V and VIII were earlier considered as sub-lineages of lineage 3 where, 3a, 3b, 3c and 3d correspond to III, IV, V and VIII respectively (Kim *et al.,* 2007).

In Africa, molecular studies on APMV-1 are emerging. However, there is still insufficient information on molecular characteristics of APMV-1 in some African countries. A study on isolates from Southern Africa as well as from Namibia, Zambia, Zimbabwe and Mozambique revealed the presence of genotypes VIII and VII (Abolnik *et al.,* 2018; Molini *et al.,* 2017). In Zambia, genotype XIII has also been isolated from poultry (Abolnik *et al.,* 2015).

A comparative analysis of APMV-1 sequences from West Africa indicated that the viruses correspond to genotypes I, VI and novel genotype XVIII (Snoeck *et al.,*

2013b). Novel genotypes XIV and XVII have also been isolated from Western and Central African countries (Snoeck *et al.,* 2013b; Cattoli *et al.,* 2010).

Isolates from Ethiopia were assigned to genotypes VII and VI (Damena *et al.,* 2016; Mulisa *et al.,* 2014; Chaka *et al.,* 2013b). Isolates from Tanzania were assigned to genotypes I, V and VIa respectively (Yongolo *et al.,* 2011). Previous studies reported that isolates from Sudan belonged to genotype VII and VI (Elmardi *et al.,* 2016; Hassan *et al.,* 2010). A study in Eastern Uganda in 2001 revealed that they belonged to genotype VIa (Otim *et al.,* 2004). A more recent study in Uganda isolated strains of genotype Vd from poultry in LBMs (Byarugaba *et al.,* 2014).

In Kenya, little is known of the APMV-1 circulating in poultry. An isolate from wild birds was assigned to genotype VI (Obanda *et al.,* 2016). Isolates from Kenya in 2010 (available in GenBank accessions: *JQ217418, JQ217419 and JQ217420*) were assigned to genotype Vd (Byarugaba *et al.,* 2014).

Another study in Kenya reported presence of APMV-2 and APMV-4 in wild water birds (Obanda *et al.,* 2016; Kasiiti, 2000). However, the diversity of APMV in domestic poultry in Kenya is poorly understood. No study has genetically characterized APMV strains present in domestic poultry in Kenya.

2.3.3 Genomic characterization of APMV

Previous studies have shown marked differences have been observed in genome lengths of various APMV serotypes as follows: 14904 nt (APMV-2), 15064 nt (APMV-4), 15186, 15192 and 15198 nt (APMV-1), 15342 nt (APMV- 8), 15438 nt (APMV-9), 15480 nt (APMV-7), 16272 nt (APMV-3), 16236 (APMV-6), and 17262 nt (APMV-5) (Suarez *et al.,* 2013). APMV-1 strains isolated globally have three genome size groups: 15,186 nucleotide (nt), 15,192nt or 15,198nt (Ganar *et al.,* 2014; Czegledi *et al.,* 2006).

Studies have shown that the APMV genome is composed of 6 to 10 tandemly linked genes that encode at least 7 to 12 different proteins and 4-57nt intergenic regions. The APMV genome 3′ end and 5′ end contain short extra-genic sequences known as 55nt 'leader' and 552nt 'trailer' sequences respectively (Noton & Fearns, 2015). Whole genome sequence analysis of APMV-1 revealed unique signatures in the genes of strains classified in the same genotype (Susta *et al.,* 2001).

In Africa, very few studies have characterized the whole genome of APMV (Cattoli *et al.,* 2011; Snoeck *et al.,* 2013). In Kenya, one study has characterized the whole genome of APMV-1 belonging to genotype VI (Obanda *et al.,* 2010). However, despite suggestion by Byarugaba *et al.,* (2014) to classify APMV-1 from Kenya into a new sub-genotype Vd, no study in Kenya has attempted to characterize the Kenyan strains of avian paramyxovirus.

2.3.4 Evolutionary dynamics of APMV-1 genes

Few studies have elucidated the evolutionary mechanisms of APMV-1 genes. In one of the studies, a high rate of evolution of $1.78x10^{-3}$ substitutions per site per year was found in the Fusion gene (Sonora et al., 2015). Fan *et al.,* (2017) revealed a rapid evolution rate of $1.059x\ 10^{-3}$ in the NP gene of APMV-1. However, Fan and colleagues noted that even with such high evolution rate, the NP gene was well conserved in structure and function (Fan et al., 2017). This indicates the presence of evolutionary forces that shape the structure of APMV. Miller *et al.,* (2009) found that both positive and negative selection were acting on APMV-1 proteins. The study by Miller *et al.,* (2009) characterized the evolutionary forces acting on all APMV-1 proteins. However, in the study, the newer genotypes of APMV-1 were not included. It would be more insightful to include the newer genotypes from Africa and other regions of the world.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Introduction

This Chapter outlines the research methods followed in the study. It provides information on the general methods including study sites, study clearance and approval procedures, research design, sample size determination and describes the poultry sampling procedure. Objective specific research methods are then described, outlining the pre-testing, validity, reliability and administration of the instrument data collection procedures and data analysis.

3.1 General methodology

3.1.1 Location of study sites

The study was carried out in smallholder poultry farms in Bungoma county in Western highland regions; Busia and Siaya Counties in Lake Victoria Basin; Mombasa, Kilifi and Lamu Counties in Coast region. The sampled wards included Chwele, Cheptais, Kimilili, Kabuchai and Malaba in Bungoma county; Bunyala, Ageng'a, Ugenya, Chakol and Amukura in Lake Victoria Basin and Kakoneni, Likoni, Mtepeni, Dabaso and Mkomani in Coastal region (**Figure 3.3**).

Live bird markets (LBM) were also sampled in five ecological zones of Kenya namely: Western Highlands, Lake Victoria Basin, Coastal region, Eastern and Nairobi metropolitan. The sampled LBMs include: Chwele, Bungoma, Kitale, Kericho, Kakamega and Bomet in Western Highlands, Bumala, Kisumu, Homabay, Migori in Lake Victoria Basin, Majengo, Marikiti, Kilifi in Coast region, Burma, Kibra, Kawangware, Machakos and Kitengela in Nairobi metropolitan and Meru, Embu, Makueni Eastern regions (**Figure 3.3**).

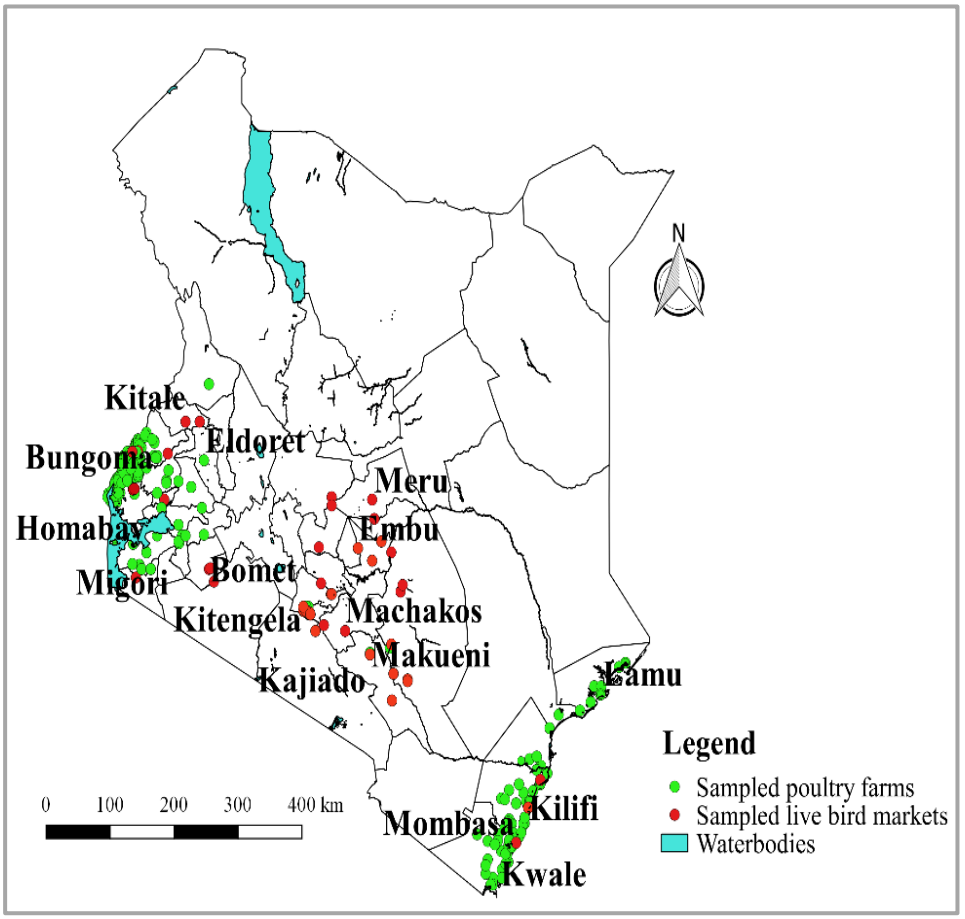

Figure 3.3: Geographical location of sampled LBMs and poultry farms

3.1.2 Description of study sites

The study collected data from five sampled ecological zones of Kenya including: Western Highlands, Coast, Lake Victoria Basin, Eastern and Nairobi metropolitan as shown on **Figure 3.4.**

The ecological zones were defined as previously described (Otolo & Wakhungu, 2013; FAO, 1996). Briefly, Western Highlands is a high potential agricultural zone with high altitude varying from 1,400 to 1,700 meters above sea level with rainfall ranging from 1,500 to 1900 millimeters per year and temperatures ranging from 5°C to 22°C (Place *et al.*, 2006). Dairy and poultry farming are common in these areas (MoALF, 2015).

Approximately 72% of households keep poultry and a third of Kenya's total poultry are kept in this region (KNBS, 2017). Poultry production is based mainly on smallholder extensive system of local poultry types.

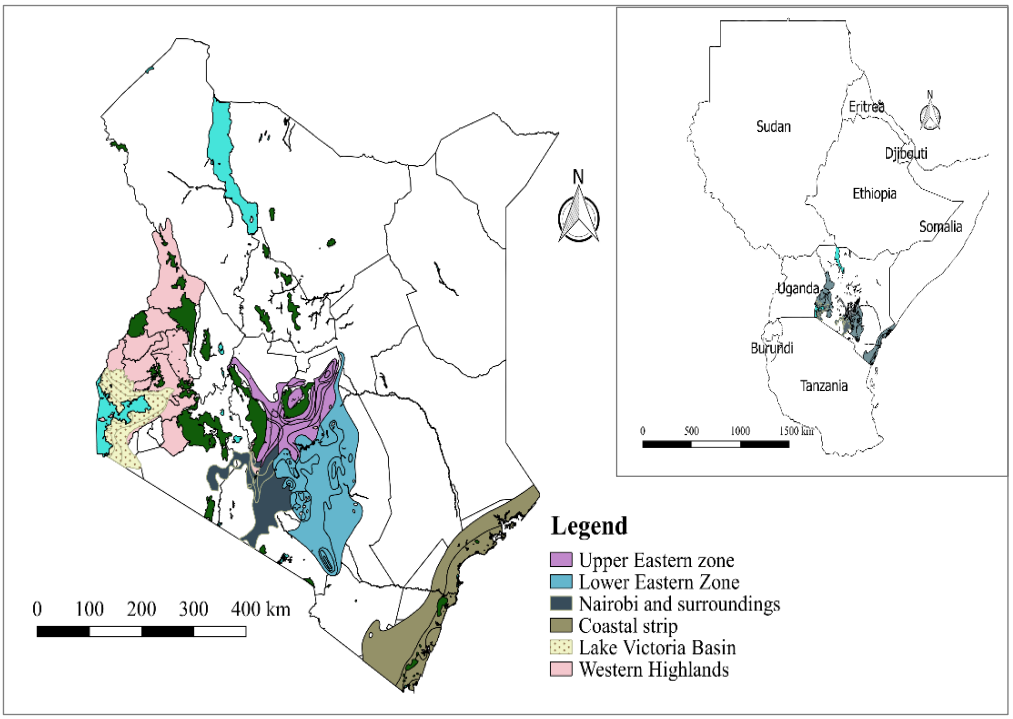

Figure 3.4: Map of selected Agro-ecological zones in Kenya

Lake Victoria Basin is located in Western Kenya within the drainage basin of Lake Victoria. It lies between the Rift Valley to the East and Lake Victoria to the West (Ochieng, 2002). It includes five counties including Busia, Siaya, Homabay, Migori and Kisumu. The rainfall ranges from 750-900mm per year and temperatures between 22-32°C (Kizza *et al.,* 2009; Ochieng, 2002). Livestock production is an important economic activity. Majority of livestock being local types and their crosses with exotic breeds. Poultry production is a very important part of the livelihoods of households in the Lake Victoria Basin (Atela *et al.*, 2016; Okeno *et al.*, 2012; Olwande *et al.*, 2010).

Coast region lies within the Coastal lowlands, which may extend from sub-humid to arid zones. The coastal region is located in the South Eastern part of Kenya, bordered to the East by the Indian Ocean. Average annual rainfall is poorly distributed and

unreliable ranging from 500 to 900 mm. The mean annual temperature ranges between 24 ºC and 33 ºC. Livestock production, especially poultry, is important in the region (Waaijenberg, 1994). Poultry production in the Coastal region is important for the hospitality industry that accrues from both domestic and international tourism (Mshenga, 2010).

Nairobi metropolitan is a rapidly growing area in Kenya that includes the counties of Nairobi City, Kiambu, Murang'a, Machakos and Kajiado (KNBS, 2016). Rapid population growth in the region offers market to agricultural produce from various regions of Kenya (GoK, 2010). Poultry from as far as Western and Eastern Kenya are sold in live bird markets in Nairobi (McCarron *et al.*, 2015). Nairobi metropolitan area is therefore a hub for poultry trade with birds sourced from different regions of Kenya for final market in Nairobi The volume of trade is large with some markets within the city center selling as much as 600 birds per day. Live bird markets in the region are both formal and informal (McCarron *et al.*, 2015).

Eastern region includes the Chalbi Desert, Mount Kenya and the Eastern part of Lake Turkana. The region covers the eight counties including Marsabit, Isiolo, Meru, Tharaka Nithi, Embu, Kitui, Machakos and Makueni. Rainfall in the lower eastern area is low and unreliable Due to the semi-arid and arid nature of most parts of the region, livestock production is also an important enterprise (Ahuya *et al.*, 2011). Households in the region commonly keep cattle, sheep and goat.

Poultry production is common with farmers in Eastern region who keep both local and exotic poultry types for close market in Nairobi City (Mailu *et al.*, 2012). In the region, poultry is kept both under extensive management and intensive commercial production systems (Kingori *et al.*, 2010; Nyaga, 2007). In total, poultry population in the region is estimated at 5 million, with majority being found around Mt Kenya and lower Eastern zones (KNBS, 2017). Live bird markets in the urban areas of Eastern region are common and serve as converging centers for birds from the rural parts in transit to Nairobi or Mombasa Cities (Mailu *et al.*, 2012).

3.1.3 Study clearance and permits of compliance

Director of Veterinary Services at the Ministry of Agriculture, Livestock and Fisheries, Kenya, approved the study. In addition, the Institutional Animal Care and Use Committee (IACUC) of KALRO approved poultry handling procedures used in the study. Permission was also sought from County Governments, poultry farmers and traders prior to their inclusion in the study and their information and responses were treated with strict confidentiality.

3.1.4 Research design

A cross-sectional research design was used where host factors, management, trade factors and ecological zones were assessed in relation to their association with avian paramyxovirus occurrence in poultry in Kenya. Data on these potential associate factors of avian paramyxovirus was collected from poultry farms and live bird markets in five regions of Kenya between November 2015 and March 2016.

Poultry were also sampled to characterize the presence and genetic nature of avian paramyxovirus. The design enabled observation of multiple variables in the poultry farms and LBMs at the specific point in time and has been used in a previous study to describe the relationship between the management practices and APMV-1 occurrence in the study (East et al., 2006)

3.1.5 Sample Size Determination

One thousand two hundred and twenty four (1,224) birds were sampled from 225 poultry farms. The minimum sample size from poultry farms for each of the three zones was calculated based on the formulae (Charan & Kantharia, 2013).

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Where n = Sample size, Z = Z statistic for a level of confidence P = Expected prevalence d = Precision. Prevalence of ND was estimated to be 20% from a previous

study (Olwande *et al.,* 2016; Njagi *et al.,* 2010b), a confidence level of 95% and precision of 5% was used. Precision indicates the level of confidence of findings. A similar precision was used in a similar study in Ethiopia(Chaka *et al.,* 2013). A target minimum sample of 320 birds per region was used. In total, samples were collected from 922 chicken, 136 ducks and 74 turkeys from BPF (**Table 3.1**).

Table 3.1: Number of birds sampled in poultry farms in three regions of Kenya

Region	**Number and species of poultry sampled on farms**				
	Poultry farms				
	Chicken	Duck	Turkey	Pigeon	**Total**
Lake Victoria Basin	288	27	24	21	**360**
Western Highlands	324	52	30	27	**433**
Coast	310	57	20	44	**431**
Total	**922**	**136**	**74**	**92**	**1224**

In LBMs, 482 birds were sampled from 124 traders. The number of birds sampled in each market was calculated assuming an average market size of 30 birds, a minimum expected prevalence of 5% and confidence interval of 95%.

However, in each of the seven live bird markets that had high bird turnover, seven traders were sampled. This included; Kisumu, Kericho, Majengo, Burma, Meru and Chwele markets. In total, 454 chicken (including tissues from 33 chicken) and 28 Ducks were sampled (**Table 3.2**).

Table 3.2: Number of birds sampled in live bird markets in Kenya

Region	**Number and species of poultry sampled in LBM**		
	Chicken	Duck	**Total**
Lake Victoria Basin	95	4	**99**
Western Highlands	122	15	**137**
Coast	71	9	**80**
Eastern	64	0	**64**
Nairobi metropolitan	102	0	**102**
Total	**454**	**28**	**482**

3.1.6 Sampling Technique

To collect data from live bird markets and poultry farms, a multistage sampling procedure was employed to ensure a fairly equal representation of the study variables. Firstly, three regions of Kenya namely: Western Highlands, Lake Victoria Basin and Coastal Strip. were selected purposively due to their high population density of poultry (KNBS, 2017). From each zone, five wards with the highest poultry population were purposively selected. Within each ward, poultry farms were selected using a simple random sampling procedure. A list of poultry farmers who did not vaccinate their flock was obtained from the County Veterinary and livestock personnel.

Simple random sampling involved writing names poultry farmers from each ward on pieces of paper, which were folded and put in a basket. The papers were then shuffled, picked, recorded and placed back into the basket until 15 farmers from each county were obtained. From each farm, four adult birds were selected using a systematic random sampling technique where a sampling interval of 2-10 was calculated based on the number of poultry of each species at the farm. Maize grain was thrown one at a time to gather the birds. Depending on calculated sampling interval, each 2^{nd} to 10^{th}

bird to eat the grains was selected for sampling. For farms with different species of poultry, two birds of species other than chicken were also sampled.

Live bird markets (LBMs) were randomly selected from five purposively selected ecological zones namely: Western Highlands, Lake Victoria Basin, Coastal region, Eastern and Nairobi metropolitan based on high poultry population and high poultry trade (in Nairobi Metropolitan and Eastern) (KNBS, 2017). Within each zone, a list of live bird markets was obtained from the County personnel. Simple random sampling was used to select live bird markets, this involved writing names of LBMs on pieces of paper, which were folded and put in a basket. The papers were then shuffled, picked, recorded and placed back into the basket until 21 LBMs were obtained. Within each selected LBMs a list of names of regular traders was obtained from the market officials, a similar simple random sampling technique was again utilized to select five traders from each market. From each trader, four (4) birds were randomly selected by assigning a number to bird. Every 2^{nd} to 5^{th} bird was the selected for sampling depending on the number of birds the trader had at the sampling time.

3.1.7 Data collection procedure

Birds selected for sampling were physically examined for any signs suggestive of ND infection (diarrhea, ocular and nasal discharge, respiratory distress, nervous signs or sudden death). Tracheal and cloacal swabs were collected from each individual bird using sterile Rayon and Dacron swabs. Two swabs each were dipped and swirled into the trachea and cloaca through the oral and anal cavities respectively. Each swab sample was collected in duplicate in 1000µl of RNAlater® (Thermo Scientific, Walton, Massachusetts, USA). A total of 1128 and 449 pairs of swab samples were collected from poultry farms and LBMs respectively.

During sampling, freshly dead birds (not more than 12 hours) were collected for a post-mortem examination at the nearest Veterinary Investigative Laboratories (VILs). One hundred and twenty nine (129) such birds (including 96 from poultry farms and 33

from LBMS), from where tissues were collected in RNAlater® (Thermo Scientific, Walton, Massachusetts, USA) were involved. Tissue samples collected included lungs, trachea, liver, spleen, proventriculus, gastrointestinal tract and brain tissue. Tissue samples from an individual bird were collected in duplicate.

During sample collection, a bar-coding labeling system was utilized. The bar code labels were pre-printed with the sample code to minimize writing errors and ink rubbing off during shipping or storage. The sample codes were serialized and each poultry species bore a unique prefix; for instance C for chicken, G for geese, GF for guinea fowls, D for ducks. On each code, the poultry species prefix was preceded by a unique code: LM for live bird markets for example LMC0400520 and PF for poultry farms for example PFG0400525. Each region also had a unique code.

Matching labels were fixed to the cryotubes for tracheal and cloacal swab collection and the sample collection form. The identity of the label was checked twice to ensure it was identical to the forms and sample vials.

The collected samples were transported a cool box and stored at -20°C and RNA extraction from one of the sample pairs was carried out within 72 hours of sample collection. The second sample pair from each bird was stored at -80°C for further laboratory analysis.

3.2 Risk factors associated with APMV occurrence

3.2.1 Characterization of risk factors

An interview schedule in the form of a semi-structured questionnaire was developed for collection of data on the management and trade practices in poultry farmers and live bird markets. Sample of the two questionnaires are attached in Appendix 1 and Appendix 2.

The questionnaire for poultry farmers captured data relating to feeding, rearing, housing practices, production parameters, chicken morbidity and mortality, common

diseases, disease control and management as well as health and disease status of birds were collected from poultry farmers (Appendix 1).

On the other hand, the poultry traders questionnaire captured data related to market characteristics, trade practices and networks, sources of birds and other trade dynamics, poultry housing and sanitation practices as well as health and disease status of birds in the market (Appendix 2). These questionnaires were piloted among farmers and traders respectively who were not part of the selected participants.

3.2.1.1 Pilot study

A pilot study involved sample of 30 poultry farmers and 20 traders of the same characteristics as the target group. The pilot test was carried out in Kilifi and Busia counties. The objective of the pilot study was to assess the reliability and validity of the questionnaire and also familiarize with the process and sampling logistics in order to improve the larger study.

3.2.1.2 Reliability of the questionnaire

For purposes of this study, the reliability of a measurement instrument was defined as the extent at which it stably assigns a similar score to subjects with common values (Drost, 2011). Responses to each question was obtained from 30 poultry farmers and 20 traders with similar characteristics with the target group. The agreement of their responses was scored on a five point Likert response scale from “strongly agrees” to “strongly disagree” (Likert, 1932).

A Cronbach’s coefficient alpha (Cronbach, 1951) was calculated for each response and overall for all the responses in SPSS Software version 21.0 (Kirkpatrick, 1958). For the management characteristics questionnaire, 19 items (questions) were obtained while the trader characteristics questionnaire had 18 items. The overall alpha for all the items for both questionnaires 0.85 and 0.76 for the farmers and traders

questionnaires respectively (**Table 3.3**). This was very high and indicated strong high internal consistency.

Table 3.3: Overall reliability statistics for questionnaires used in the study

Tool	**No of items**	**[1]Cronbach Alpha**	**[2]Scale statistics**		
			Mean	**Variance**	**SD**
Poultry farmers questionnaire	19	0.85	62.41	73.05	6.23
Traders questionnaire	18	0.76	51.21	67.23	7.19

[1]Overall Cronbach's alpha coefficient is shown for both questionnaires;[2]Scale statistics shows the overall Likert scale on the agreement of all responses

3.2.1.3 Validity of the questionnaire

Six experts familiar with poultry farmer characteristics, traders' dynamics and livestock disease epidemiology, assessed the validity of questionnaire. These experts reviewed the questionnaire and scored each question for readability, clarity and comprehensiveness on a dichotomous score: 0-unfovarable and 1-favourable.

The rate of agreement between the raters was then assessed using as described previously (Sangoseni *et al.*, 2013). A content validity Index (CVI) score was then generated where a score greater that 0.78 (Sangoseni *et al.*, 2013) was indicative of a significant level for inclusion of an item (question). The questions retained in both questionnaires had CVI score greater than 0.81.

3.2.1.4 Interview procedures

After piloting the questionnaires selected study sites were visited to obtain prior permission from the county officials, county livestock and veterinary officials, market officials, community leaders, farmers and traders to collect necessary data. Subsequently details about the investigations, nature and purpose of the study were discussed with the county officials, community leaders, farmers and traders.

After establishment of good rapport with the county officials, community leaders, farmers and traders, a management practice and trade practices characterization. In the second phase of the study, the two questionnaires were administered to individual selected farmers and traders. Each session to the questionnaire interviews ended with a vote of thanks to the farmer/trader. Questionnaires were administered to two hundred and twenty five (225) poultry farmers in three regions and one hundred and twenty four (124) traders in five regions of Kenya.

3.2.2 Screening for APMV presence

3.2.2.1 Reliability of the laboratory techniques

Optimization of each process was done to ensure their reliability. In addition, to overcome the issue of RNA contamination and ensure maximum yield, a conventional phenol-chloroform based RNA extraction method as utilized. Two additional ethanol washes were done to remove residual salts from isopropanol and improve on purity. To maximum yield and specificity, primers optimization was done by Gradient PCR on a Bio-Rad cycler to determine the annealing temperature for each primer pair.

3.2.2.2 Validity of laboratory techniques

To ensure validity of the PCR, materials used were sourced from renowned companies that provide quality reagents. Similarly, the study utilized Large polymerase protein gene primers published by Tong *et al.,* (2008) and fusion gene primers published by Liu *et al.,* (2008). The F gene primers were validated by (Samar *et al.,* 2017). In addition, before their use, these primers were subjected to a primer blast analysis (Ye *et al.,* 2012) to establish whether they could detect a broad range of APMV viruses.

3.2.2.3 Viral RNA extraction

In the laboratory, 1128 tracheal and cloacal swab samples from poultry farm as well as 449 swab samples from markets were processed separately. In addition, the one hundred and twenty-nine (129) pools of tissue samples were also processed separately. To process swab samples, the cryotubes with swabs were vortexed vigorously for 2

minutes and then centrifuged at 300 x g for 10 minutes in a pre-chilled centrifuge at 4^{o}C. The supernatant was aliquoted in 500 microliters (µl) volumes and transferred into sterile 2000µl cryotubes. To one aliquot, 1000 microliters (µl) of Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added.

One hundred milligrams (100mg) of each pooled tissue sample was crushed using a sterile mortar and pestle and each crushed tissue sample was aliquoted into two and placed in two sterile 2000µl cryotubes. RNA extraction was done using one of the aliquots into which 1000µl of Trizol reagent was added. The other aliquot was stored at -80°C. Trizol LS reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction following the manufacturer's instructions.

Briefly, 1000µl of Trizol LS reagent (Invitrogen, Carlsbad, CA, USA) was added to the processed samples and vortexed vigorously for 10 minutes at room temperature. To the mixture, 200µl of chloroform was added and vortexed vigorously for 10 minutes at room temperature and then centrifuged at 12,000 rtp at 4°C for 15 minutes in a pre-chilled centrifuge. The aqueous form was then transferred into a labelled sterile cryotube and 500 µl of Isopropanol-2 was then added to it, incubated at room temperature for 10 minutes, and centrifuged at 12000 rtp at 4°C for 10 minutes.

The supernatant was then discarded and the RNA pellet air-dried for 15 minutes. The RNA pellet was re-suspended in 70 µl of DEPC-treated water. The quantity of the extracted RNA was determined using a Nano Drop® ND-1000 spectrophotometer (Thermo Scientific, Walton, Massachusetts, USA) and the integrity of RNA was visualized by electrophoresis in a 1.2 % formaldehyde agarose gel stained with Gel Red. The RNA was stored at -80°C in aliquots of 100µl until use.

3.2.2.4 Reverse transcription

Complementary Deoxyribonucleic acid (cDNA) was synthesized using 5microlitre of eluted RNA with random hexanucleotides and Superscript® III (M-MLV) reverse transcriptase of the First strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA)

following the manufacturer's instructions. Briefly, for each reaction, 5µl of sample total RNA was combined with 3 µl of 50ng/µl of random hexamer; 1µl of 10 mmoles dNTP mix, 2µl of DEPC treated water and heated at 65°C for 5 minutes. The mixture was placed on ice immediately for 1 minute to anneal the primers to the 3' terminal sequence of the RNA.

The Superscript™ III reverse transcription mix was then prepared according to the manufacturer's instructions. Each reaction containing; 1µl of 50 U of Superscript™ III Reverse Transcriptase, 1µl of 40 U RNaseOUT™ Recombinant Ribonuclease Inhibitor, 2µl of 0.1 M DTT, 2µl of 10X First strand Buffer and 4µl of 25mM $MgCl_2$ was annealed at 25°C for 10minutes, extended at 42°C for 50 minutes. Thereafter, the reverse transcription enzyme was inactivated by incubating at 70°C for 15 minutes and chilling at 4°C. To digest the remaining RNA from the synthesized cDNA, 1µl of RNAse H was added to cDNA template and incubated at 37°C for 20 minutes.

3.2.2.5 APMV amplification

The complementary DNA samples were then tested for the presence of APMV by two methods. Firstly, a semi-nested Polymerase Chain Reaction (PCR) was carried out to partially amplify the Large polymerase protein gene (L gene) of APMV as a 221 base pair (bp) fragment using a set of previously published APMV universal primers (Tong *et al.,* 2008). The PCR primers are shown on **Table 3.4**.

PCR reactions were carried out in a volume of 50µl containing 25µl of 2X DreamTaq™ Green PCR MasterMix (Thermo scientific). DreamTaq™ contains DNA polymerase, 2X DreamTaq Buffer, 4mmoles $MgCl_2$ and 0.4 mmoles each of dNTPs. To PCR MasterMix, the 0.5µl of either Forward primer, 0.5µl of reverse primer, 19µl of PCR water and 5µl of cDNA or first round PCR product were added.

Table 3.4: Primer sequence information for detection of APMV

Forward primer sequence (5'-3')	**Reverse primer sequence (5'–3')**	**Gene**	**Size (bp)**
[1]GGTTATCCTCATTTGTTTGAGTGGATACA	[1]GCAATTGCTTGATTTTCICCGTCAAC	L-	221
[2]ACACTCTATGTTGGCGACCCATTCAACCC	[2]GCAATTGCTTGATTTTCICCGTCAAC	L	221
[3]ATGGGC(C/T)CCAGAC(C/T)CTTCTAC	[3]CTGCCACTGCTAGTTGTGATAATCC	F	535

[1]Primer set for first PCR reaction for universal APMV L gene amplification (Tong *et al.,* 2008); [2]Primer set for second semi-nested PCR reaction for universal APMV L gene amplification (Tong *et al.,* 2008);[3]Primer set for PCR reaction for APMV-1 F gene amplification (Liu *et al.,* 2008)

The cycling conditions used were as specified by Tong et al (2008) which included: an initial denaturation at 95°C for 3 min, 40 cycles of amplification at 95°C for 30s, 49°C for 30s and 72°C for 1 min and final extension at 72°C for 10min for both the first and second PCRs. The amplified products were visualized by agarose gel (1.5% w/v) electrophoresis and UV illumination after staining with Gel Red™ (Biotium). One kb and100 bp DNA Ladder was used to identify the approximate size of the molecule run on a gel.

Secondly, a conventional PCR was used to test samples for the presence of APMV-1. This involved partial amplification of the Fusion gene using degenerate primers previously published by (Liu et al., 2008) which targets the cleavage site of the Fusion protein gene (**Table 3.4**). The fusion gene region amplified by this primer set has been used previously to detect a wide range of APMV-1 (Shabbir et al., 2013).

The PCR was performed using Taq DNA polymerase and 5µl of cDNA as described by Liu et al (2008). Briefly, the cycling parameters started with a denaturation step of

95°C for 3 min and subsequent 35 cycles of 94°C for 1 min, 50°C for 1 min 72° C for 3 min and a further extension of 72° C for 10 min. The final amplified products (1 µl) were visualized by agarose gel (1.5% w/v) electrophoresis and UV illumination after staining with Gel Red™ Nucleic acid gel stain (Biotium).

3.3 Diversity of Fusion and Large polymerase protein genes

3.3.1 Purification of PCR products

Amplified partial APMV L gene and APMV-1 F gene products were purified. These products were obtained from poultry samples collected from farms and live bird markets (LBMs) in five regions of Kenya. Purification was done using a QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instruction.

Briefly, 50µl of the PCR products were combined with 50µl DNA binding buffer after addition of 10µl 3M sodium acetate and thorough mixing, the mixture was added to a purification column. The binding buffer contains an agent that denatures proteins and promotes the binding of DNA to the silica membrane in the column. Impurities were then removed from the silica membrane by washing the column twice with wash buffer. With each wash centrifugation were done at 12000rpm for 1 minute at room temperature and the flow-through discarded.

Purified DNA was then eluted from the column using 30µl of elution buffer and the DNA concentration and quality was checked using Nano drop® ND-1000 Spectrophotometer (Nanodrop Technologies, DE, USA). The concentration of all purified products were above the recommended 35ng/ul for sequencing. In addition, approximately 5ul of the aliquot was electrophoresed in a 2% agarose gel containing GelRed and prepared in 1 x TAE buffer to confirm presence of purified amplicons. The bands were visualized under a UV transilluminator.

3.3.2 Sequencing of the large polymerase (L) and Fusion (F) gene

Purified amplified fragments of the L gene and F gene were sequenced on an automated ABI 3730 DNA Analyzer using dye terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, U.S.A.) (Church & Gilbert, 1984; Maxam & Gilbert, 1977; Sanger & Coulson, 1975). For each PCR product, sequencing was done in both directions using the same primers used for PCR reactions.

3.4 Determination of genome characteristics

The samples that tested positive for APMV-1 following amplification of the partial Fusion gene and successful sequencing of the partial F gene were selected for whole genome sequencing. Two samples of each identified unique haplotype were selected from the samples that tested positive with the Fusion gene assay. For genome characterization, the samples were selected based on the Fusion gene assay because of its higher sensitivity ((Roy, 2012). The selected samples were those that had good quality RNA whose quality was based on the following measures:

1) 260/280 purity index ≥ 2.0 on spectrophotometer (ND-1000, Nanodrop Technologies, USA);
2) RNA quantities ≥ 35ng/ul on Qubit fluorimeter (Thermo Scientific, Walton, Massachusetts, USA) using Quant-IT RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and the
3) good integrity as visualized by electrophoresis in a 1.2 % formaldehyde agarose gel stained with GelRed
4) Bioanalyzer measurements was greater than (RIN> 8).

3.4.1 Viral RNA extraction

The selected swab and tissue samples were processed for RNA extraction. The cryotubes with swabs were centrifuged at 300x g for 10 minutes in a pre-chilled centrifuge at 4^{o}C, vortexed and 1ml of the supernatant was aliquoted in 0.5ml volumes and transferred into sterile 2ml cryotubes. To one aliquot, one milliliter of Trizol

reagent was added. RNA was extracted using Trizol LS reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

The concentration and purity of extracted RNA were measured using a spectrophotometer (ND-1000, Nanodrop Technologies, USA) and Qubit fluorimeter (Invitrogen, Carlsbad, CA, USA) using Quant-IT RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and the integrity of RNA was visualized by electrophoresis in a 1.2 % formaldehyde agarose gel stained with GelRed and also using the Bioanalyzer. RNA extracts were selected for library preparation when the 260/280 purity index was equal to or greater than 2.0 and the integral RNA in electrophoresis and Bioanalyzer measurements was greater than (RIN> 8). The concentration and purity of the extracted RNA was determined using a Qubit fluorimeter (Thermo Scientific, Walton, Massachusetts, USA) before storage at -80°C until use.

3.4.2 Library preparation

Library preparation was done on total RNA using TruSeq RNA Sample Preparation Kit v2 (Illumina, USA) according to the manufacturer's instructions. Briefly, RNA was prepared by purification and fragmentation of 2ug of total RNA using oligo (dT) magnetic beads and used as a template for cDNA synthesis by random hexamer priming followed by end repair, A-tailing and ligation of Illumina adaptors. DNA particles with adaptors were then amplified using PCR After various steps, cleanup of the template was done using Agencourt Ampure RNAClean XP (Beckman Coulter, Pasadena, CA, USA). These clean-up steps included: post fragmentation clean up, post-cDNA synthesis; post end repair, A-Tailing and adapter ligation and post-PCR library clean-ups. Amplified libraries were analyzed for size distribution using the Agilent Tapestation 2200 DNA kit. Libraries were quantified using Quant-IT Qubit dsDNA High sensitivity Assay (Invitrogen, Carlsbad, CA, USA).

3.4.3 Whole genome Sequencing

Each library was prepared to an equimolar concentration of four nanomolar and pooled. Pooled libraries were sequenced in Macrogen Inc (Korea) using the MiSeq (Illumina, USA) platform for 300 cycles (2 x151 paired-end reads)**.** Three samples, representing three different haplotypes, were successfully sequenced. An average of 0.5 Gigabytes (GB) of sequence was produced per sample. To confirm the NGS results, the sequences obtained by conventional Reverse-Transcription polymerase chain reaction (RT-PCR) with virus specific primers of the Fusion gene were compared to the NGS results. The sequences obtained by Sanger method were identical to the NGS sequences.

3.5 Data Analysis methods

3.5.1 Analysis of association of independent factors and APMV occurrence

Data on poultry management and trade dynamics captured through the questionnaires were entered in a spreadsheet (Microsoft Excel). These were linked with the APMV laboratory results. Descriptive analysis on management and market practices and APMV molecular prevalence was carried out using R version 3.2.3 (R CRAN) and Excel sheet embedded with formulae. Avian paramyxovirus prevalence was calculated as the proportion of the number of APMV/APMV-1 positive birds on PCR to the total number of samples tested. Frequencies (percentages) for categorical variables were calculated and Chi-square test was used for comparison.

The association between avian paramyxovirus (APMV) occurrence and management and trade factors was assessed by logistic regression analysis. In the logistic regression, APMV occurrence was used as the binary value indicating the presence (1) or absence (0) of APMV (based on L gene and F gene PCR assay test result). The APMV positivity was determined at farm (flock)/trader level. A farm/trader who had at least one bird that tested APMV positive was considered to have a positive flock or stock

of poultry. For the logistic model, APMV occurrence in poultry farms and trade stock was presented as;

$$\text{logit(p)} = \beta_o + \beta_1 X_2 + \beta_2 D_2 + \ldots \beta_k X_k$$

Where p is the probability of presence of APMV (positive PCR assay result) which is the dependent variable; β_o is the estimated intercept term, β_1 , β_2 and β_k are the regression coefficients of the independent variables X_1 , X_2 , X_k the independent variable *k* is the number of variables. The logit transformation was defined as the logged odds presented in an equation as:

$$odds = \frac{p}{1-p} = \frac{probability\ of\ APMV\ presence}{probability\ of\ APMV\ absence}$$

The independent variables in the poultry flock included: poultry flock size, breeding system, frequency of disease control and disease control methods, feeding system and confinement, waste/carcass disposal methods, movement of birds/ bird products in and out of farm.

At the LBMs, independent variables included market location, frequency of operation (daily or weekly), volume of trade and presence of structures for example, slaughter slabs, enclosures/ fixed bird-holding areas. Trade factors included: volume of trade, frequency and distance of trade, source of poultry stock, relationship of trader with other traders/poultry farmers, disposal method of sick birds in stock, poultry examination for disease at sourcing point, waste/carcass disposal, type and frequency of cleaning bird holding cages and bird transport methods to market.

Multivariable association analysis was done by backwards stepwise logistic regression analysis. Starting with all potentially significant, independent variables were sequentially dropped if their effect on the model was not significant ($p>0.05$). Statistically significant effect was accepted at a probability (p) of $p<0.05$.

3.5.2 Genetic diversity and phylogenetic analysis of APMV

3.5.2.1 Genetic diversity analysis of Fusion and Large polymerase protein

The chromatograms partial sequence of the Fusion and Large polymerase protein gene were visualized using ChromasLite version 2.6 and those of low quality were removed. The consensus nucleotide sequences were aligned using MUSCLE v. 3.8.31 (Edgar & Batzoglou, 2006) and visualized using SeaView v.4 (Gouy *et al.*, 2010).The Primer sequence was trimmed in MEGA (Molecular Evolutionary Genetics Analysis) version 7.0 (Tamura *et al.*, 2013).

Unique sequences, herein referred to as haplotypes, were identified from the aligned F and L gene sequences using DNA sequence polymorphism (DnaSP v 6.0). DnaSP implements statistical methods to infer haplotype phase and prepares the phased data for subsequent analysis. This includes reconstructing the haplotype phase by applying algorithms (PHASE v2.1, fastPHASE v1.1 and HAPAR) differing in the underlying population genetic assumptions. Haplotype distributions were analyzed using Microsoft excel and mapped using QGIS version 2.16.2 (QGIS Development Team, 2009).

To examine the genetic variation of the F and L gene, genetic parameters were computed for each of the two genes. The two parameters used were haplotype diversity(H_d) and nucleotide diversity(π) using DnaSP v 6.0 (Rozas *et al.*, 2017). Haplotype diversity is the probability that two randomly chosen haplotypes are different and refers to the frequency and number of haplotypes in the population (Nei & Tajima, 1981). On the other hand nucleotide diversity(π) is the average number of nucleotide difference per site between two randomly chosen sequences and is used to measure genetic variation and polymorphism in population (Nei & Li, 1979).

The nucleotide diversity of the two genes in the different sampled populations was estimated using Jukes and Cantor model in DnaSP. In addition, the variation between

haplotypes was assessed through by calculation of evolutionary distances by Pairwise Distance method using Maximum Composite Likelihood method in MEGA 7.0 (Tamura *et al.,* 2013).

3.5.2.2 Whole genome analysis

The quality of the MiSeq sequencing reads was assessed based on Phred quality scores. This was performed using fastqc/0.11.5. The raw reads were trimmed to remove adapters and duplicate reads using Trimmomatic/0.33. The trimmed sequence reads were mapped to the host genome sequence using Bowtie2/2.2.8. Reads from which the host genome had been trimmed (unmapped to host genome) and which passed the quality check were considered useful and were subjected to *de novo* assembly using SPAdes/3.10.1 (Bankevich *et al.,* 2012).

Complete APMV-1 sequences including current vaccine strains and reference strains for each APMV-1 genotype were obtained from GenBank. The obtained whole genomes of APMV-1 strains from poultry in Kenya were also compared to whole genomes of characterized APMV-1 strains isolated from Africa. Details of the APMV-1 strains used in the analysis are included in Appendix 3.

These sequences and the complete nucleotide coding sequences and amino acid sequences of the APMV genes for the three APMV-1 strains and reference strains were aligned using MUSCLE v. 3.8.31 (Edgar & Batzoglou, 2006; Edgar, 2004). Highly polymorphic sites were detected on a sliding window in DnaSP and thereafter visualized using Jalview v 2.9.0 (Waterhouse, 2009).

Genetic distance analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version X.0 (Tamura *et al.,* 2013). Genetic distance is the degree of genetic/ genomic difference between species or populations. It is measured as the average number of codon or nucleotide differences per gene. Genetic distance metrics summarizes the genetic differences for a pair of populations. At a given locus, the

distance is 1 if both individuals have the same genotype, 0 if they have no allele in common.

The Nei's standard genetic distance (Nei, 1972, Nei, 1978) was used to estimate the genetic distance. Nei's genetic distance assumes that genetic distances are due to accumulation of mutations and genetic drift (Nei *et al.*, 1983). To calculate the proportion of variation, R^2, that is presented in the genetic distance matrix, the equation used was:

$$R^2 = 1 - \sum \frac{(D_{ij} - d_{ij})}{(D_{ij} - \overline{D})^2}$$

Where D_{ij} represent the observed genetic distance between populations i and j d_{ij} represents the distance between populations i and j and $\overline{D}$is the mean distance.

3.5.2.3 Phylogenetic analysis

Phylogeny was constructed using the F and L gene sequences. Prior to this, multiple alignment and comparison of the nucleotide and amino acid sequences of the F and L gene fragments of the study and reference sequences was performed using MUSCLE v.3.8.31 (Edgar & Batzoglou, 2006). In order to classify the haplotypes to serotypes and genotypes, they were compared with at least two representative F and L gene sequences of known APMV serotypes/genotypes reported to date from GenBank. To identify other sequences for comparison with the obtained haplotypes, sequences similar to the haplotypes were selected from GenBank using the BlastN algorithm (Benson *et al.*, 2017).

The selected references included closest matches to the haplotypes which had an expectation value greater than 1e-10. The Accession numbers of the strains included in the analysis are summarized in Appendix 3.

Phylogenetic inferences for the partial F and L gene were done using Maximum Likelihood (ML) method based on a Kimura 2-parameter model (Kimura, 1980) with gamma parameter to model the nucleotide substitution pattern and rate of evolution. For statistical support of internal tree branch values and to assess their statistical reliability, bootstrap resampling process (1000 replications) was done (Seo, 2008; Soltis & Soltis, 2003; Hillis & Bull, 1993). The resultant trees were viewed and edited in FigTree v 1.4.3 (Rambaut, 2016). The robustness of the phylogenetic analyses was also evaluated using phylogenetic networks. The latter were inferred based on uncorrected p-distances with phylogenetic split Phylogenetic analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 7.0 (Tamura *et al.,* 2013).

Analysis of the phylogenetic relationship between APMV-1 strains isolated from poultry in Kenya and global APMV-1 isolates of class II was also carried out using both the genomic and protein-coding sequences. This was done using maximum likelihood (ML) method. The tree used a best-fit general time-reversible (GTR) model of nucleotide substitution with gamma distributed rate variation among sites. A bootstrap resampling process (1000 replications) was employed to assess the robustness of individual nodes of phylogeny

3.5.2.4 Assessing signatures of selection on APMV-1 genes

This involved selection of three full coding region sequences representing each genotype. This included the coding sequences of the six structural genes (NP, P, M, F, HN and L) of APMV-1 strains. Sequences of APMV-1 strains from Africa and the Kenyan strains from this study were also included.

3.5.2.4.1 Detection of selection pressure

Sequences of the full coding region of the six structural genes (NP, P, M, F, HN and L) of APMV-1 strains of Class II were included in the analysis. At least three sequences representing each genotype were included. In addition, where available

sequences of APMV-1 strains from Africa were included in the analysis. The accession numbers of the APMV-1 strains included in the analysis are shown in Appendix III. The nucleotide and amino acid sequences of each gene were aligned in a multiple sequence alignment using MUSCLE v. 3.8.31 (Edgar & Batzoglou, 2006; Edgar, 2004;). Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version X.0 (Tamura *et al.,* 2013).

Phylogenetic trees were constructed using Maximum Likelihood (ML) method and general time-reversible (GTR) model with gamma distributed rate variation among sites was selected as the best-fit evolutionary model to estimate the optimal nucleotide substitution (Tamura et al., 2013). Model selection was done using the Akaike Information Criterion (AIC) (Posada & Buckley, 2004). Phylogenetic analysis was also carried out using amino acid sequences of the six genes. Phylogenetic trees construction with amino acid sequences was also done using ML method and Jones, Taylor, Thorton (JTT) model with gamma distributed rate variation among sites (Jones *et al.*, 1992). In all phylogenetic analysis, a bootstrap resampling process (1000 replications) was employed to assess the robustness of individual nodes of phylogeny. The trees were visualized using Figtree v1.4 (Rambaut, 2016).

To estimate the selective pressures on the six proteins of the APMV-1 genome, the CODEML program of the PAML package was used (Yang, 2010). In the programme, ML method was used to identify sites under positive selection (adaptive evolution) in genes encoding functional proteins on the APMV-1 genome. Sites and lineages under positive selection were identified by calculating the number of synonymous (d_S) and nonsynonymous (d_N) substitutions per site and their ratio (d_N/d_S) which is termed ω. Where ω was less than one, it was interpreted as a purifying (negative) selection, while $\omega > 1$ indicated positive selection. The ML method uses codon-based substitution models (Yang, 2010). Specifically, these are two nested site-specific models, a neutral model that does not allow for existence of positively selected sites ($\omega<1$) and an

alternative model that permits positive selection (ω>1). The models used included M0 (one-ratio) compared to M3 (discrete) and M7 (beta) compared to M8 (beta & ω).

M0 assumes a constant ω for all codons. M3 allows for discrete classes of sites with differing ω ratios. These two models were used to detect selection in lineages. On the other hand, M7 assumes a beta distribution with 10 categories of ω over sites, each corresponding to a unique value that is always less than 1 while M8 has an extra category with ω>1 and are used to detect selection in amino acid sites. The log likelihood test (LRT) for each pair of the nested models was used to compare the two nested models against a Chi-square distribution to assess whether the model allowing for positive selection was significantly more suitable for the data (Yang, 2010).

Inference on the particular codons under positive selection was done using the Bayes Empirical Bayes (BEB) procedure (Ziheng *et al.*, 2005) which also calculated their posterior probabilities. Amino acid sites with posterior probabilities greater than 95% were identified to be under positive selection while those with posterior probabilities equal to 90% were identified as good candidates of positive selection.

3.5.2.4.2 Visualization of sites under selective pressure on 3D protein structure

The 3D structure of the proteins encoded by the six APMV-1 genes were obtained from the Protein Data Bank (PDB) in the National Centre for Biotechnology Information (NCBI) (Madej *et al.*, 2014; Parasuraman, 2012) and the conserved domains of the proteins were identified from the Conserved Domains Database (CDD). The position of the positively selected sites obtained using BEB analysis was then selected and visualized on the 3D structure of the protein using Pymol version 1.7.4.5 (Schrödinger, 2015; DeLano, 2002).

CHAPTER FOUR

RESULTS

4.0 Introduction

This chapter presents the data gathered, results of statistical analysis done. First, the general results are described followed by sequential presentation of results obtained under each objective.

4.1 General results

This sub-section gives results of the characteristics of management and trade practices observed in the study sites and health status of sampled birds.

4.1.1 General characteristics of sampled poultry farms and LBMs

Chicken were the predominant poultry species, kept by 98.2% (221/225) of sampled poultry farms. Two-thirds (65%) of the households also kept other poultry species including ducks, guinea fowls, turkeys, pigeons and geese. Similarly, sampled live bird sellers predominantly sold chicken (98.4%:122/124). While all sampled poultry farms kept only local chicken ecotypes, almost half of market sellers (46%) sold both local chicken ecotypes and exotic chicken **(Table 4.5).**

Chicken flock size in the sampled poultry farms ranged from 2 to 120. Majority (60%) of poultry farms had a flock size of less than 10 birds. The flock size varied by zone with the Coastal zone having higher average farm chicken flock size of 20 compared to Lake basin and Western Highlands with an average flock size of 8 and 10 respectively. To restock or increase their poultry flocks, half of sampled farms (54%) relied on purchases/gifts from neighbouring farms and almost a quarter (36%) bought replacement birds from LBMs in the region. Only 10 % of the flocks consistently bred their own birds. The latter farms had breeding stock kept aside from the other flock. (**Table 4.5**).

Table 4.5: Characteristics of sampled poultry farms

Characteristics	**No. households (N=225)**	**[1]Proportion [2]sampled (95% CI)**	**SE**
Farm type			
Chicken only	113	50.2(37.3-61.8)	0.04
[1]Mixed species	112	49.8(38.2-62.7)	0.04
Chicken flock size			
<10	136	60.4(45.9-72.3)	0.03
11-30	62	27.6(18.8-34.1)	0.04
>30	23	10.2(7.1-24.8)	0.04
Breeding method			
Own breeding	22	9.8(3.3-21.5)	0.04
Source from market	82	36.4(23.7-51.6)	0.04
Neighbouring farms	121	53.8(39.0-64.7)	0.05
Mode of disposal of sick/dead birds			
Consume	116	51.6(32.3-63.9)	0.03
Sell	15	6.7(2.8-12.5)	0.02
Throw/give dogs	94	41.8(33.3-51.4)	0.02
Confinement			
Yes	53	23.6(13.8-38.9)	0.05
No	172	76.4(63.9-88.5)	0.05

[1]Proportion of farms with the described characteristic; in paracenteses is the 95% confidence intervals of proportion of farms the described characteristic.
[2]Sampling Standard error

Majority of farms (76%) managed their flock entirely extensively with no form of confinement and little or no supplementation as compared to 24% of farms that

confined their flock within a chicken house. Carcass disposal on the farms was generally poor. Majority of farms reported to consume meat from sick birds while 41% reportedly buried or threw the carcasses to dogs. On most farms, faecal wastes from poultry were dispose on farms.

Table 4.6: Characteristics of sampled live bird markets

Market characteristics	**No of markets (N=20)**	**[1]Proportion (%) sampled (95% CI)**	**[2]SE**
Market enclosure			
Open air	12	60.0 (46.6-71.8)	0.11
Enclosed	8	40.0 (38.2-53.5)	0.11
Bird population			
<100	5	25.0 (16.8-32.7)	0.12
100-200	7	35.0 (22.6-41.9)	0.11
>200	8	40.0 (28-53.1)	0.11
Slaughter services			
No	8	40.0 (35.2-52.5)	0.11
Yes	12	60.0 (48.2-69.7)	0.11
Category of market			
Trading centre	4	20.0 (15.5-27.4)	0.13
Major town	9	45.0 (37.3-57.9)	0.11
City market	7	35.0 (29.0-41.8)	0.11

[1]Proportion of markets with the described characteristic; in paracenteses is the 95% confidence intervals of proportion of markets the described characteristic. [2]Standard error

Live bird markets (LBMs) were categorized as city, major town or trading centre markets Twenty percent (20%) of the LBMs studied were trading center markets (**Table 4.6 above**). These were located in the rural areas in close proximity to the poultry keepers and served as the primary bird bulking points.

The trading centers were the primary tier markets and received birds directly from the farms. Major town markets were the largest (45%) sampled markets and served as second tier markets receiving their bird supplies mainly from brokers who bought birds from the farmers with only few birds being sold directly by the farmers themselves. City markets accounted for 35% of the studied LBMs.

Birds found in the city markets were supplied from all over the country. In Nairobi city markets for instance, birds were supplied from Western Kenya, Rift Valley (Bomet and Kericho) and Eastern regions. Two-thirds of the sampled live bird markets routinely offered slaughter services (**Table 4.6** above) and traded both, live and slaughtered birds. However, only 17.7% of these had designated slaughter facilities. A majority (64.7%) of the sampled markets had between 100 and 200 birds.

Two market types were encountered; open air and enclosed. The two market types were found in all the sampled regions. In the open-air markets (**Plate 4.1**), birds were placed outdoors either in cages, traditional baskets or tethered to a pole. Majority of the sampled markets (64.7%) were open-air markets.

Plate 4.1: Open-air live bird markets of Chwele (A) and Bumala (B). Photo courtesy of Irene Ogali

Enclosed markets had semi-permanent to permanent bird-holding stalls or cages (Plate 4.2). Thirty-five percent (35%) of the sampled markets were enclosed. These include Kitale, Kericho, Kisumu, Kawangware, Meru, Embu, Marikiti and Majengo markets. These markets were mostly daily markets and birds were left in the cages overnight.

Plate 4.2: Meru (A) and Kitale (B) live bird markets with bird holding structures; Photo courtesy of Irene Ogali

Each trader was allocated a cage by the market authorities and was responsible for its cleaning. Cleaning of cages was done irregularly with many traders cleaning once to twice in a month. However, on observation, all cages were littered with faecal matter. Sickly birds were also observed inside the cages.

Majority (89%) of interviewed poultry traders reported to receive bird supplies from a single source while 11% reported receiving their bird supplies from multiple sources (**Table 4.7**). A sizeable (61%) proportion of traders interviewed during market visits reported that they regularly examined birds for disease signs before purchase of their stock (**Table 4.7**). They reported that they were able to differentiate sick birds from apparently healthy ones by keenly observing for breathing anomalies, presence of discharges, walking and standing disorders as well as signs of diarrhea.

Table 4.7: Characteristics of sampled poultry traders in five regions of Kenya

Characteristic	Description	[1]Count	Percent
Trader's source of birds	Single area source	110	89
	Multiple areas/counties	14	11
Traders examine for disease while purchasing stock	Yes	76	61
	No	48	39
Traders took precaution in bird sourcing; avoiding disease outbreak areas	Yes	104	84
	No	20	16
Traders sold bird of mixed species	Yes	21	17
	No	103	83
Traders sold mixed breed chicken	Yes	73	59
	No	51	41

[1]Number of traders with the described characteristic

Majority of the traders 84% (104/124) reported that they avoided sourcing birds from certain areas during disease outbreaks. Similarly, majority (83%) of the traders reported to trade in a single species of birds as opposed to mixed bird species (17%) which included chicken, doves, ducks, turkeys and guinea fowls.

Traders who stocked chicken dealt with a variety of breeds. A sizeable proportion (59%) reported buying and selling mixed breeds while only 41% dealt with a single breed (Table 6). When interviewed for losses of birds attributed to disease outbreaks, 44% of traders (55/124) reported losing at least 1-5 birds from their stock monthly.

On how they handled sick birds, 56% (69/124) of the traders reported that they slaughtered the sick birds for consumption while 44% (55/124) slaughtered such birds and sold them at reduced prices to hotels or local consumers

4.1.2 Health status of sampled birds

During sampling, birds selected for sampling were examined to establish their health status before sampling. Clinical signs such as diarrhea, difficulty breathing, lethargy, paralysis were among the key clinical signs observed for in the birds.

Post mortem examination was carried out in dead or dying birds collected on both poultry farms and LBMs. Plate 3 shows the clinical signs commonly observed and post mortem lesions in the birds that were indicative of APMV infection. Birds showed signs of neck paralysis/torticollis (A on **Plate 4.3**), severe diarrhea (B), and head edema (C).

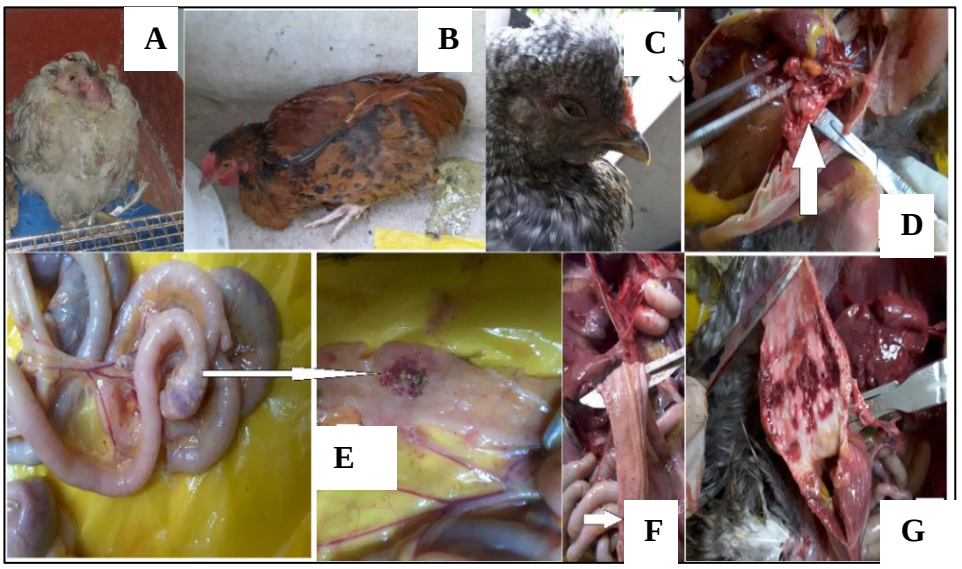

Plate 4.3: Symptoms and post-mortem lesions indicative of APMV infection in sampled chicken; Courtesy of Irene Ogali

Others clinical symptoms observed included leg paralysis. Mouth and nasal discharge among others respiratory distress. On postmortem examination, lesions were observed in the lungs (D), small intestines (E and F) and in the proventriculus (G) (**Plate 4.3** above).

Among the different poultry species, clinical disease was only observed in sampled chicken. Compare to other regions, Lake Victoria Basin had a slightly higher proportion (40%) of sick birds from poultry farms. Western Highlands and Coastal Region had 26% and 34% respectively (**Figure 4.5**).

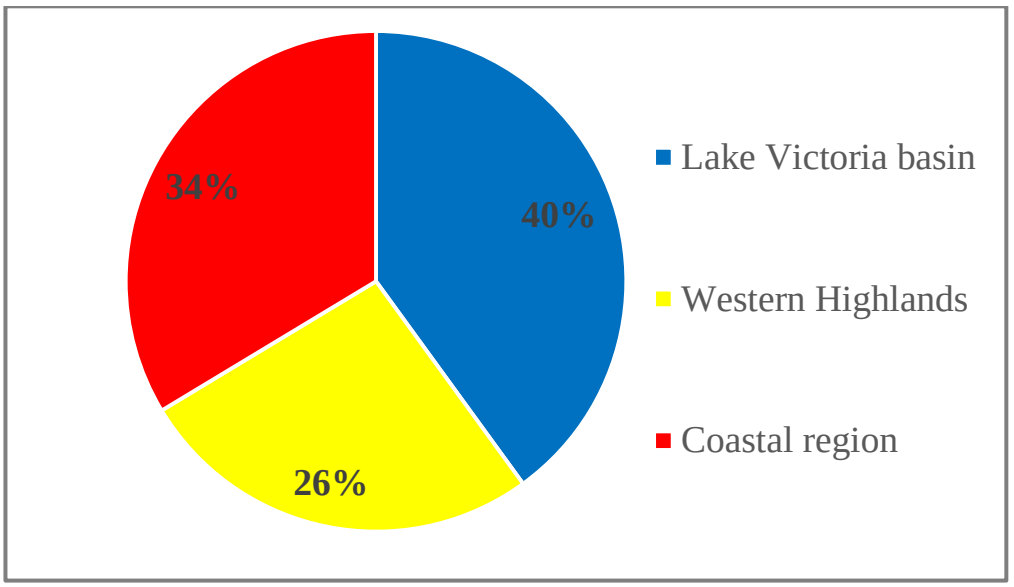

Figure 4.5: Proportion of chicken with clinically disease in poultry farms by region

Compared to other regions, live bird markets in Nairobi metropolitan had the highest proportion (30%) of sick birds followed by Eastern region (25%). Proportion of clinically sick birds in Western highlands (17%), Coastal region (15%) and Lake Victoria Basin (13%) were similar (**Figure 4.6**).

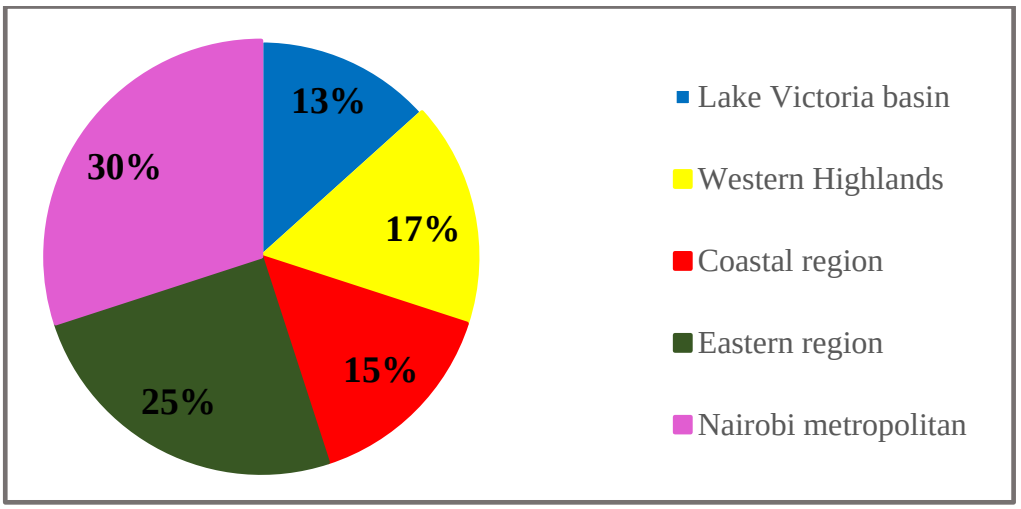

Figure 4.6: Proportion of chicken with clinically disease in live bird markets by region

4.1.3 Risk factors of avian paramyxovirus

4.1.3.1 Amplification of the L gene

Polymerase Chain Reaction amplification using APMV L gene universal primers yielded a fragment size of 221bp on 1.5% agarose gel (Figure 7)

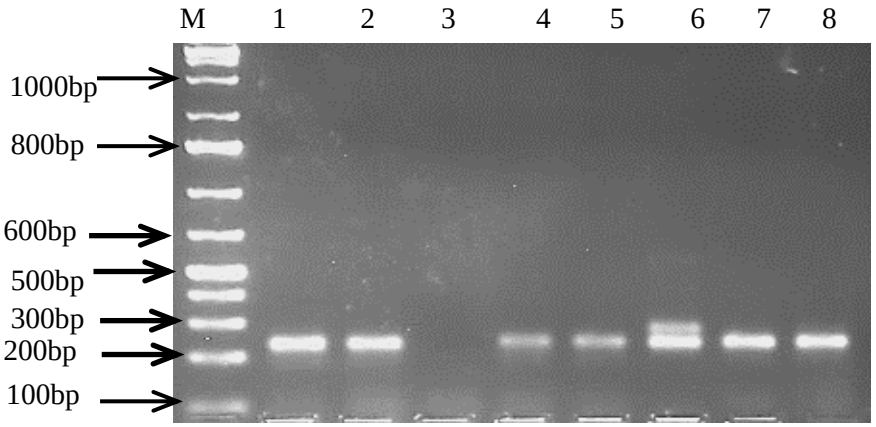

Figure 4.7: Image of amplified L gene fragments on 1.5% agarose gel
M-DNA marker; 1-positive control (*LaSota* strain); lane 2-7-samples from different regions; lane 3 negative control

The amplified PCR products of the L gene 221 bp fragments. However, other samples had double **(Figure 4.7)**. Samples with double bands (lane 6 of Figure 4.7) were run again on agarose gel to allow complete separation and incision of the bands for gel purification and sequencing.

Majority of the L gene positive samples were obtained from birds that presented with clinical disease. The L gene was detected in a very small proportion of healthy birds except in Lake Victoria Basin where L gene was detected in 5% of healthy birds (**Figure 4.8**)

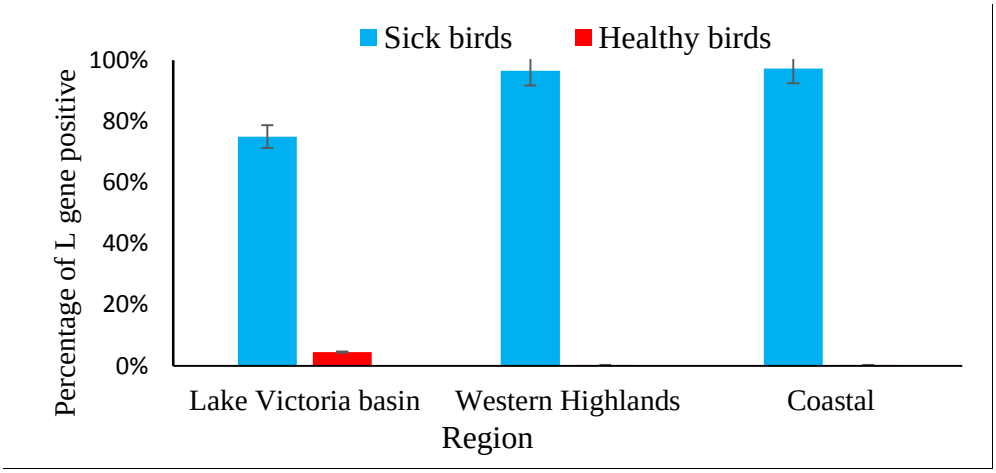

Figure 4.8: Proportion of flock L gene positivity by health status in sampled regions

A very small proportion of healthy birds were L gene positive in all region except in Nairobi metropolitan followed by Western Highlands, and Lake Victoria Basin L gene was detected in 11% and 4% of healthy birds respectively (**Figure 4.9**).

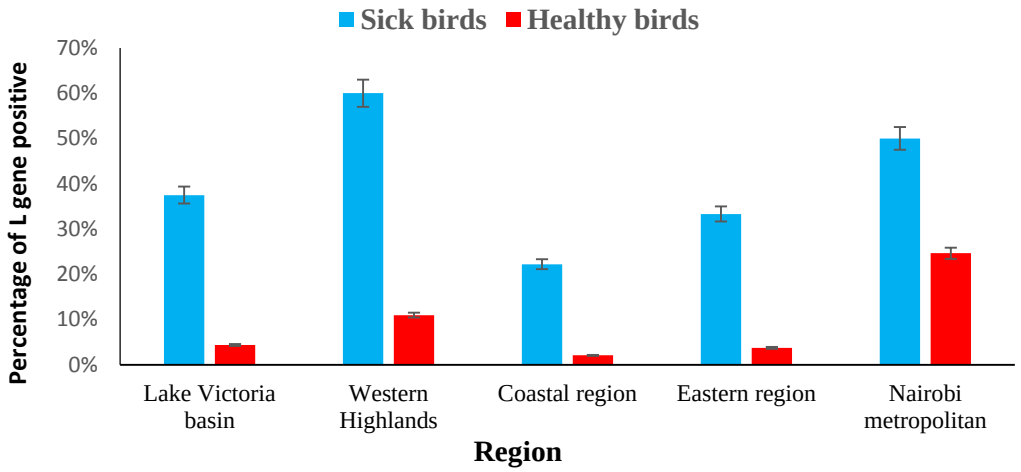

Figure 4.9: Proportion of L gene positive birds by health status in LBMs

4.1.3.2 Amplification of the F gene

Amplification of the fusion gene showed positive samples with the expected band size of 535bp as (**Figure 4.10**). The samples selected were those with clear bands when viewed on UV transilluminator

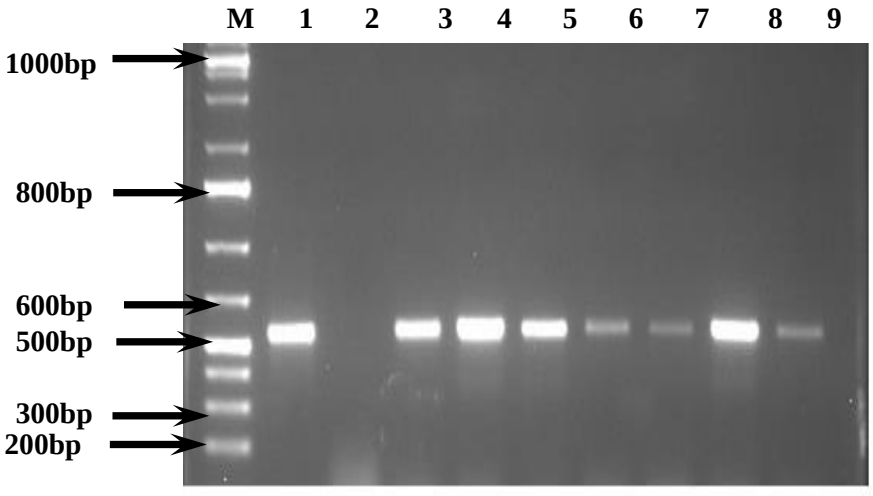

Figure 4.10: Image of amplified F gene fragments on agarose gel
M- 100bp DNA marker; Lane 1 positive control (*LaSota strain*), lane 2-negative control; lanes 3-9-Samples

The F gene positive samples varied largely based on the health status of birds. In all regions, F gene detection was higher in birds that showed clinical disease as compared to healthy birds in poultry farms **(Figure 4.11).** Detection of APMV by the F gene assay was negligible in healthy birds except in Lake Victoria Basin where F gene was detected in 5% of healthy birds.

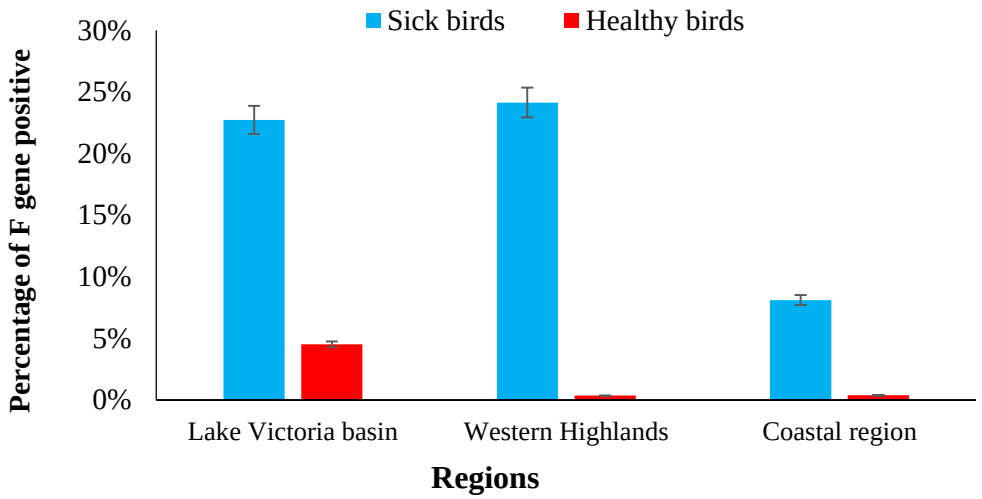

Figure 4.11: Proportion of flock F gene positivity by health status in sampled regions

Overall, even in LBMs, the proportion of F gene positive birds was higher in sick birds (42%) compared to healthy birds (9%). **Figure 4.12** shows the proportion of F gene positive samples in LBMs in different regions by the clinical status of the birds. F gene detection was higher in birds that showed clinical disease in all regions. However, in Nairobi markets, APMV was detected in significant proportion (25%) of healthy birds

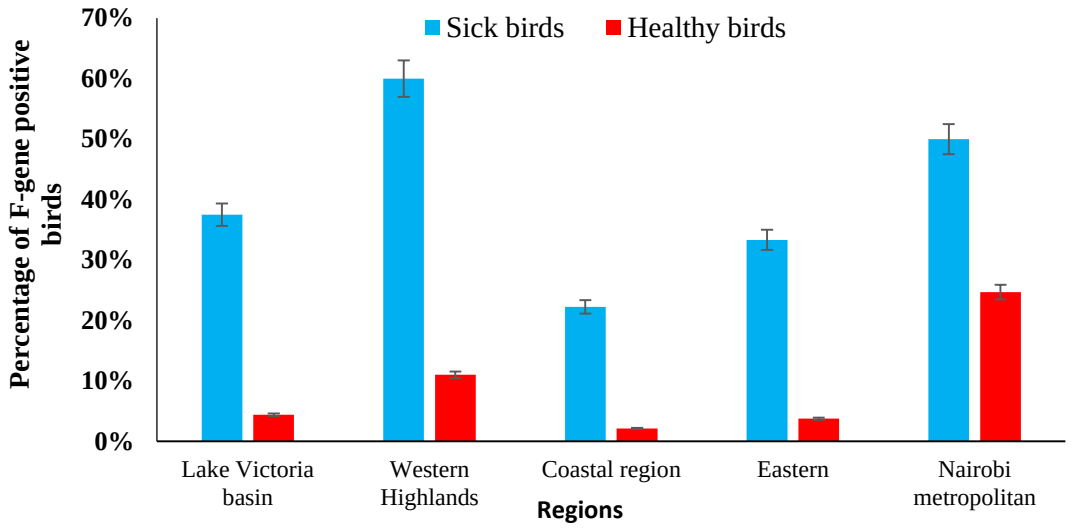

Figure 4.12: Proportion of F gene positivity by health status in LBM

4.1.4 Factors associated with APMV occurrence in poultry

4.1.4.1 Occurrence rate of APMV in sampled location

Overall, the L gene was amplified in 5.8% (71/1224; range 3.3-9.5) of the samples from poultry farms (**Table 4.8**). There was variation across region in the number of L gene positive samples.

Table 4.8: Number and proportion of L gene positive samples from poultry farms

Region	**No. of samples (N)**	**No. positive (n)**	**[1]Proportion (%) positive (95% CI)**	**[2]p-value**
L. Victoria basin	360	34	9.4 (5.0-13.4)	0.03*
Western Highlands	433	23	5.3 (2.1-7.2)	0.477
Coast	431	14	3.3 (1.8-4.5)	Ref
Total	1224	71	5.8 (3.3-9.5)	

[1]Proportion of L-gene positive poultry; in paracenteses is 95% confidence intervals of proportion of L gene positive poultry; [2]p-value indicating the level of significance of differences by ecological zones; *-significant difference in proportion of APMV positive farms ($p<0.05$)

The L gene detection rate in poultry farms from Lake Victoria Basin (9.4%; 34/360) was significantly higher than the other two regions (p=0.03). There seemed to be no difference in the APMV detection rate (p=0.477) in Western Highlands (5.3%; 23/433) and Coastal region (3.3%; 14/431).

Overall, on poultry farms, 99% (70/71) of the L gene positive samples were from chickens. In the ducks sampled, APMV was detected in only one sample representing 4.8% (1/21) of sampled ducks. The L gene was not amplified in the samples from guinea fowls or pigeons. Likewise, in LBMs, APMV positive samples were from chicken and one duck. In LBMs, the L gene was amplified in 23% (111/482; range 18.2-29.9) of sampled poultry. There was regional variation in the detection rates of APMV in sampled LBMs (**Table 4.9**).

Table 4.9: Number and proportion of L gene positive samples from live bird markets

Region	**No. of samples (N)**	**No. of positive (n)**	**Proportion(%) positive (95% CI)**	**p-value**
L. Victoria basin	99	16	16.2 (9.8-25.3)	0.03*
Western Highlands	137	30	21.9 (16.4-28.7)	0.097
Coast	80	15	18.8 (14.6-20.8)	0.035*
Eastern	64	14	21.9 (15.5-29.6)	0.135
Nairobi	102	36	35.3 (26.7-41.6)	Ref
Total	482	111	23.0 (18.2 to 29.9)	

[1]Proportion of L gene positive poultry in LBMs; in paracenteses is the 95% confidence intervals of proportion of positive poultry; [2]p-value indicating the level of significance of differences by ecological zones; *-significant difference in proportion of APMV positive farms (p<0.05)

Nairobi metropolitan, Western Highlands and Eastern regions had significantly higher proportion ($p<0.05$) of L gene positive samples (35.3%, 21.9% and 21.9% respectively). Market L gene detection rates in all the regions was above 15%. (**Table 4.9**). The L gene was detected in all the sampled LBMs, however, the proportion of the positive birds in the sampled markets varied. Generally, all markets in Nairobi metropolitan had higher APMV detection rate. In particular, Kibra market seemed to have the highest L gene detection rate (87%) of samples were positive) (**Table 4.10**).

Table 4.10: Number and proportion of L gene positive samples in live bird markets

Markets	**No. birds sampled**	**No. APMV positive birds**	**Proportion (%) positive birds (95% CI)**
Bumala	24	5	20.8 (6.2-58.6)
Homabay	23	6	26.1 (8.1-44.7)
Migori	22	3	13.6 (3.5-33.4)
Kisumu	30	2	6.7 (1.2-45.6)
Bungoma	16	7	43.8 (11.7-66.8)
Chwele	19	3	15.9 (4.5-46.9)
Kitale	23	4	17.4 (4.1-53.0)
Bomet	22	6	27.3 (9.8-47.4)
Kakamega	22	2	9.1 (2.7-30.5)
Kericho	35	8	22.9 (7.1-49.5)
Kilifi	24	8	33.3 (10.4-63.1)
Marikiti	27	4	14.8 (4.5-36.9)
Majengo	29	3	10.3 (6.6-37.8)
Kitengela	15	6	40.0 (9.6-63.0)
Kawangware	20	3	15.0 (2.6-33.4)
Kibra	15	13	86.7 (27.7-99.0)
Burma	20	8	40.0 (12.3-65.1)
Machakos	32	6	18.8 (6.1-37.6)
Makueni	22	7	31.8 (4.4-45.6)
Meru	21	4	19.5 (6.3-34.2)
Embu	21	3	14.3 (5.7-30.6)

[1]Proportion of L gene positive poultry in LBMs; in paracenteses is the 95% confidence intervals of proportion of positive poultry

The other LBMs with high L gene detection rates included: Bungoma, Kitengela, Kilifi, Burma and Makueni markets. On the other hand, the markets with comparatively Kisumu and Kakamega markets had lower detection rates of 6.7% and 9.1% respectively.

The Fusion gene was amplified in 2.7% (33/1224; range 1.9-3.8) of samples from poultry farms (**Table 4.11**), a detection rate that was lower than was observed with the L gene assay. Of these samples, 32 were from chicken and one from a duck.

There were regional variations in the poultry samples from farms for which the F gene was amplified. Lake Victoria basin (5.8%) had significantly higher number of samples in which the F gene was amplified (p=0.024) compared to Coast and Western Highlands which had detection rates of 1% and 2% respectively.

Table 4.11: Number and proportion of F gene positive samples from poultry farms

Region	**No. NDV positive (n)**	**Proportion (%) positive (95% CI)**	**p-value**
L. Victoria basin	21	5.8 (2.9.-9.8)	0.024*
Western Highlands	8	1.9 (1.1-8.2)	0.257
Coast	4	0.9 (0.4-2.5)	Ref
Total	33	2.7 (1.9-3.8)	

[1]Proportion of F gene positive poultry in poultry farms; in paracenteses is the 95% confidence intervals of proportion of positive poultry [2]p-value indicating the level of significance of differences by ecological zone; *-significant difference in proportion of APMV positive farms (p<0.05)

In LBMs, the F gene was amplified in 11.9% (54/482; range 8.2 to 13.9) of the samples (**Table 4.12**). Nairobi metropolitan had the highest proportion of F gene positive samples (23.5%), followed by Western Highlands (11.7%) and Eastern (6.3%). The

difference in the proportion of F gene positive samples was significantly higher in Nairobi region compared to other regions ($p<0.05$)

Table 4.12: Number and proportion of F-gene positive samples from live bird markets

Region	**No. NDV positive (n)**	**Proportion (%) positive (95% CI)**	**p-value**
L. Victoria basin	6	6.1 (2.4-14.3)	0.003*
Western Highlands	16	11.7 (6.1-19.1)	0.04*
Coast	4	5.0 (1.7-13.8)	0.004*
Eastern	4	6.3(2.1-16.9)	0.01*
Nairobi	24	23.5(15.7-30.6)	Ref
Total	54	11.9(8.2 to 13.9)	

[1]Proportion of F gene positive poultry in LBMs; in paracenteses is the 95% confidence intervals of proportion of positive poultry; [2]p-value indicating the level of significance of differences by ecological zones; *-significant difference in proportion of APMV positive farms ($p<0.05$)

Avian paramyxovirus was detected in all the sampled LBMs, however, the proportion of the positive birds in the sampled markets varied. Kibra seemed to have a higher F gene detection rate compared to the others.

The other LBMs with fairly higher F gene detection rates included: Bungoma, Kitengela, Burma, Machakos and Burma markets. On the other hand, the markets that seemed to have the lowest detection rate included: Marikiti, Majengo, Kisumu, Meru and Embu (**Table 4.13**).

Table 4.13: Number and proportion of F-gene positive birds in live bird markets

Market	Number of. birds sampled	Number of positive birds	Proportion (%) positive (95% CI)
Bumala	24	2	8.3 (3.2-38.6)
Homabay	23	2	8.7 (11.1-44.8)
Migori	22	1	4.5 (1.5-23.4)
Kisumu	30	1	3.3 (1.2-15.6)
Bungoma	16	4	25 (9.1-46.3)
Chwele	19	2	10.5 (2.3-26.8)
Kitale	23	2	8.7 (2.1-43.0)
Bomet	22	3	13.6 (7.8-45.7)
Kakamega	22	1	4.6 (2.7-20.9)
Kericho	35	4	11.4 (3.0-48.8)
Kilifi	24	2	8.3 (1.4-27.1)
Marikiti	27	1	3.7 (1.2-37.8)
Majengo	29	1	3.4 (1.4-33.4)
Kitengela	15	6	4.0 (8.9-39.0)
Kawangware	20	1	5.0 (2.6-33.4)
Kibra	15	7	46.7 (17.7-65.0)
Burma	20	4	20 (6.9-35.1)
Machakos	32	6	18.8 (3.1-49.6)
Makueni	22	2	9.9 (3.4-45.6)
Meru	21	1	4.8 (1.3-32.9)
Embu	21	1	4.8 (1.3-32.9)

. [1]Proportion of F gene positive poultry in LBMs; in paracenteses is the 95% confidence intervals of proportion of positive poultry

4.1.5 Management and trader factors associated with APMV occurrence

4.1.5.1 Management factors associated with APMV

Overall, 18.2% (range 14.1-23.1) of sampled poultry flocks had at least one bird whose sample was positive for APMV by L gene amplification.

Large flock sizes, introduction of birds from market or neighbors, and keeping a mixed poultry species had high odds ratios; 3.4, 2.7 and 2.07 respectively (**Table 4.14**).

Significant p-values of 0.034, 0.005, 0.006, and 0.016 were observed for keeping mixed species, large poultry flocks, introduction of poultry and confinement of birds respectively.

Table 4.14: Management factors associated with APMV detection at flock-level

Variable	**[1]Odds ratio**	**SE**	**p-value**
Keeping mixed poultry species	2.07	0.711	0.034*
Introduction of birds from outside farm	2.76	0.983	0.005*
Confinement of birds	0.246	0.126	0.006*
[2]Poor disposal of sick/dead birds	0.691	0.351	0.467
[3]Large chicken flock size	3.41	1.75	0.016*
Routine treatment of flock	0.715	0.271	0.377
Number of observations	221		

*asterisk represent factors with significant association on flock APMV occurrence at ($p<0.05$); [1]Odds ratio strength of association between APMV occurrence in a flock and an independent factor; [2]consumption and selling; [3]flock sizes ranging from 30-100 birds;

4.1.5.2 Risk factors associated with APMV occurrence in sampled LBMs

Location of market in the City or big town had an odd's ratio of 2.98, and p-value =0.044 **(Table 4.15)**. The results indicated that, the practice, by traders, of examining birds for disease before purchasing significantly lowered (p value=0.025, odds ratio=0.55) the risk of APMV occurrence in birds.

At trader level, the sourcing birds from multiple sources had an odds ratio of 3.15 and p-value =0.04. The odds ratio of examining birds **(Table 4.15).**

Table 4.15: Regression estimates for market/trade factors against APMV positivity

Variable	Odds ratio	p-value
Location of market in Major town/Cities	2.98	0.044*
Open air opposed to enclosed market	0.88	0.67
Presence of slaughter slab	0.723	0.481
Trader characteristics		
Trader source birds from multiple sources	3.15	0.004*
Traders examine for disease while purchasing stock	0.547	0.025*
Traders selling mixed species	1.09	0.751

*asterisk represent factors with significant association on flock APMV occurrence at ($p<0.05$); [1]Odds ratio strength of association between APMV occurrence in a flock and an independent factor;

4.2 Diversity and phylogeny of L and F gene sequences

4.2.1 Sequence analysis of L gene

Seventy-seven (77) partial L gene sequences sequenced. These included 42 sequences from LBMs and 35 sequences from poultry farms. Out of the 77 sequences, seven were of poor quality and were discarded from the analysis. The remaining 70 good quality sequences were used for the analysis. Sixty-eight of these sequences were obtained from chicken while two sequences were from ducks (Appendix 4).

The partial large polymerase protein (L) gene sequences were classified into 18 distinct haplotypes with unique sequences. The sequences were deposited in the GenBank with accession numbers ranging from *MG471393* to *MG471410*.

Similarity search on the nucleotide databases through the BLAST algorithm yielded a best hit of 172bp sequence highly similar (97%) to Newcastle disease virus (APMV-1) *isolate, A148,* partial L gene sequence isolated by Ommeh *et al.,* (2010). However,

haplotype 10, had higher similarity (99%) to Newcastle disease virus (APMV-1), *isolate, LaSota* (**Table 4.16**).

Table 4.16: BlastN search results of partial L gene haplotypes from poultry in Kenya

Accession #	**H#***	**Location**	**Highest Blast Match**	**Accession # match**	**E value**	**% Similarity**
MG471393	1	All regions	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
MG471394	2	NM, Eastern	*Newcastle disease virus isolate, A148,*	JQ217420	0.0	99%
MG471401	9	WH, Coast	*Newcastle disease virus isolate, A148*	JQ217420	0.0	99%
MG471402	10	WH	*Newcastle disease virus strain, LaSota,*	AF077761	0.0	99%
MG471403	11	Coast	*Newcastle disease virus isolate, A148*	JQ217420	0.0	98%
MG471405	13	Eastern, NM,WH	*Newcastle disease virus isolate, A148*	JQ217420	0.0	99%
MG471407	15	Coast	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
MG471408	16	Coast	*Newcastle disease virus isolate, A148*	JQ217420	0.0	98%
MG471409	17	Eastern	*Newcastle disease virus isolate, A148*	JQ217420	0.0	98%
MG471410	18	Eastern	*Newcastle disease virus isolate, A148*	JQ217420	0.0	99%

H#-Refers to Haplotype identity; NM-Nairobi metropolitan; WH-Western Highlands; LVB-Lake Victoria basin

Similarity search in the protein database using a translated nucleotide query (BLASTX) yielded a partial RNA-dependent RNA polymerase protein of APMV-1 with 98% sequence identity to that of the APMV-1 isolate (*JQ905275).* Multiple sequence alignment of L gene. Sequence alignments of partial L gene showed various

polymorphic sites. The region between 30-100bp (shown by red arrows-A) of the L gene nucleotide sequence, was the most variable region while the region between 100-172 was conserved in the different APMV haplotypes (**Figure 4.13**).

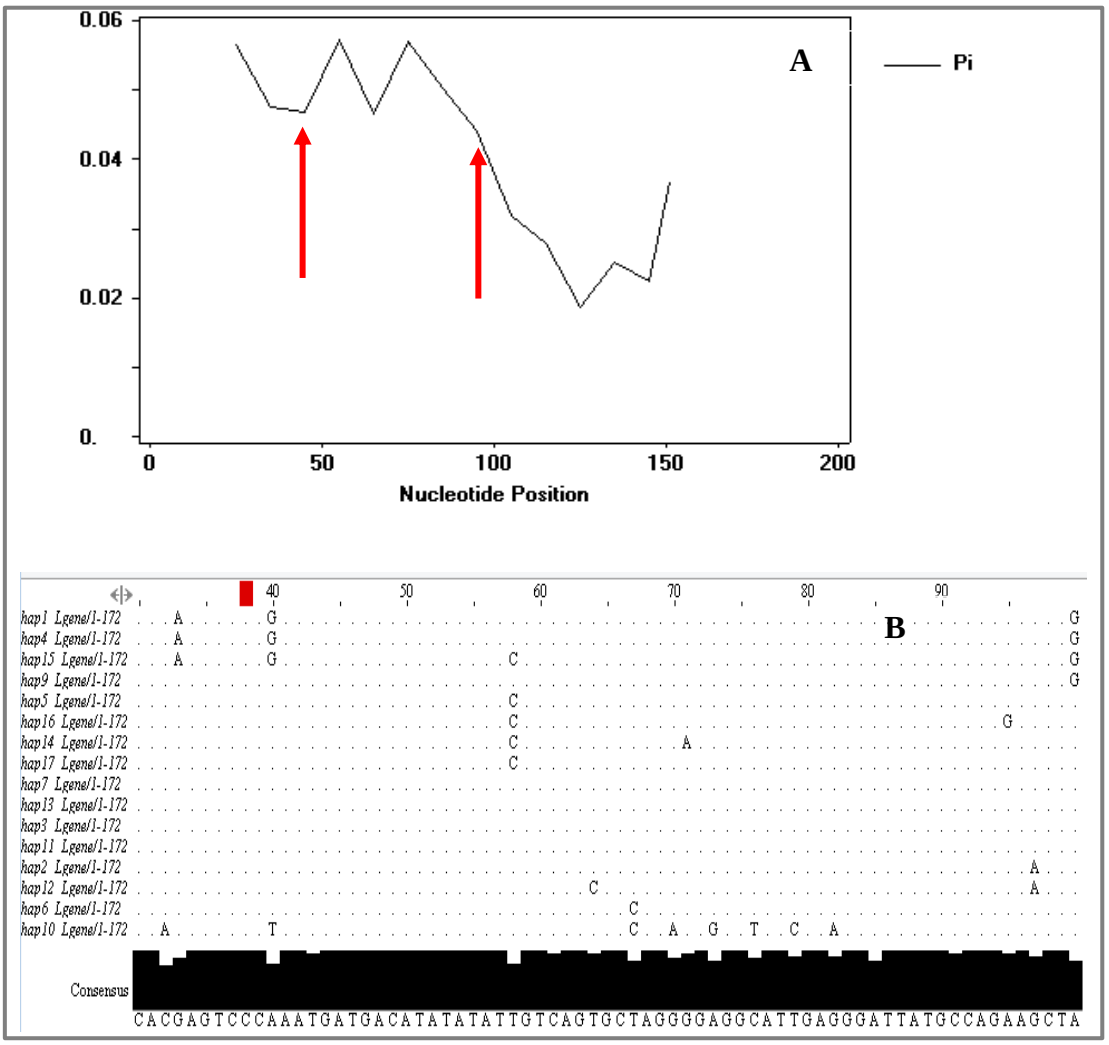

Figure 4.13: Polymorphic sites on partial L gene nucleotide sequences of APMV
A- region 30-100bp of L gene; B -100-172bp –conserved region

The eighteen L gene haplotypes were identified in chicken and two haplotypes were found in ducks. The eighteen haplotypes were distinct from each other as polymorphisms. These differences were seen in the partial L gene sequence

chromatograms. The chromatogram result indicate nucleotide T/C substitution at position 87, nucleotide A/G substitution at position 126 and another A/G substitution at position 129 (**Figure 4.14**). Polymorphism was seen at nucleotide positions 87, 126 and 129 on the L gene haplotypes. The chromatogram result indicate nucleotide T/C substitution at position 87, nucleotide A/G substitution at position 126 and another A/G substitution at position 129 (**Figure 4.14**).

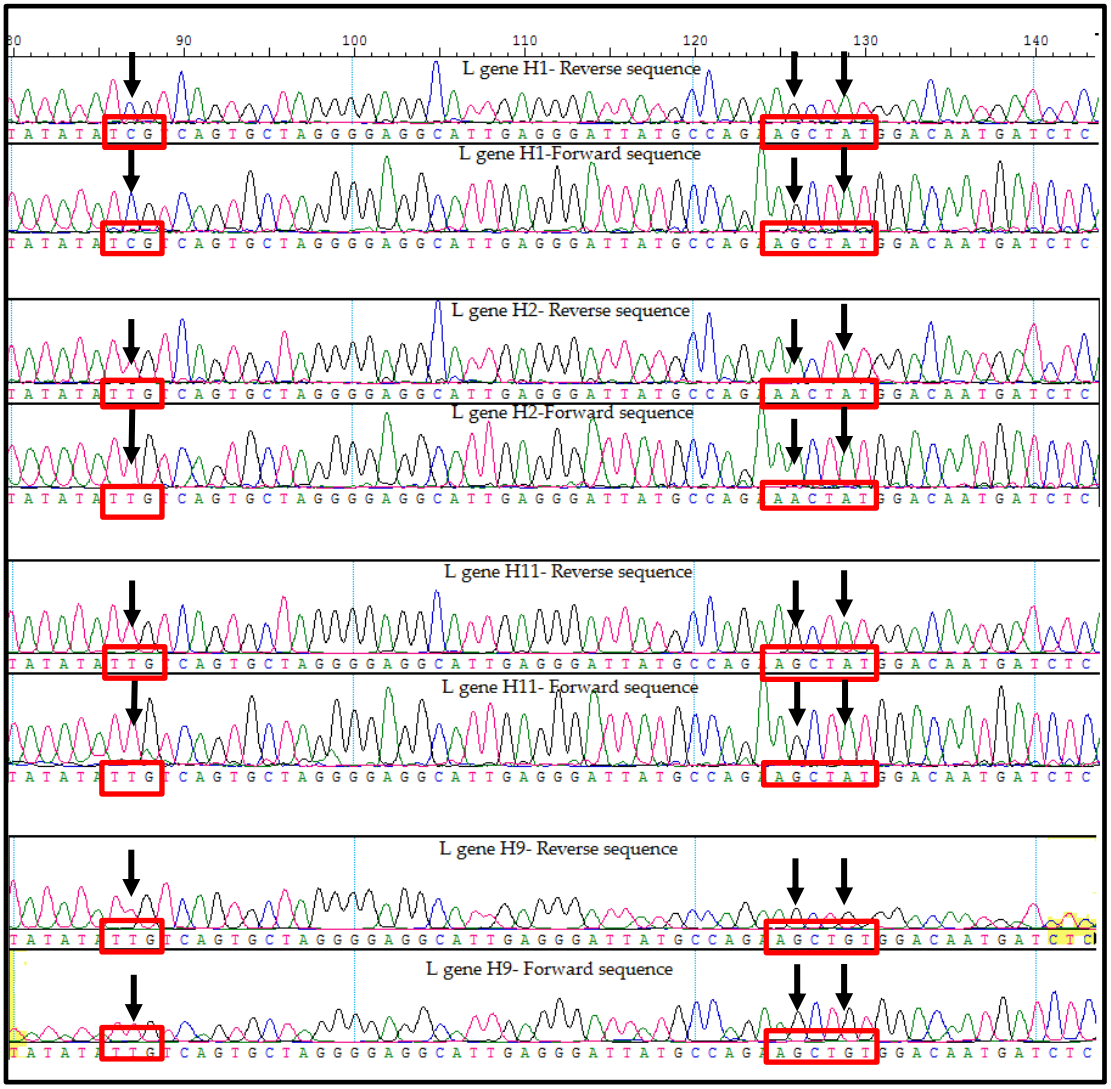

Figure 4.14: L gene sequence chromatograms of APMV haplotypes from poultry
The black arrows show areas where nucleotide substitutions occurred.

Multiple alignment of L gene showed that polymorphic regions were also observed. The polymorphisms were mainly substitution or single point. (**Figure 4.15).**

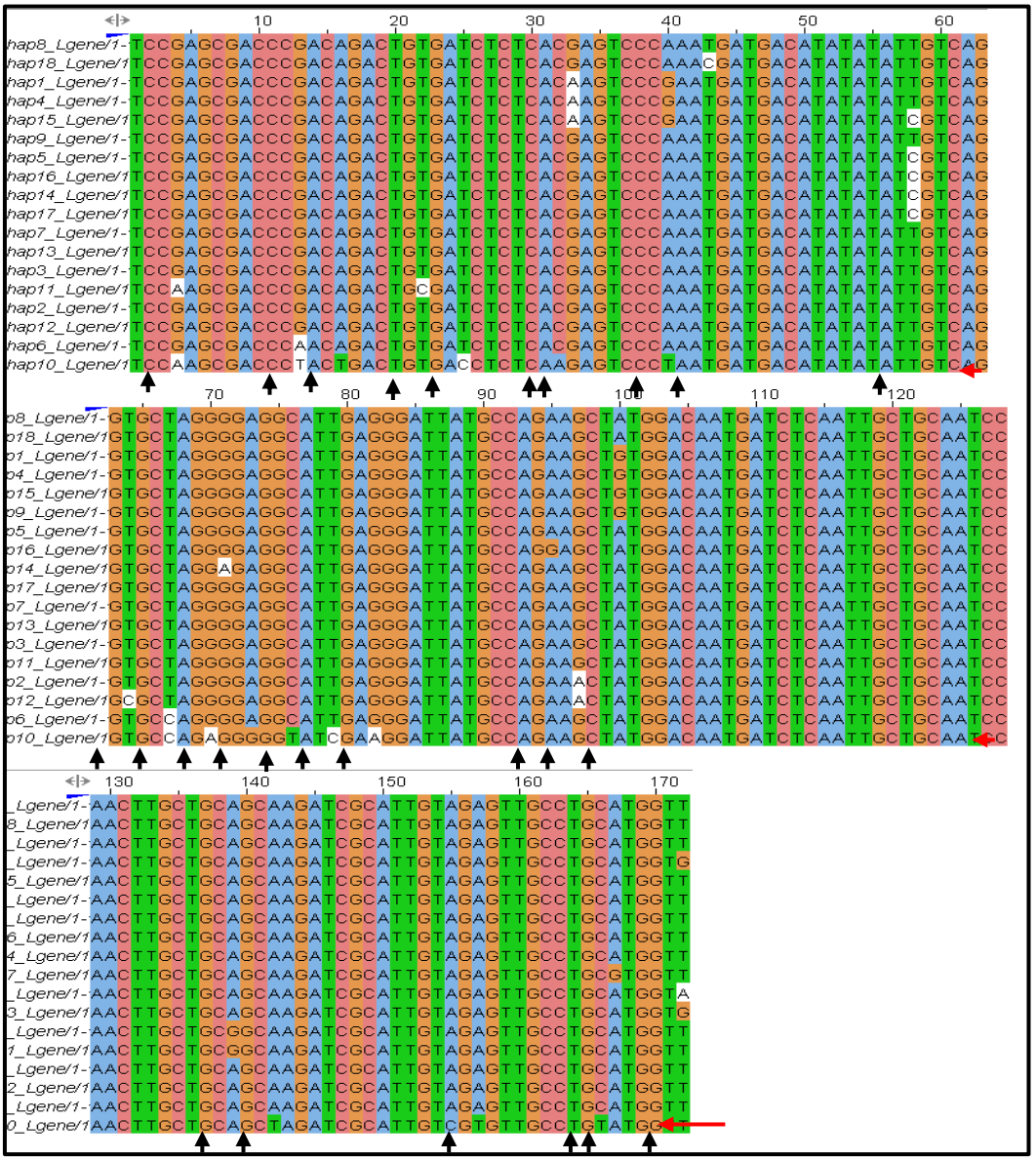

Figure 4.15: Alignment of partial L gene sequences of obtained APMV haplotypes Black arrow shows point of mutations; Haplotype 10 with highest number of polymorphic sites

4.2.2 Distribution of L gene haplotypes in sampled zones

Haplotypes of the L gene were distributed unevenly in the five sampled regions of Kenya. Two haplotypes were found in all the five regions; haplotype 1 and 13). Western Highlands had seven haplotypes, but majority of the sequences were classified under haplotype 1. Four haplotypes were found in Lake Victoria Basin. Four haplotypes were found in both Nairobi metropolitan and Eastern regions. Five out of ten of the unique haplotypes were found in Coastal region **(Figure 4.16).**

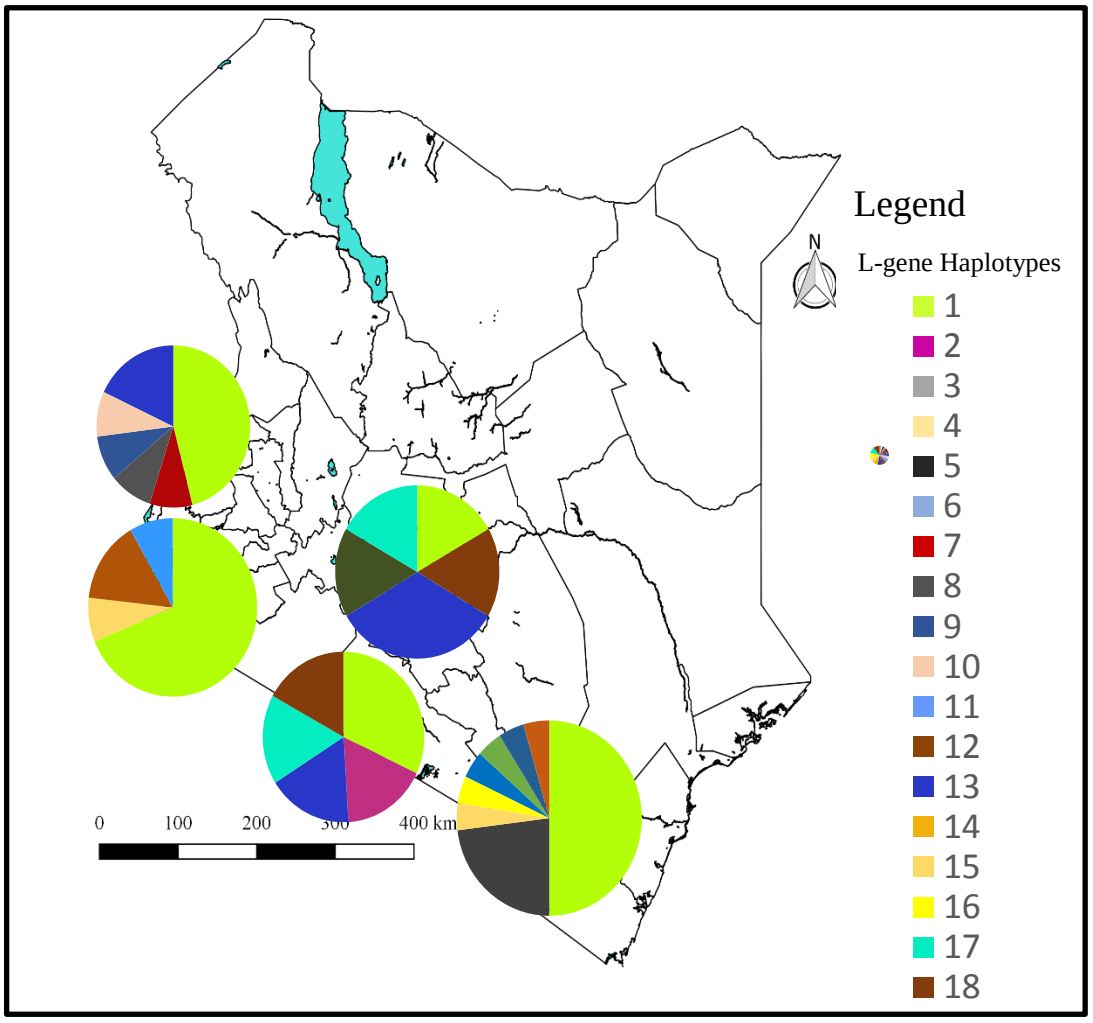

Figure 4.16: Map of L gene haplotype distribution and their proportions in Kenya

Coast region had the highest number of L gene haplotypes (8) followed by Western Highlands (7). The least number of haplotypes was observed in Lake Victoria basin (4). In terms of haplotype frequency in the populations, L gene haplotype 1 was highly distributed in all the five regions and consisted of the majority of sequences except in Eastern region where most sequences were classified as haplotype 13. The haplotype 1 proportion consisted of 33%, 50%, 46%, 69% and 17% in Nairobi, Coast, Western Highlands, and Lake Victoria basin and Eastern regions respectively.

4.2.3 Sequence analysis of F gene

Fifty-seven (57) partial F gene sequences were obtained from Macrogen® Inc (South Korea). These included 31 and 26 sequences from LBMs and poultry farms respectively. Out of the 57 sequences, five were of poor quality and were discarded from the analysis. The remaining 52 good quality sequences were used for the analysis. Details of these sequences are shown in Appendix 5. The obtained partial F gene sequences were classified into 22 distinct haplotypes. The sequences were deposited in the GenBank with accession numbers ranging from; *KY007043* to *KY007063*. The 52 sequences were obtained from chicken samples and one duck sample. The sequence obtained from the duck sample was identical to the sequence from a chicken sample (sample KE0697/2015-Accession number *KY007050)* obtained from the same farm).

Similarity search on the nucleotide databases through the BLAST algorithm yielded a best hit of a 535bp sequence highly similar (98%) to Newcastle disease virus (APMV-1) *isolate, A148,* partial F gene sequence published by Ommeh et al., (2010) and *Newcastle disease virus strain MU013/2011* published by Byarugaba et al., (2014) classified as Genotype V clade II **(Table 4.17)**. Similarity search in the protein database using a translated nucleotide query (BlastX) yielded a partial Fusion glycoprotein of APMV-1 with 92% sequence identity to that of the glycoprotein (*AAR17528)* of APMV-1 isolate which belongs to classified as Clade II published by Pedersen et al (2004).

Table 4.17: BlastN search results of partial F gene sequences from Kenya

Accession #	H#	**Location**	Highest blast match	Accession #	E Value	%Similarity
KY007043	1	All regions	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
KY007044	2	NM	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
KY007045	3	NM	*Newcastle disease virus isolate, A148*	JQ217420	0.0	98%
KY007049	7	All regions	*Newcastle disease virus strain MU013/2011*	HG937571	0.0	97%
KY007050	8	Eastern	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
KY007051	9	WH	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
KY007052	10	LB	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
KY007053	11	Eastern, WH	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
KY007054	12	LB	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
KY007060	18	NM	*Newcastle disease virus isolate, A148*	JQ217420	0.0	98%
KY007061	19	NM	*Newcastle disease virus isolate, A148*	JQ217420	0.0	97%
KY007062	20	Eastern	*Newcastle disease virus isolate, A148*	JQ217420	0.0	96%
KY007063	21	WH	*Newcastle disease virus isolate, A148*	JQ217420	0.0	96%
KY007064	22	LB	*Newcastle disease virus strain MU013/2011*	HG937571	0.0	97%

*H#-Haplotype identity; NM-Nairobi metropolitan; WH-Western Highlands; LVB-Lake Victoria basin

4.2.4 Multiple sequence alignment of F gene

Consensus sequences alignment showed polymorphism in different sites of the F gene of study strains. Fifty nine (59) polymorphic sites were observed. The region analyzed is localized between 1-535bp.

The region between 10-200bp of the partial F gene nucleotide sequences showed the greatest variability **(Figure 4.17** region marked A, and its alignment is marked B).

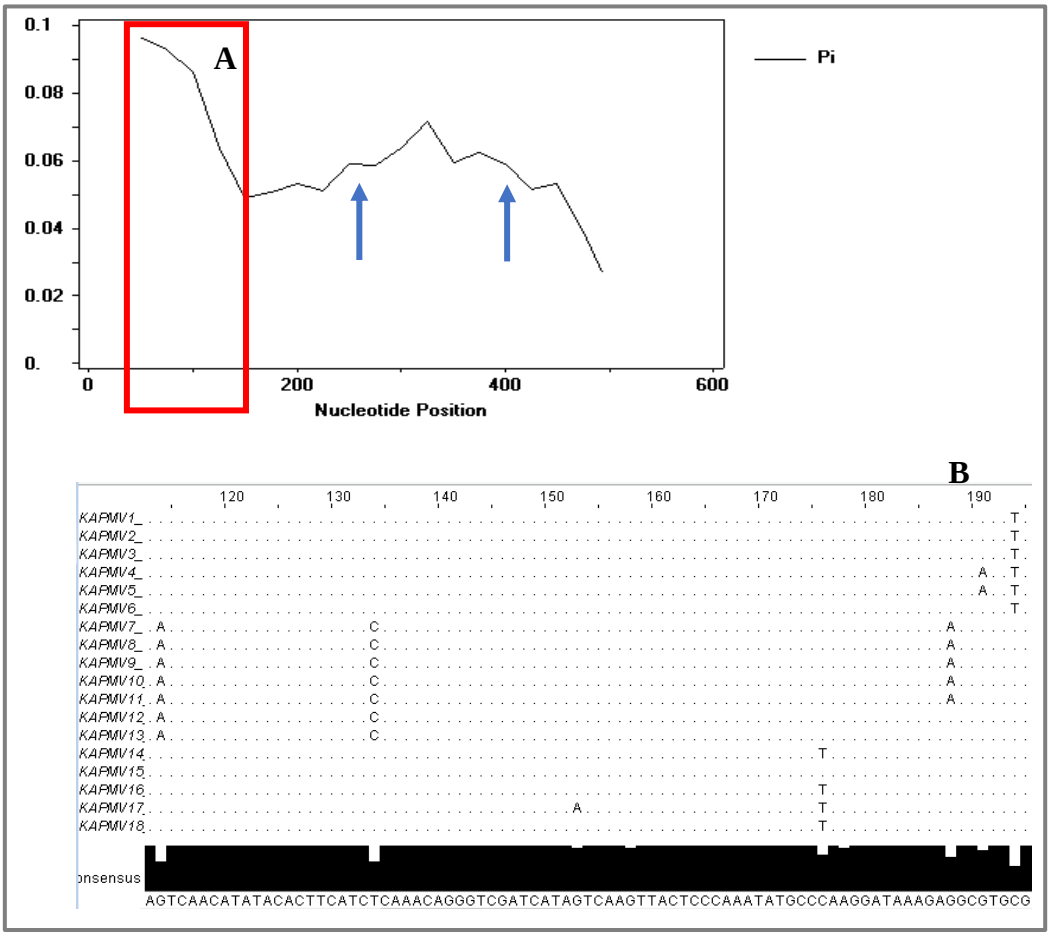

Figure 4.17: Polymorphic sites on obtained APMV F gene nucleotide sequences A is graph of nucleotide diversity against the nucleotide position; B is the sequence alignment of polymorphic area (120-190). Red arrows showing variable region

The region between 275 to 400bp showed slight variability (**Figure 4.17**; region marked with blue arrows). This region includes the F gene cleavage site (336-345bp) whose amino acid (aa) motif is the determinant of virulence for APMV-1 strains (**Figure 4.17**).

Chromatograms of the haplotypes were distinct from each other and substitutions were observed. The position with the high polymorphism was observe at the F gene cleavage site (nucleotide 330-350); nucleotide: A/T substitution at position 334, A/G substitution at position 346 and C/T substitution at nucleotide 350 (**Figure 4.18**).

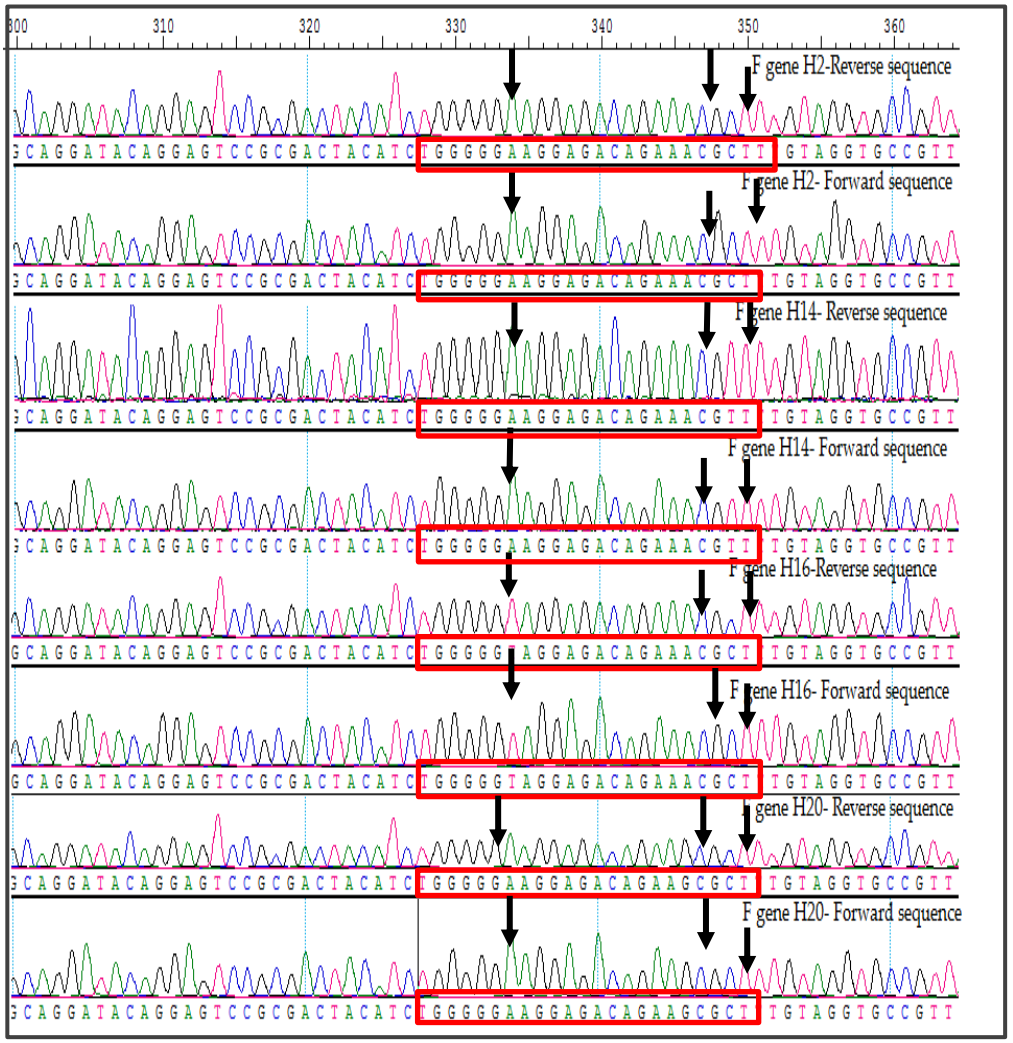

Figure 4.18: F gene sequence chromatograms with cleavage site polymorphisms Black arrow shows point of mutations in the region highlighted by red square

Multiple alignment of the F gene haplotypes shows substitution or single point mutations were common 19. Haplotype 20 (shown by the red arrows on **Figure 4.19**) had the highest number of polymorphic sites was observed across the fusion gene.

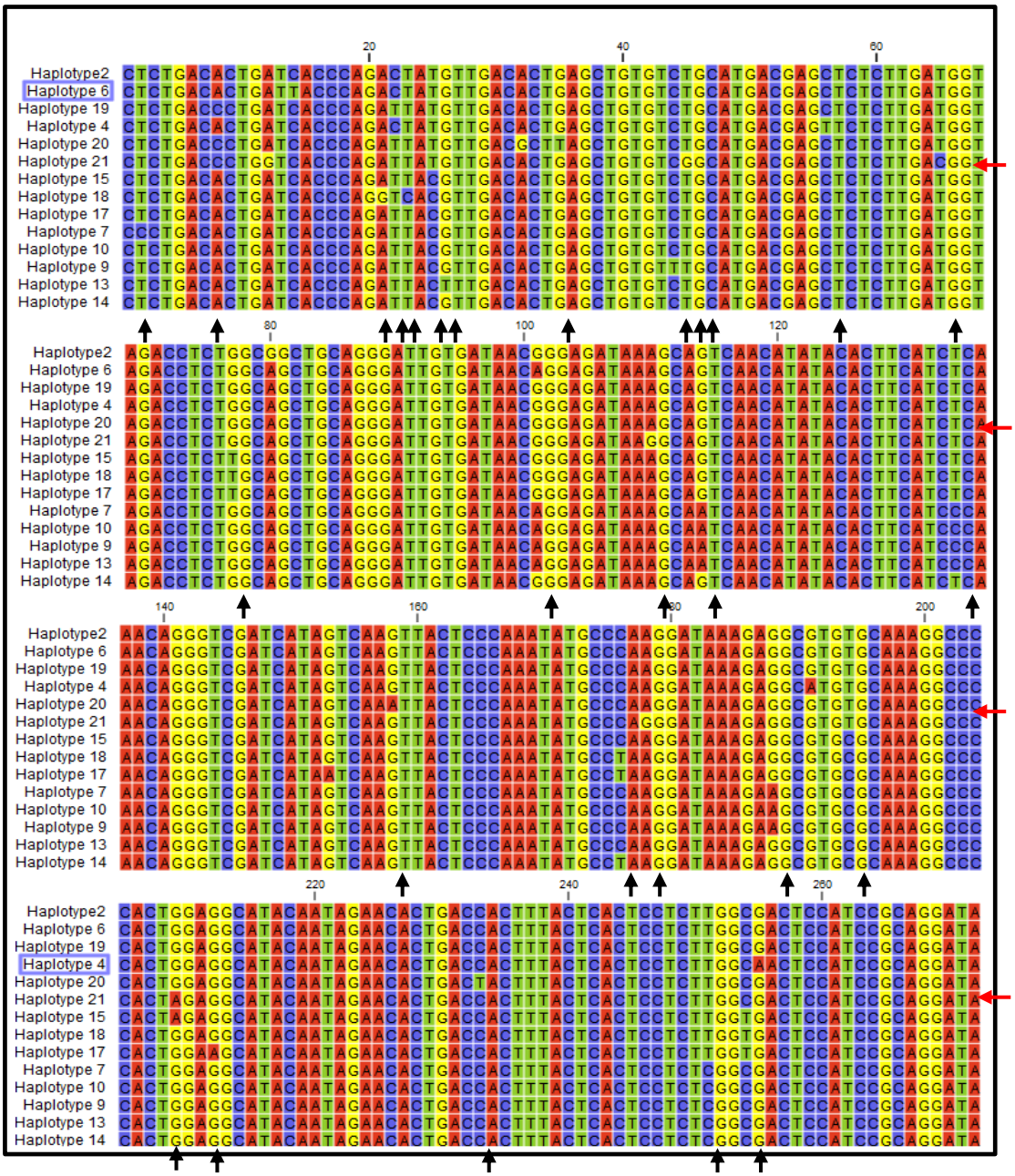

Figure 4.19: Alignment of APMV F gene sequences showing polymorphic sites. Black arrow shows point of mutations

4.2.5 F gene haplotype distribution analysis

The 22 F gene haplotypes were distributed in different proportions in the five regions of Kenya. Two haplotypes (1 and 7) were common in all the regions (**Figure 4.20**). The haplotype frequency per region seemed to differ between the L gene and F gene.

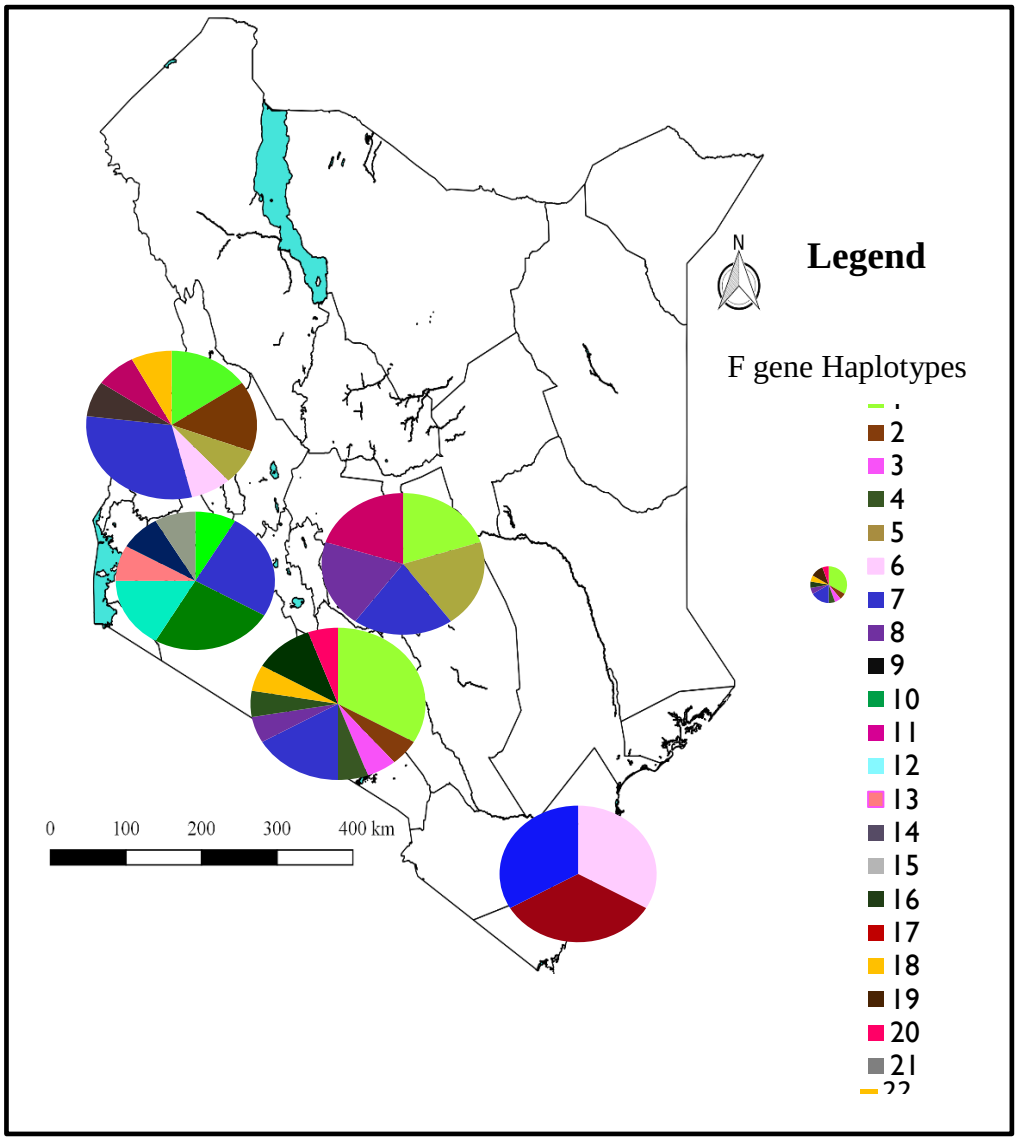

Figure 4.20: F gene haplotype proportions and distribution across Kenya

For the F gene, Nairobi, Western highlands and Lake Victoria Basin had the highest number of F gene haplotypes; 10, 8 and 7 respectively (**Figure 4.20**). On the other hand, the haplotype frequency per region seemed to differ between the L gene and F gene. For the F gene, Nairobi, Western highlands and Lake Victoria Basin had the highest number of F gene haplotypes; 10, 8 and 7 respectively (**Figure 4.20**). On the other hand, Coastal region had the lowest number of haplotypes.

4.2.6 Phylogenetic and diversity analysis

Results of the genetic diversity of avian paramyxovirus is presented using both the Fusion and L genes whose findings were similar in characterizing both the diversity and phylogeny of APMV

4.2.6.1 Diversity and phylogeny of the L gene

Eighteen haplotypes were assigned from seventy-one L gene sequences based on 28 polymorphic sites, 10 of which were parsimony informative. The overall L gene haplotype diversity was 0.759 (±0.02) while the nucleotide diversity was 0.018 (± 0.001). All the ecological zones had haplotype diversity greater than 0.5 (**Table 4.18**).

Table 4.18: L gene haplotype and nucleotide diversity among poultry populations in sampled regions

Population	**Nucleotide diversity(π)**	**Haplotype number**	**Haplotype diversity**
Coastal region	0.016	9	0.743
Lake Victoria basin	0.011	4	0.526
Nairobi metropolitan	0.019	5	0.933
Western Highlands	0.029	6	0.800
Eastern region	0.016	8	0.972

The highest L gene haplotype diversity was in the Eastern region and the least was seen in the Lake Victoria Basin. However, the nucleotide diversity was low and varied from 0.011in Lake Victoria Basin to 0.029 in Western Highlands (**Table 4.18**).

The overall mean number of nucleotide differences between the L gene haplotypes from Kenya was 3.059 (±0.50). The average pairwise distances between the L gene haplotypes from poultry in Kenya varied from 1-12% (0.01 to 0.12). Haplotype 10 was the most divergent from the other L gene haplotypes and diverged from the other haplotypes by 9-12% (0.09 to 0.12). The other haplotypes were less divergent from each other with pairwise distance estimates ranging from 1-4% (**Table 4.19**).

Table 4.19: Pairwise nucleotide divergence estimates between L gene haplotypes

	Average nucleotide differences															
H#	**H3**	**H11**	**H1**	**H15**	**H4**	**H9**	**H2**	**H12**	**H18**	**H8**	**H5**	**H17**	**H14**	**H16**	**H13**	**H7**	**H6**
H3																	
H11	0.01																
H1	0.02	0.04															
H15	0.03	0.04	0.01														
H4	0.03	0.04	0.01	0.01													
H9	0.01	0.02	0.01	0.02	0.02												
H2	0.01	0.02	0.02	0.03	0.03	0.01											
H12	0.02	0.03	0.03	0.04	0.04	0.02	0.01										
H18	0.01	0.02	0.02	0.03	0.03	0.01	0.01	0.02									
H8	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01								
H5	0.01	0.02	0.02	0.02	0.03	0.01	0.01	0.02	0.01	0.01							
H17	0.02	0.03	0.03	0.02	0.04	0.02	0.02	0.02	0.02	0.01	0.01						
H14	0.02	0.03	0.03	0.02	0.04	0.02	0.02	0.02	0.02	0.01	0.01	0.01					
H16	0.02	0.03	0.03	0.02	0.04	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01				
H13	0.01	0.02	0.02	0.03	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.02	0.02			
H7	0.01	0.02	0.02	0.03	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.02	0.02	0.01		
H6	0.02	0.03	0.03	0.04	0.04	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	
H10	0.11	0.11	0.11	0.12	0.12	0.11	0.11	0.11	0.11	0.10	0.11	0.11	0.11	0.11	0.11	0.11	0.09

Phylogenetically, the partial L gene nucleotide sequences were grouped in several clusters: Cluster 1 contained all the sequences from various regions belonging to haplotype 1, 4, 9 and 15 (**Figure 4.21**).

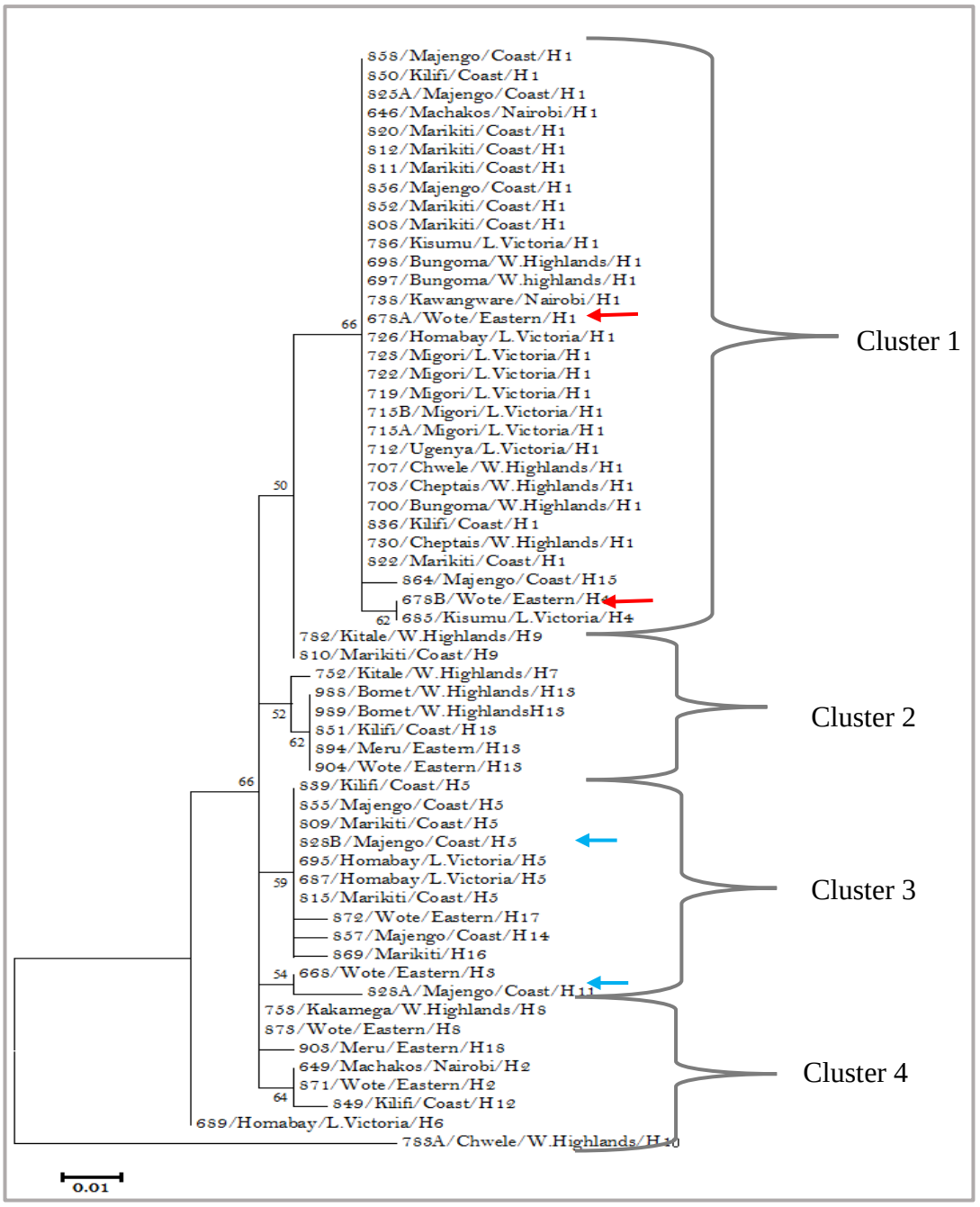

Figure 4.21: Phylogenetic relationship of L gene sequences from poultry in Kenya
The tree was constructed using the Maximum Likelihood method based on Kimura-2 parameter. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (only >50% is shown). The tree was unrooted. Red and blue arrows show two strains from the same bird

Cluster 2 contained sequences that from all regions belonging to haplotype 7 and 13. Cluster 3 contains sequences of haplotype 5, 14, 16 and 17. Cluster 4 contains sequences of haplotypes 3, 8, 11 and 18. Cluster 5 contains sequences of haplotype 2 and 12. Haplotype 6 and 10 were clustered separately from the other sequences (**Figure 4.21).**

Sequences belonging to APMV strains obtained from the same bird were grouped in different haplotypes but similar clusters for example 678A-haplotype 1 and 678B-Haplotype 4 from Eastern region (shown in red arrows-Figure **4.21**), 828A-Haplotype 11 and 828B-Haplotype 5 from Majengo market in Coastal region. From the tree no regional clustering of the sequences was observed, rather sequences, from various regions clustered together.

To determine the phylogenetic relationship between the L gene haplotypes and other APMV-1 strains of class II that have been isolated globally, a phylogenetic tree of L gene haplotypes from Kenyan poultry and reference strains of APMV-1 class II was constructed using the Maximum Likelihood method (**Figure 4.22**).

The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (only >50% is shown). The rate of heterogeneity among the species was high with gamma shape parameter of 0.3261.

The phylogenetic tree (**Figure 4.22**) classified the APMV-1 strains of class II into two main branches: Branch 1 consisting of cluster 1 to 5 and Branch 2 consisting of cluster 6-11. Further, the tree reveals that L gene haplotypes form Kenyan poultry (Cluster 1) and APMV-1 strains classified under class II genotype isolated from wild birds in the USA and Canada (Cluster 2) originate from the same branch.

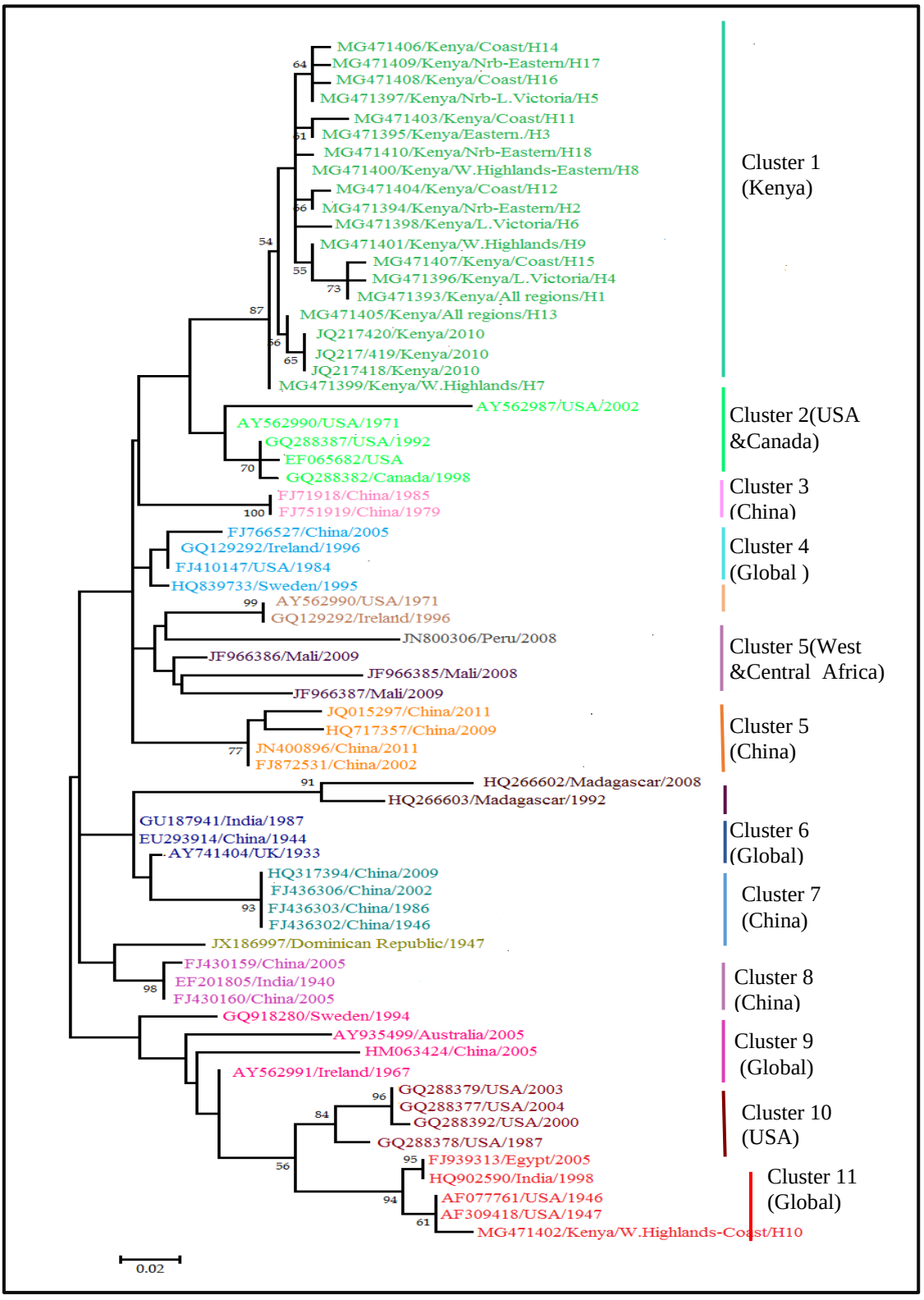

Figure 4.22: Phylogenetic tree of Kenyan L gene and references sequences

The Kenyan haplotypes seem to form a distinct cluster (Cluster 1) that branched off from cluster 2. In cluster 1, the L gene haplotypes clustered together with APMV-1 strains (*JQ217418, JQ217419, and JQ217420*) previously isolated in Kenya (**Figure 4.22** above). However, one haplotypes (Haplotype 10) clustered in a different group from other haplotypes. It clustered with APMV-1 belonging to class II genotype II (Cluster 11).

4.2.6.2 Diversity and phylogeny of the F gene

Twenty-two haplotypes were assigned from fifty-two F gene sequences based on 59 polymorphic sites, 35 of which were parsimony informative. The overall F gene haplotype diversity was 0.906 (±0.03) while the nucleotide diversity was 0.021 (± 0.001). The F gene haplotype diversity among all the sampled regions was 0.97. The haplotype diversity in the different regions was high and ranged from 0.95 to 0.82 (**Table 4.20**). The highest F gene haplotype diversity was observed in Eastern region and the least was seen in the Coastal region. The nucleotide diversity on the other hand varied from 0.01in Lake Victoria Basin and coastal region to 0.023 in Western Highlands.

Table 4.20: F gene haplotype and nucleotide diversity in sampled populations

Population	**Nucleotide diversity(π)**	**Haplotype number**	**Haplotype diversity**
Coastal region	0.01	3	0.820
Lake Victoria basin	0.01	7	0.873
Nairobi metropolitan	0.02	10	0.890
Western Highlands	0.023	9	0.912
Eastern region	0.021	5	0.95

The average number of nucleotide differences between F gene haplotypes was 10.6 (±0.79). **Table 4.21** shows the pairwise distances between the F gene haplotypes from

poultry in Kenya based on the number of base substitutions per site over all sequence pairs (pairwise distance). The pairwise distance between haplotypes ranged from 1%-6% (0.01-0.06). Haplotypes 21 and 19 seemed to be most divergent with their pairwise distance estimates ranging from 3% to 6%.

Table 4.21: Pairwise sequence divergence estimates between F gene haplotypes

H#	Pairwise distance estimates																		
	H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**	**H13**	**H14**	**H15**	**H16**	**H17**	**H18**	**H19**	**H20**
H1																				
H2	0.00																			
H3	0.00	0.00																		
H4	0.01	0.01	0.01																	
H5	0.01	0.01	0.01	0.00																
H6	0.01	0.01	0.01	0.01	0.02															
H7	0.03	0.03	0.03	0.04	0.04	0.03														
H8	0.03	0.04	0.04	0.04	0.04	0.03	0.00													
H9	0.03	0.04	0.04	0.04	0.04	0.03	0.00	0.00												
H10	0.03	0.04	0.04	0.04	0.04	0.03	0.00	0.00	0.00											
H11	0.03	0.03	0.03	0.03	0.04	0.03	0.00	0.00	0.00	0.00										
H12	0.03	0.03	0.03	0.03	0.04	0.03	0.00	0.00	0.00	0.00	0.00									
H13	0.03	0.03	0.03	0.04	0.04	0.03	0.00	0.01	0.01	0.01	0.01	0.00								
H14	0.03	0.03	0.03	0.03	0.04	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02							
H15	0.02	0.02	0.02	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03						
H16	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.01					
H17	0.03	0.03	0.03	0.04	0.03	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.04	0.03	0.02	0.01				
H18	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.01	0.01	0.01			
H19	0.03	0.03	0.03	0.03	0.03	0.03	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.03	0.04	0.05	0.05		
H20	0.01	0.01	0.01	0.01	0.02	0.01	0.03	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	
H21	0.03	0.03	0.03	0.04	0.04	0.03	0.06	0.06	0.06	0.06	0.05	0.05	0.06	0.06	0.05	0.05	0.06	0.05	0.05	0.02

Phylogenetically, the obtained F-gene sequences were grouped into five clusters. No cluster belonged to a particular region, each cluster 1-5 contained viruses from all the five regions. (is shown on **Figure 4.23**)

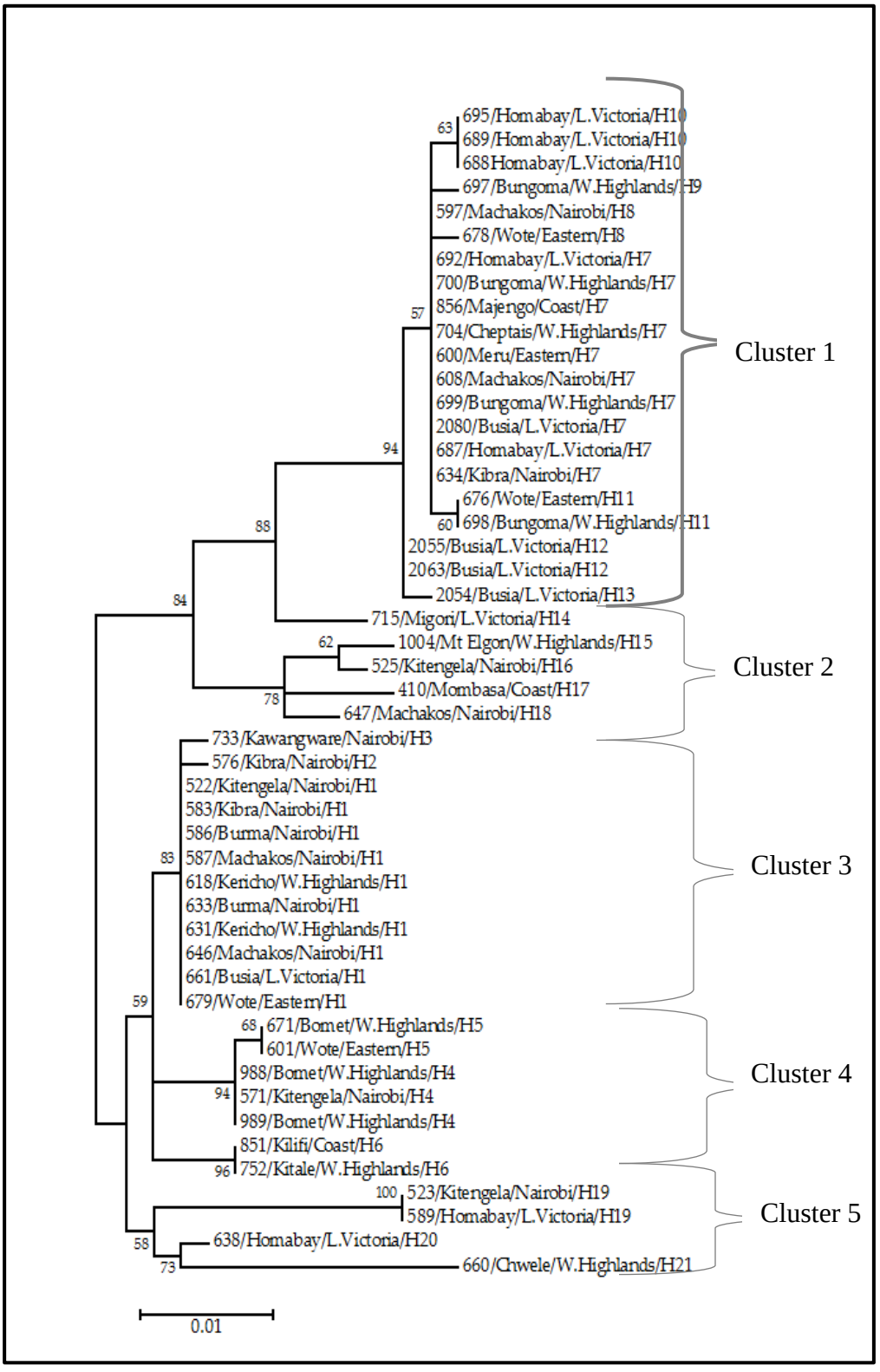

Figure 4.23: Phylogenetic relationship of F gene sequences from poultry in Kenya

From Figure **4.23** above, Cluster 1 contained all the sequences from various regions belonging to haplotype 7, 8, 9, 10 11, 12, 13 and 22. Cluster 2 contained sequences from all regions belonging to haplotype 14, 15, 16, 17 and 18. Cluster 3 contained sequences of haplotype 1, 2 and 3. Cluster 4 contained sequences of haplotypes 4, 5 and 6. Cluster 5 contained sequences of haplotype 19, 20 and 21. Cluster 5 seemed to be somewhat distant from the other clusters.

To determine the phylogenetic relationship between the F gene haplotypes and other APMV-1 strains of class II that have been isolated globally, a phylogenetic tree of F gene haplotypes from Kenyan poultry and reference strains of APMV-1 class II was constructed using the Maximum Likelihood method (**Figure 4.24**).

The tree was constructed based on Kimura 2-parameter model. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (only >50% is shown). APMV-1 strain of Class I was used as an out-group. The gamma correction for rate heterogeneity was 0.4292. The analysis involved 112 nucleotide sequences. The phylogenetic tree (**Figure 4.24**) classified the APMV-1 strains of class II into two main branches: One branch containing Clusters 1 to eight, which are 'recent' genotypes isolated worldwide and another branch containing .clusters 9 to 16, which are 'earlier' genotypes.

All the F gene haplotypes from Kenyan poultry, previous isolates from Kenya *(JQ217418, JQ217419, and JQ217420*) and Uganda (*HG937580*) (cluster 1) together with isolates from North, South America and Europe (Cluster 2) formed a separate branch (bootstrap value =0 92) within the 'recent' genotype's branch. Indicating the close relatedness between the Kenyan and Ugandan strains to cluster 2 strains isolated from Europe, South and North America. The Kenyan and Ugandan strains seemed to form a distinct cluster from cluster 2 (branch bootstrap value=100) (Cluster 2 on **Figure 4.24**) and seemed to cluster separately from isolates from other regions of Africa.

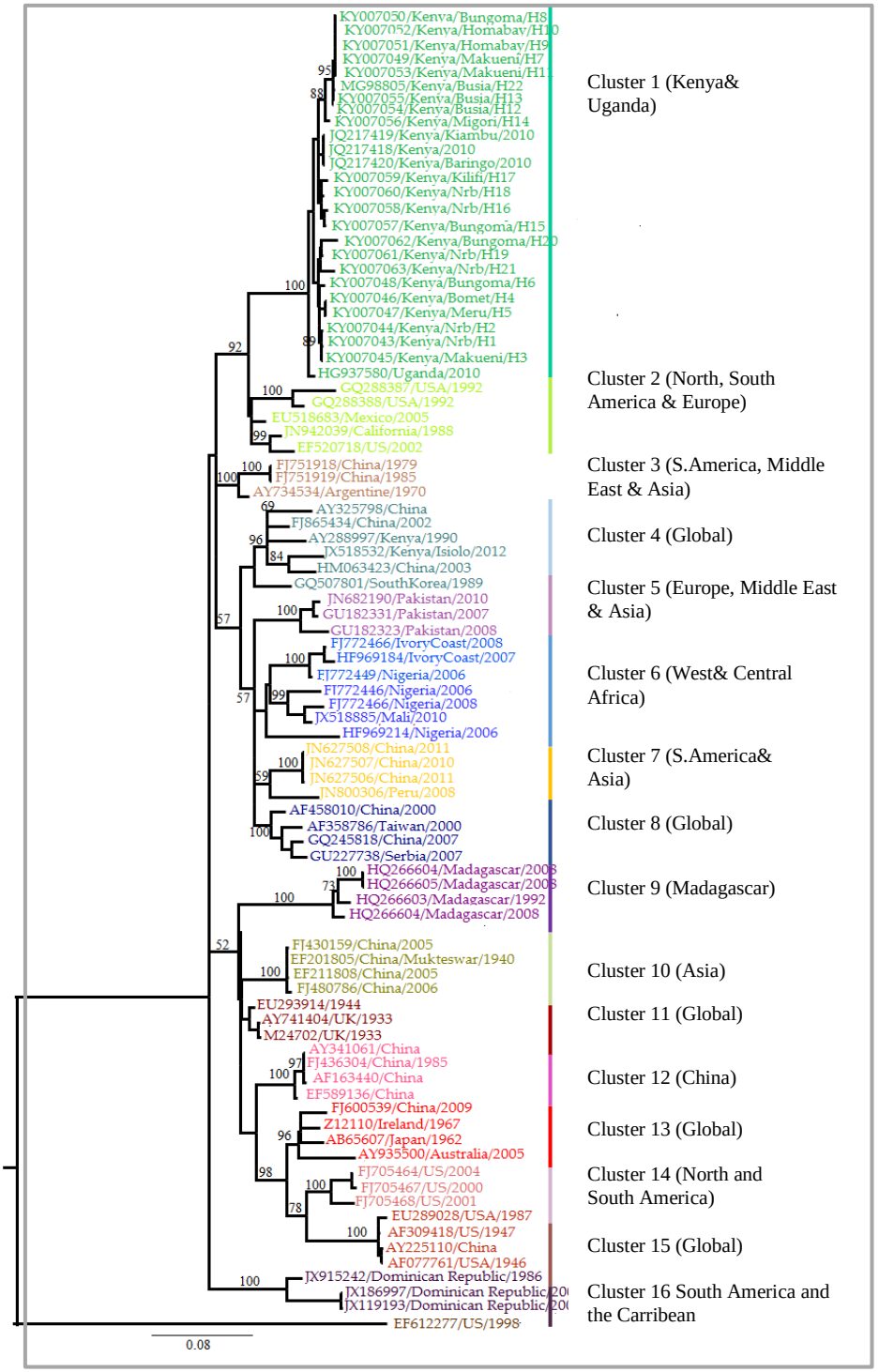

Figure 4.24: Phylogenetic tree of partial F gene sequences of APMV-1 Class II

To characterize the virulence of the F gene haplotypes, the amino acid motif at the cleavage site of the haplotypes was compared in an amino acid (aa) multiple sequence alignment (**Figure 4.25**). At the cleavage site (outlined by a yellow rectangle on Figure 19), all the haplotypes had the aa motif: ^{112}GRRQKR^{116} * F^{117} which is indicative of virulent APMV-1 strains. However, one haplotype (Haplotype 20: *KY007062)* had a G/R substitution at amino acid 112 thereby harboring the motif; ^{112}GRRQRR^{116} * F^{117}

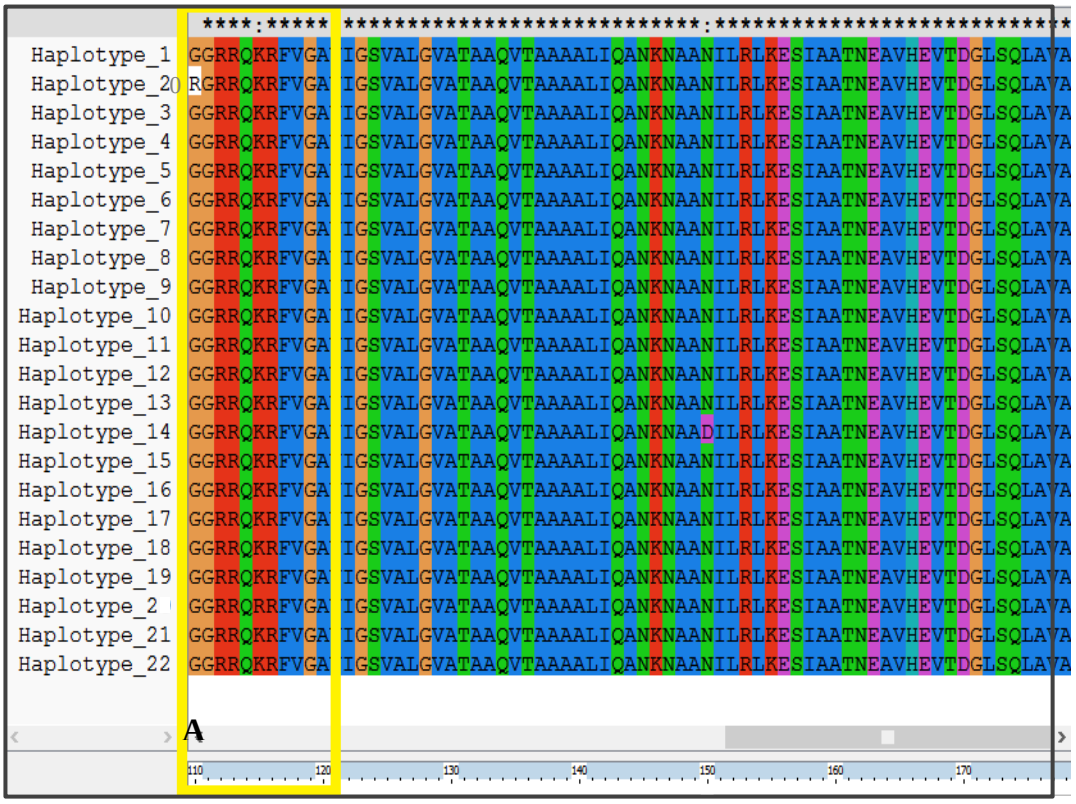

Figure 4.25: Amino acid motif at Fusion gene cleavage site of F gene haplotypes. A-shows the F gene cleavage site amino acid motif

4.3 Whole genome analysis

4.3.1 Identity of obtained genome sequences

The full genomes of three APMV-1 strains were successfully sequenced. The strains represented F gene haplotypes 1, 6 and 21. In addition, the partial genome of two APMV-1 strains representing haplotypes 7 and 14 were also sequenced.

However, the latter two were excluded from the whole genome analysis since they lacked some of the protein coding sequences. H1 was commonly found in chicken in four of the five sampled regions in both live bird markets and poultry farms. However, the sequenced strain was obtained from Burma market in Nairobi. H6 was common in both LBMs and poultry farms in Coast and Western Highlands region. The sequenced H6 strain was obtained from chicken in a poultry farm in Bungoma while H21 was found in chicken in Western Highlands the sequenced strain was obtained from chicken in a market in Malaba.

Prior to library preparation for sequencing, the quality of all the RNA was checked on agarose gel. For most samples, good quality RNA with 28S/18s ribosomal RNA ratio of 2.1 was obtained and used for library preparation (**Figure 4.26**).

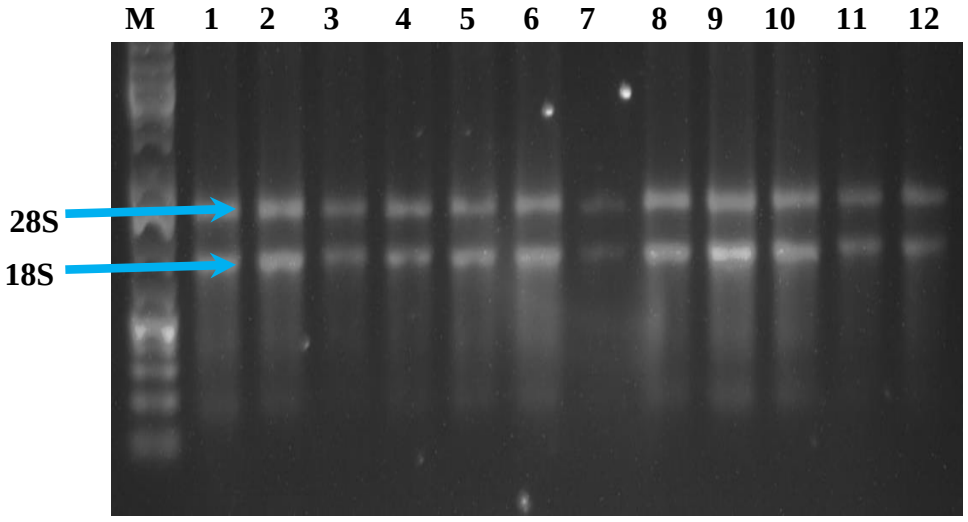

Figure 4.26: RNA gel images of samples for whole genome sequencing
M-marker; Samples 1-11 with clear 18s and 28s bands.

Prior to sequencing, the quality of libraries was also checked using the bioanaylzer. **Figure 4.27** shows the quality and fragment sizes of the DNA libraries for sequencing. The DNA fragments ranged in size from 250bp and 300bp. The concentration of the libraries ranged between 35-50ng/µl.

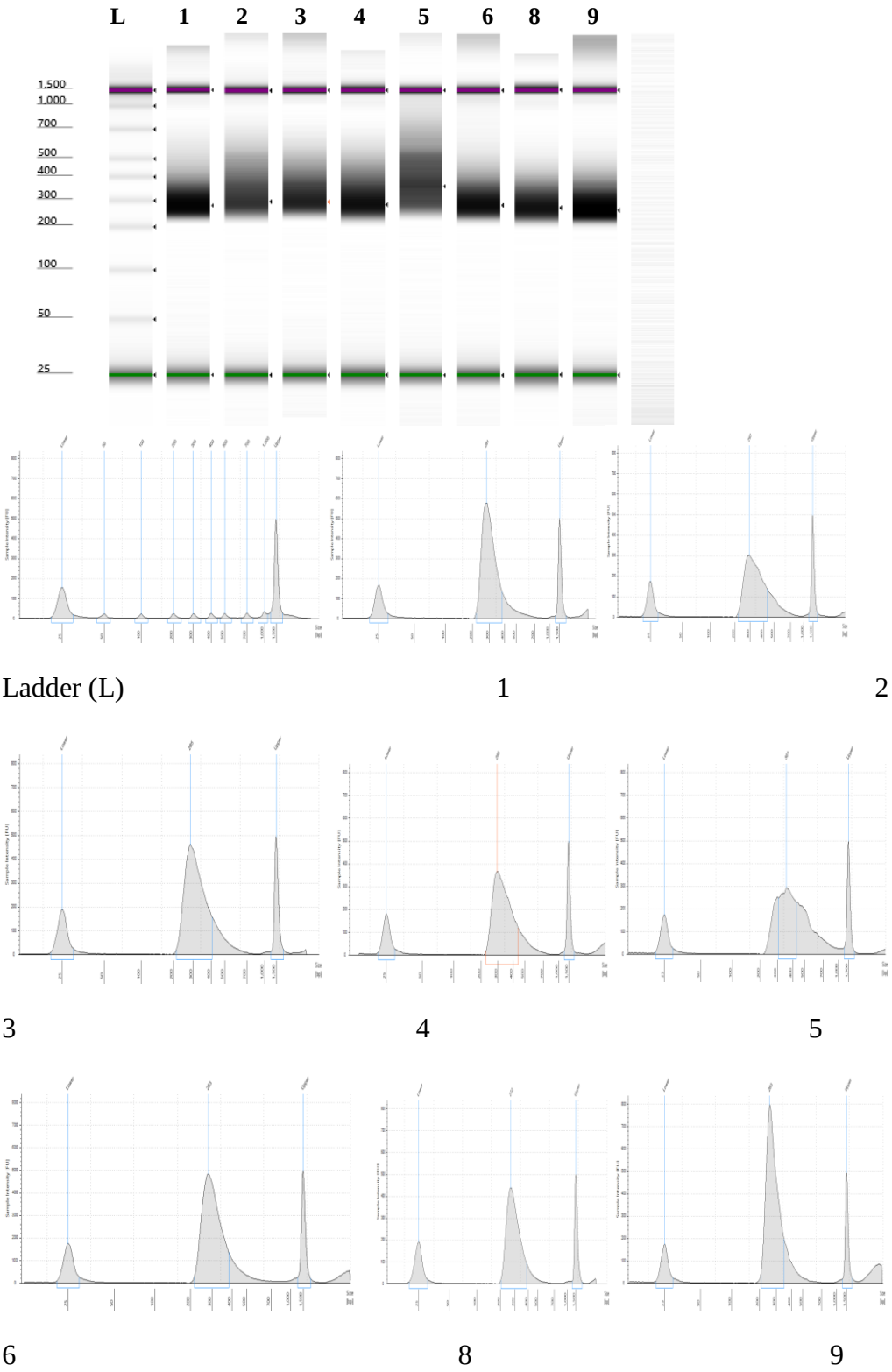

Figure 4.27: Agilent bio-analyzer quality for DNA libraries

4.3.2 Genomic and non-coding sequence analysis

Details of the three genome sequences are shown on Table 21. Similarity search on the nucleotide databases through the BLAST algorithm yielded high similarity (97%) to a partial genome sequence of *Newcastle disease virus (APMV-1) isolate, A48,* submitted to the GenBank by Ommeh et al., (2010). The haplotypes also showed high similarity (92%) similarity to a complete genome sequence *Newcastle disease virus strain, Largo/71* from the USA classified as Genotype V clade II **(Table 4.22.)**.

Table 4.22: BlastN search results of genome sequences of APMV-1 from Kenya

H#	Location	Year of collection	Highest blast match(Accession)	E Value	%Similarity
1	Makueni	*2015*	*A48 (JQ217419)*	0.0	97%
			Largo/71(AY562990)	0.0	92%
6	Marikiti	*2016*	*A48(JQ217419)*	0.0	97%
			Largo/71(AY562990)	0.0	92%
21	Malaba	*2016*	*A48(JQ217419)*	0.0	97%
			Largo/71(AY562990)	0.0	92%

H#-F gene haplotype number. In parenthesis- Accession numbers of highest match

The genomes of the study strains (H1, H6 and H21) had a length of 15192 nt. The genomes were organized, as 3´-NP-P-M-F-HN-L-5´ (**Figure 4.28**). The GC content was 47%. Their genomes started with a leader and trailer sequences at the 3´ and 5´ end respectively. Similarly, the study strains had a considerably conserved sequence at the start of every gene (GS) and another one at the end (GE) of each gene. The open reading frame of each gene was flanked with 3´ and 5´ end untranslated regions (UTRs) on each end. Between GE of one gene and GS of the next gene is a conserved sequence, intergenic sequence (IGS).

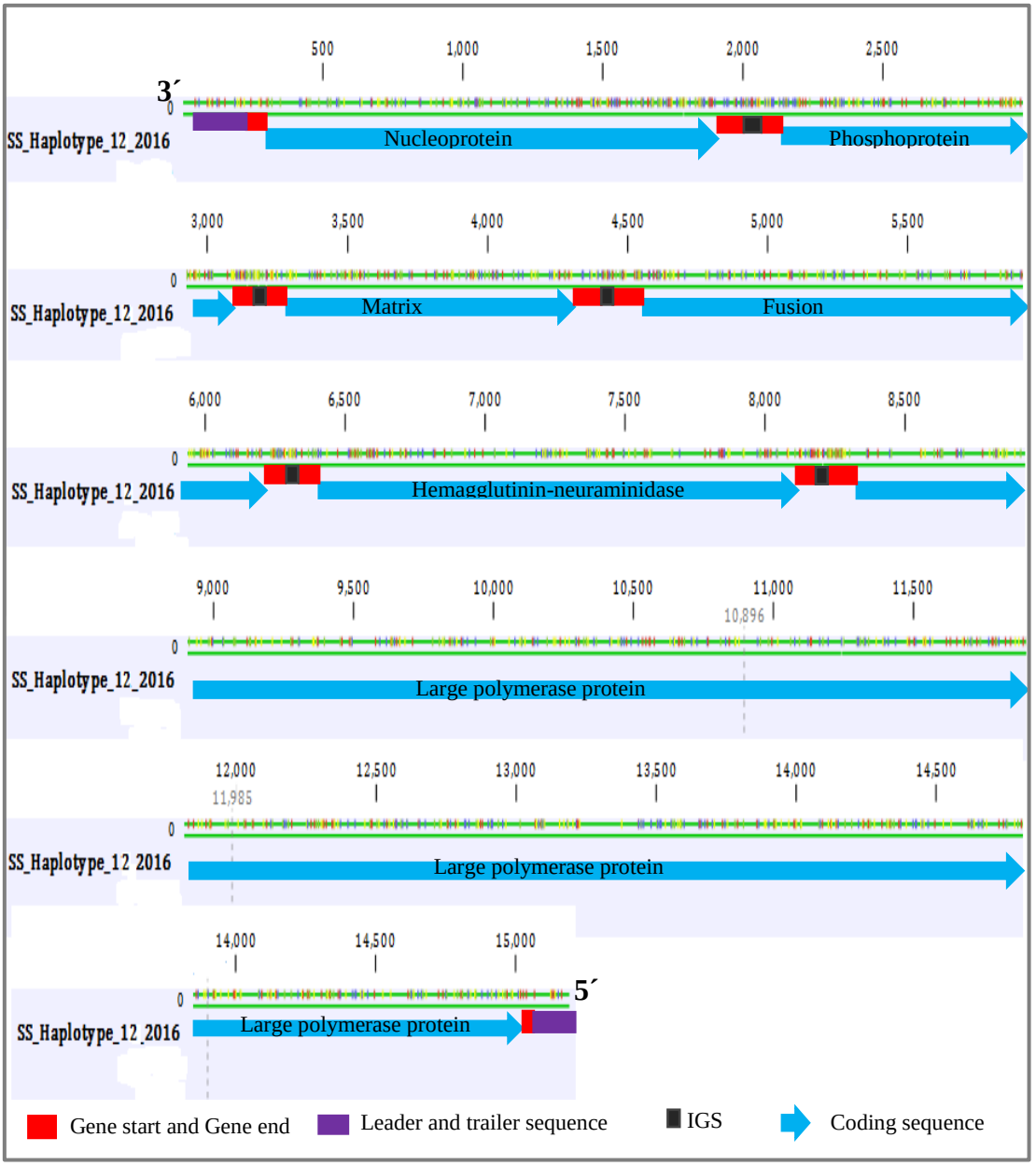

Figure 4.28: Map of the genomic regions of APMV-1 strain obtained from Kenya

The large polymerase protein was the largest gene with a protein coding sequence of 6114bp in length while the Matrix protein was comparatively smaller (1074bp) (**Table 4.23**). There were intergenic regions (IGS) comprised of short sequences of 1-3bp The 3´UTR before each gene ranged between 11 and 91 bp and were shorted than the

5´UTR, which ranged between 77 and 216bp. The largest protein was the largest protein was the large polymerase protein (2204aa). The M protein had the amino acid shortest sequence.

Table 4.23 Genome characteristics of APMV-1 strains from poultry in Kenya

ORF	Genome characteristics					IGS	Gene length	Protein length
	Gene start	3 UTR	Coding sequence	5UTR	Gene end			
NP	56-65	66	122-1591	216	1798-1807	2	1752	489
P	1810-1819	83	1893-3080	180	3250-3260	1	1451	395
M	3262-3271	34	3296-4390	112	4493-4501	1	1241	364
F	4504-4513	46	4550-6211	84	6285-6295	31	1791	553
HN	6327-6336	91	6418-8133	195	8319-8328	47	2002	571
L	8376-8385	11	8387-15001	77	15069-15078		6702	2204

NP-Nucleoprotein; P-phosphoprotein; M-matrix gene; F-fusion gene; HN-Hemagglutinin Neuraminidase; L- Large RNA polymerase; IGS-intergenic region sequence; ORF-open reading frame; UTR- untranslated region.

Several polymorphic sites were observed across the genome of the study strains. The most variable region is between 1000 and 2500bp (area marked A in **Figure 4.29**), a region that also contains the 5´UTR region of the NP gene. In this non-coding region of the NP gene, the study strains, compared to other strains of APMV-1 (Genotype I (*AF077761*), II (*AF309418*) and Genotype III (*EF201805*), had an insertion of six nt (Cytosine: ‘CCCCC (T) C’) between position 1643nt and 1650nt (Marker with a blue arrow on **Figure 4.29**).

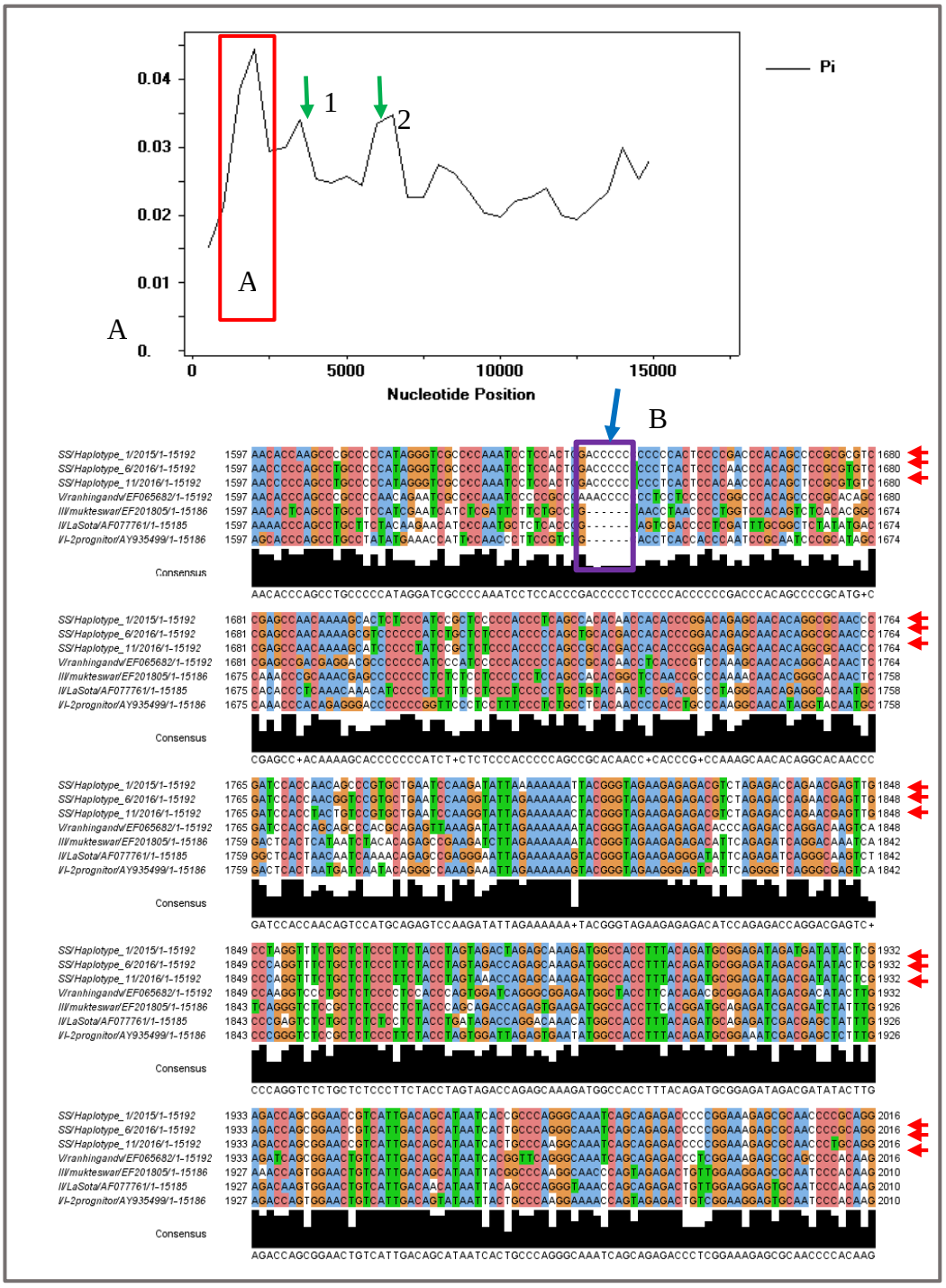

Figure 4.29: Polymorphic sites in the genomic sequence of Kenyan APMV-1 strains A-peak of polymorphism corresponding to NP gene insertion (arrow B); Green arrows-other peaks of polymorphism (F and P gene)

On **Figure 4.29** above, the area marked with green arrows 1 and 2 show other areas of polymorphism on the genome of the study strains between nucleotides 2500 to 4000bp and between 5500 to 7000bp respectively. These two areas correspond with the coding sequences of the P and F genes respectively. The area marker B is an alignment of region 1650 to 2480bp showing the polymorphic sites. Variation in the genomes of the three study sequences (marked by red arrows) was mainly due to substitutions (mainly A/G and C/T substitutions).

4.3.3 Genomic features of the protein coding regions

The multiple alignment of aa sequence of the NP protein (**Figure 4.30**) showed that the sequence of the study strains was similar except at aa 50, which had a N/S substitution and aa 480 with I/T substitution. The NP aa sequence of SS was similar to the Kenyan isolate *A48*. Like other paramyxoviruses, the NP protein of SS had a conserved domain in the amino acid sequence between aa 322 and 336 with the motif: ^{322}FAPAEYAQLYSFAMG^{336} **(Figure 4.30).**

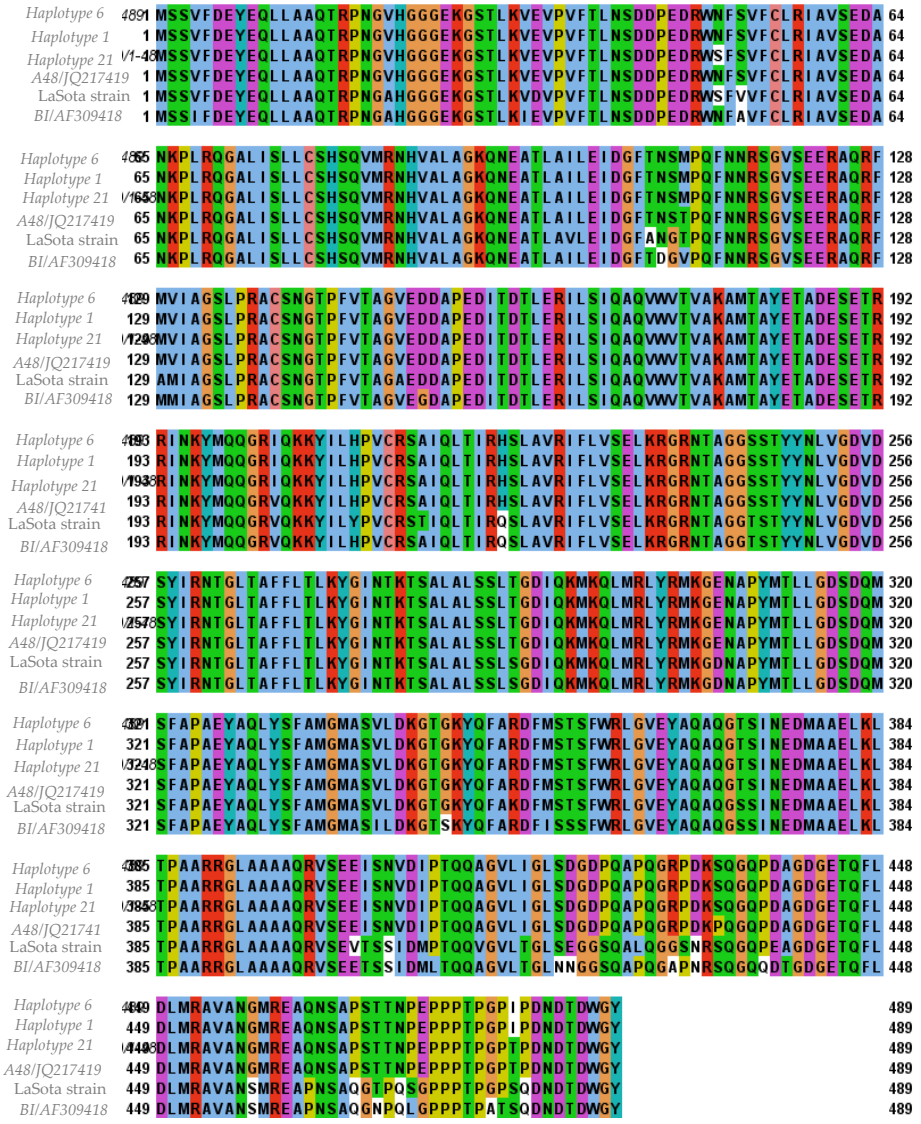

Figure 4.30: Multiple alignment of amino acid sequences of the NP gene

The multiple alignment of aa sequence of the phosphoprotein (**Figure 4.31**) showed that the protein was highly polymorphic. Several aa substitutions were observed among the study strains. For example, at aa 54 Q was observed in strains H1 and H21 while H was observed in H6. This was also the case for aa 45 where N was observed for H1 and H21 while H was observed for H6. The phosphoprotein of the study strains seemed to have unique aa at some sites that differentiated them from vaccine strains (*LaSota* and *B1*). The nucleotide sequence of the P gene of study strains had a putative editing

site that occurs in all APMV-1 strains to allow for RNA editing (5'-AAAAAGGG-3'). This was found in our study sequences between nucleotide 394-401 of the P-gene and nucleotide 2286-2293 of the genome.

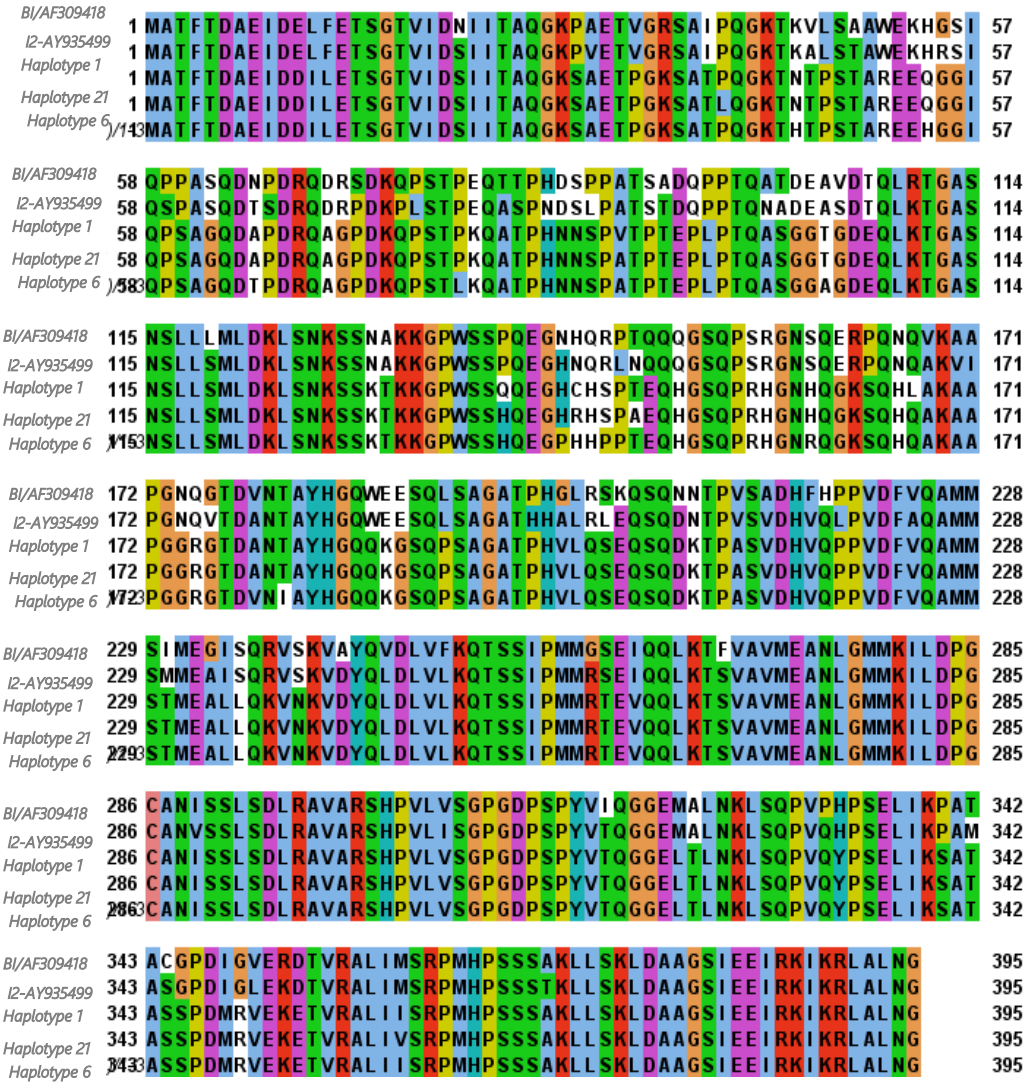

Figure 4.31: Multiple alignment of amino acid sequences of the phosphoprotein gene

The multiple alignment of the aa sequences of the matrix gene (**Figure 4.32**) showed less variability between the study strains. However, sequence variations were observed

between the study strains and the vaccine strains but these were lesser for the Matrix gene than observed for other genes.

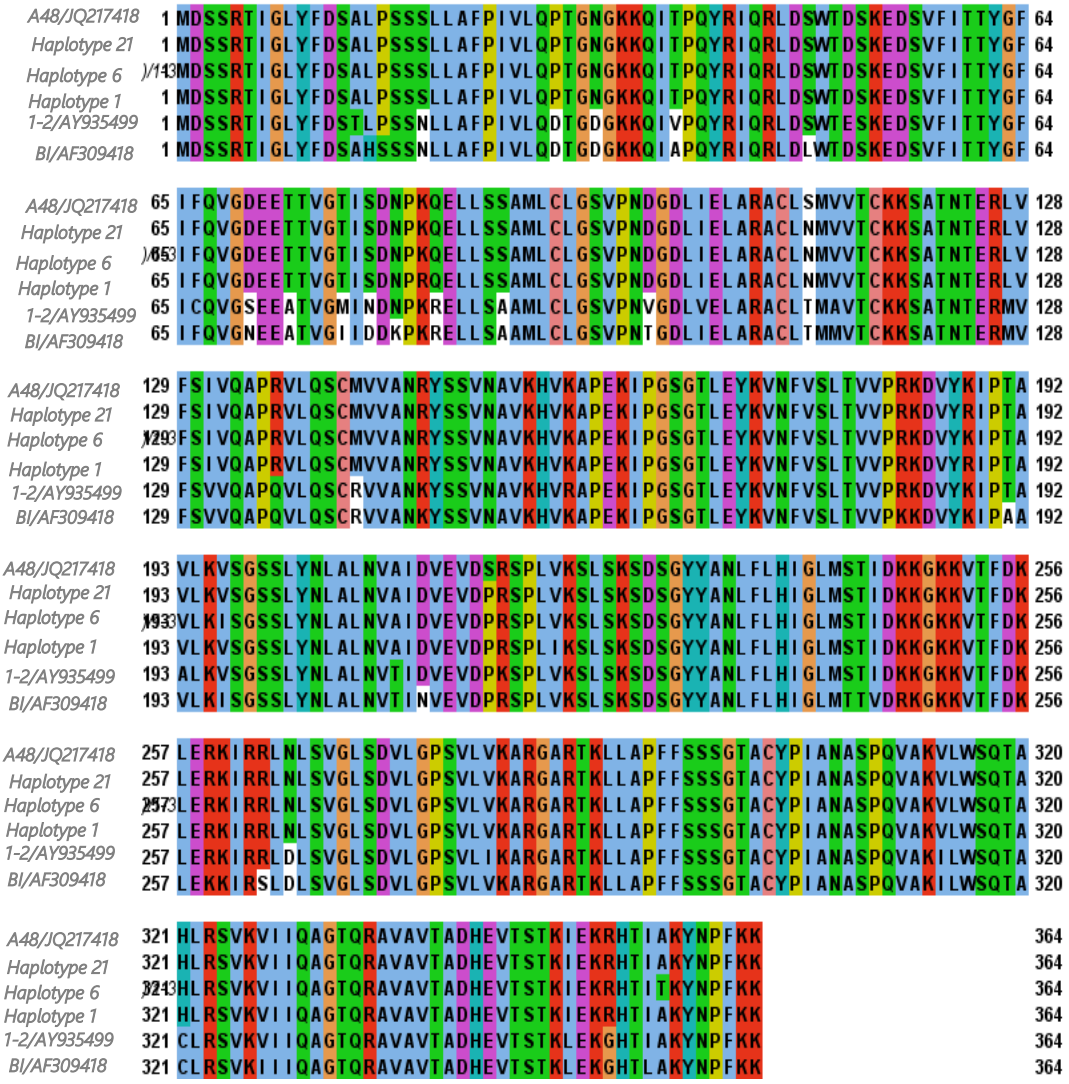

Figure 4.32: Multiple alignment of amino acid sequences of the matrix gene

The multiple alignment of aa of Fusion gene (**Figure 4.33**) shows that Kenyan stains like the vaccine strains had 553aa in length. Study strains and vaccine strains shared similar neutralizing antibody epitope aa residues at the aa positions 151 to 171; ^{151}ILRLKESIAATNEAVHEVTDG^{171}. However, there were aa residues in the protein that seemed unique for the study strains these include: M28, V121, and K146. Other

polymorphic sites that were identified in study strains and were different from vaccine strains included residues: A29, N192, R195, T203, H420, L450, S476, N489, V516 and T553.

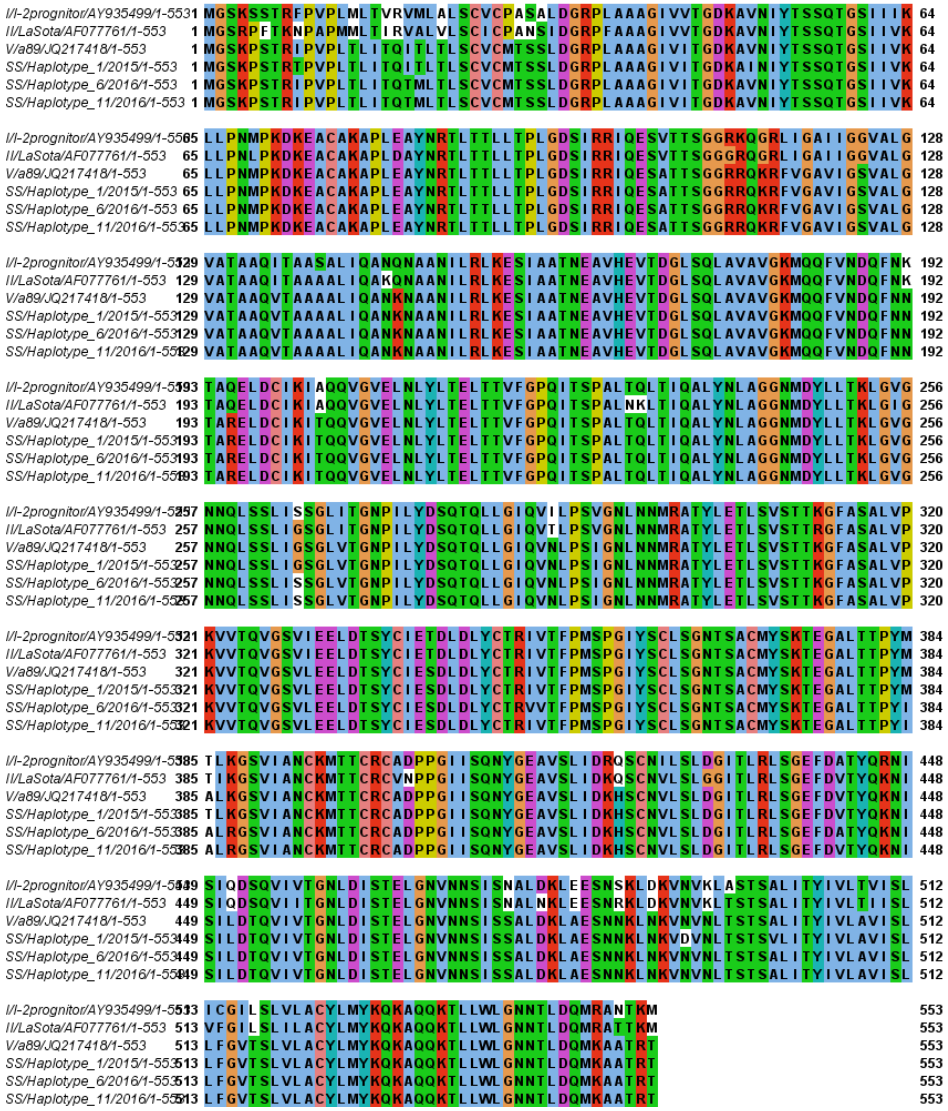

Figure 4.33: Multiple alignment of amino acid sequences of the fusion gene

The HN protein had 571aa in length. The HN protein of SS and Vaccine strains had a conserved sequence: [79]DVIDRVYKQVALESPLALLSTESIIMNAITSLSYQIN[115] except three substitutions at residues V81I, N98S and T102I (**Figure 4.34**). This site

corresponds to the Neuraminidase active site that binds the Fusion protein. Other aa residues that form HN antigen epitopes were similar for both vaccine and study strains: these include K138, P244, F277, F307, T380, T409 and H482 (**Figure 4.34**).

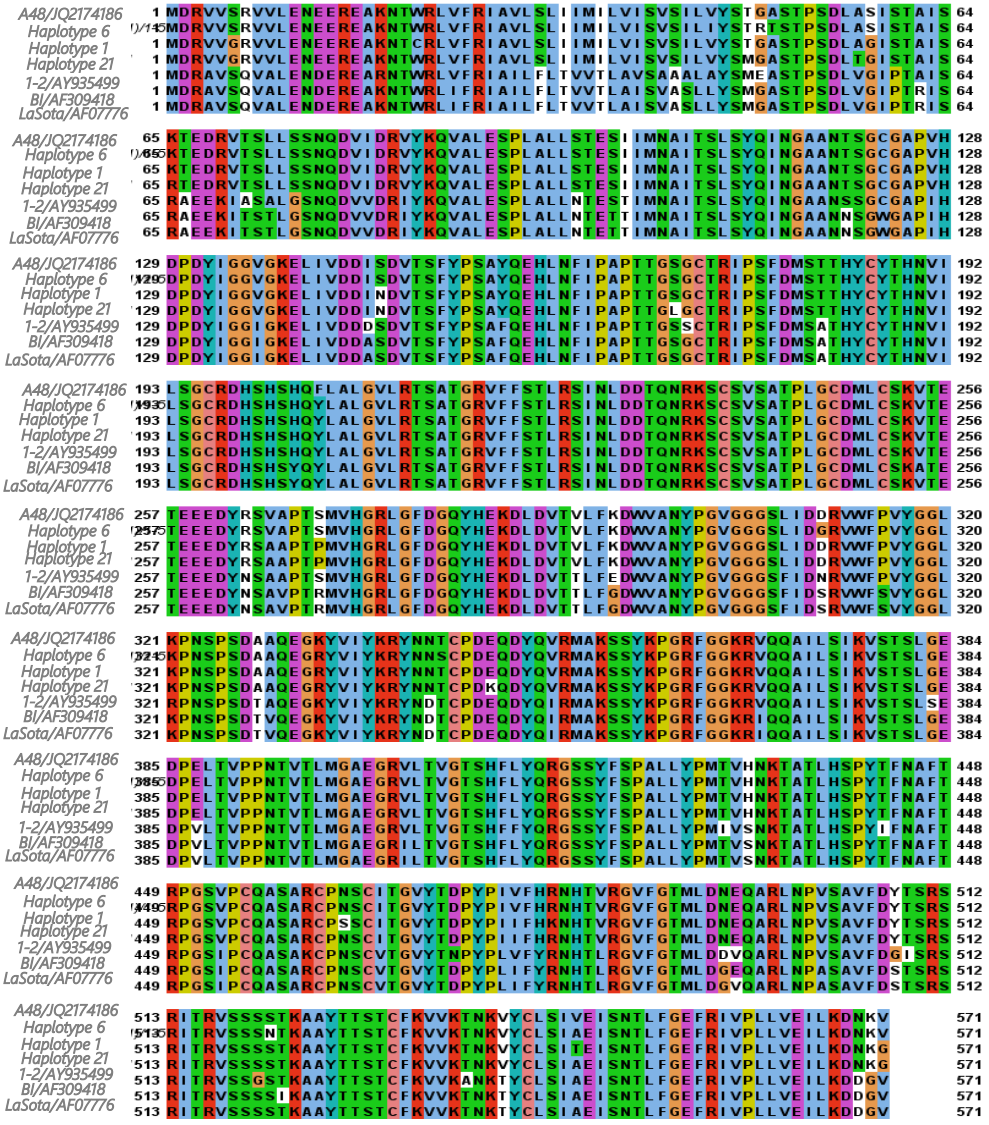

Figure 4.34: Multiple alignment of amino acid sequences of the HN gene

Across the large polymerase protein, there were unique aa markers for the study strains that differed from the APMV-1 strains belonging to other genotypes as well as the vaccine strains.

These included residues: R159, K426, P897, K1077, N1174, A1237, Q1346, N1495, E1567, H1738, H1845, T1869, Q1983, V2056, I2094, K2118 and R2156. The later residues seemed to be unique for Kenyan APMV-1 strains. APMV-1 strains of Genotype V including our study strains had the unique aa residues: S287, P897, Q1241, P1408 and. A1476.

4.3.4 Genomic similarity and divergence

Among the full-length sequences of APMV-1 the study strains (SS) showed highest nucleotide sequence similarity (96%) to a previous Kenyan isolate (strain A48: GenBank accession number *JQ217418*), followed by strain rAnhinga (89%), both classified in Class II Genotype V (**Table 4.24**).

Table 4.24: Percentage (%) similarity between genomes of APMV-1

Strain name	Comparative similarity (%)					
	Study strains (SS)			Vaccine strains		
	H1	H6	H21	LaSota	I-2	BHG
H1-Study strain		96.7	96.6	82.2	82.7	82.4
H6-Study strain	96.7		98.9	82.1	82.7	82.3
H21-Study strain	96.6	98.9		82.1	82.6	82.3
LaSota-II	82.2	82.1	82.1		87.6	87.4
I-2-I	82.7	82.7	82.6	87.6		90.7
BHG-I	82.4	82.2	82.3	87.4	90.7	
Mukteswar-III	85.8	84.8	84.8	87.7	88.7	88.1
Italien-IV	84.6	84.7	84.6	87.8	88.2	88.1
ZJ/1/86/Ch-IX	84.5	84.6	84.5	87.8	88.2	88.1
rAnhinga-Va	89.4	89.2	89.1	83.2	84.1	84.1
A48-Vd	95.7	95.6	95.6	81.4	81.9	81.6
AV324/96-VI	85.6	85.5	85.5	83.5	84.8	84.8
ZA/AL495/04-VII	84.2	84.2	84.3	82.5	83.1	82.8
QH1-VIII	86.6	86.5	86.5	84.4	85.4	85.3
MG/1992/08-XI	82.9	82.8	82.8	83.5	84.1	84.2
Mali/ML007/08-XVII	84.5	84.5	84.5	82.5	83.3	83.2
499-31/2008-XVI	83.4	83.4	83.4	82.6	83.1	82.9

H1, H6,H21-Kenyan strains of APMV-1 obtained in this study

There was high similarity (96-99%) between the genomes of the three haplotypes (Study Sequences). The similarity between the genomes of vaccine strains and SS ranged from 82-83% (**Table 4.24)**.

The percentage similarity for nucleotide sequences encoding the different APMV-1 genes were compared between the H1 study strain and other APMV-1 strains including the other two study strains and vaccine strains. The similarity of the nucleotide sequence of Haplotype 1 genes to vaccine strains (*LaSota, BHG and I-2)* ranged from 77-85%. (**Table 4.25**). The similarity in nucleotide sequences of the study strains ranged from 96-97%. The study strains were highly similar to strain *A48*, a previous Kenyan isolate belonging to Genotype V sub-genotype Vd. The lowest similarity between the study and vaccine strains was observed on nucleotide sequence the P gene.

Table 4.25: Percent (%) similarity of the nucleotide coding sequences

Strain	***Comparative similarity(%) with H1-SS***					
name	***NP***	***P***	***M***	***F***	***HN***	***L***
H6-SS	96.4	95.7	96.8	96.4	96.7	97.1
H21-SS	96.3	95.7	96.8	96.5	96.3	97.1
A48-Vd	91.1	-	97.2	97.1	96.9	97.7
LaSota-II	80.5	77.9	79.9	82.6	80.0	85.2
I-2-I	81.1	76.9	81.8	82.9	80.4	85.4
BHG-I	81.9	76.9	82.8	82.7	79.2	85.3

H1, H6,H21-Kenyan strains of APMV-1 obtained in this study

The amino acid sequences of Haplotype 1 were highly similar (96-99%) to those of the other two strains (H6 and H11) from poultry in Kenya. The genes of haplotype 1 were highly similar to those of *strain A48* (**Table 4.26**). The similarity in the amino acid ORFs between vaccine strains and study strains ranged from 80-96%. The least similarity between Study strains and vaccine strains was observed in the aa ORF of the P gene.

Table 4.26: Percent (%) similarity of amino acid ORFs

Strain name	***Comparative similarity(%) to H1-SS***					
	NP	***P***	***M***	***F***	***HN***	***L***
H6-SS	99.4	96.7	98.5	98.4	97.7	99.3
H21-SS	99.4	97.2	98.3	98.6	97.5	99.3
A48-Vd	98.6	-	98.8	99.3	99.1	99.4
LaSota-II	93.5	79.5	88.5	91.9	91.1	96.1
I-2-I	93.0	79.2	90.8	93.1	90.7	96.0
BHG-I	94.1	80.5	89.5	92.4	91.1	96.2

NP-Nucleoprotein; P-Phosphoprotein; M-matrix gene; F-fusion gene, H- Hemagglutinin neuraminidase. L- Large RNA polymerase

4.3.5 Phylogenetic analysis

To establish the phylogenetic relationship of the SS and other available APMV-1 strains, a phylogenetic tree (**Figure 4.35**) was drawn using full genome sequences of SS and 30 other APMV-1 strains representing known APMV-1 genotypes available in the GenBank. The tree was drawn in MEGA X.O using maximum likelihood method with a GTR model. The rate of heterogeneity among the genotypes was high with a gamma shape parameter of 0.44. Numbers on the branches indicate the bootstrap values based on 1000 replicates. The tree grouped the APMV-1 strains of class II that were used in the analysis alongside study strains into two clusters. Cluster I were the recent genotypes : V, VI, VII, VIII, XII, XIII, XIV, XVII and XVIII while cluster II consisted of earlier genotypes of APMV-1 (I, II, III and IV) and their closely related strains, IX, X and XI. The three study strains (underlined in **Figure 4.35**) obtained from poultry in Kenya clustered with APMV-1 strains of genotype V. However, together with an APMV-1 strain *(A48)* previously isolated from Kenya, the three study strains formed a distinct cluster separate from the other strains of Genotype V (**Figure 4.35**).

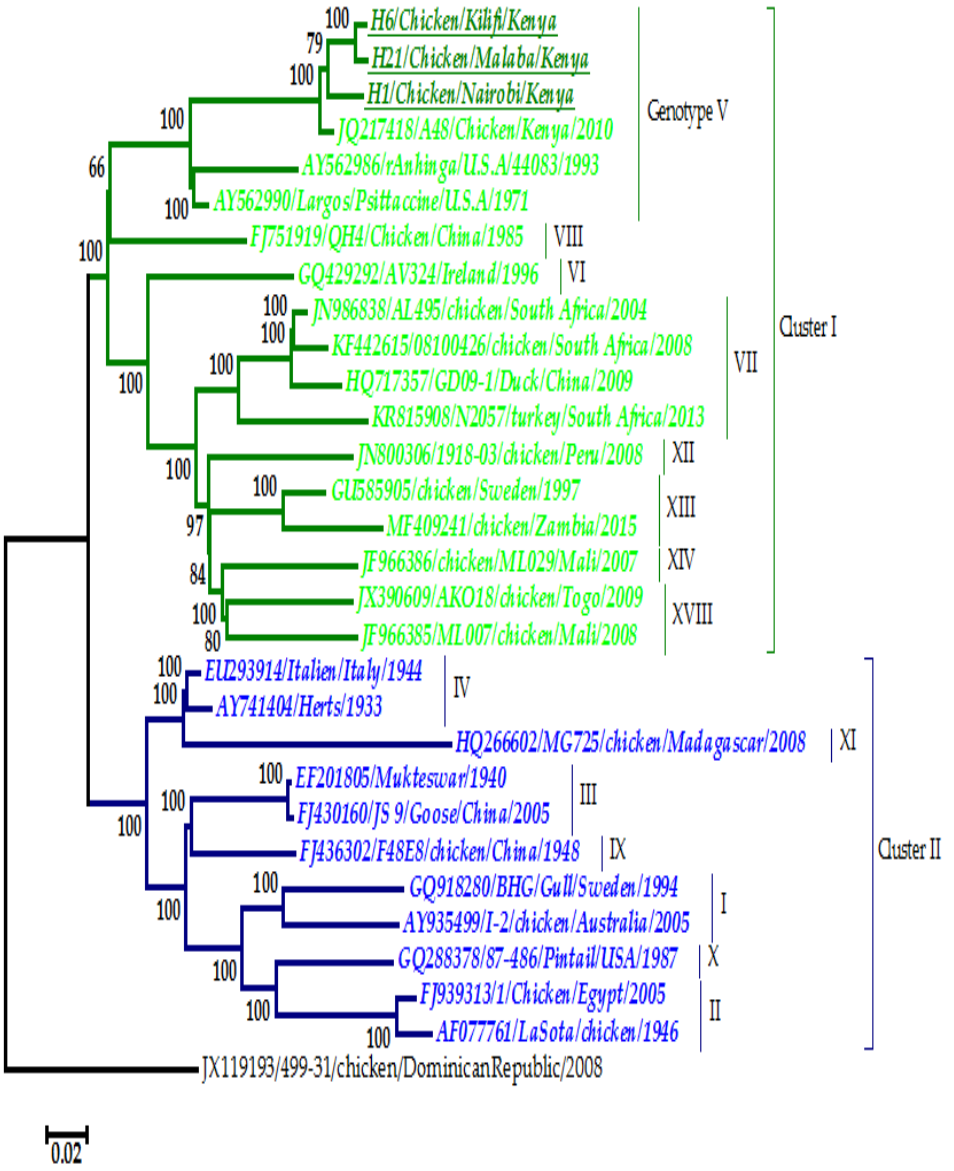

Figure 4.35: Phylogenetic tree of APMV-1 complete genome sequences

Phylogenetic analysis was also carried out using the full nucleotide coding sequences of the six genes (NP, P, M, F, HN and L) of the APMV-1 strains from poultry in Kenya and representative APMV-1 strains. The phylogenetic trees based on full coding nucleotide sequence of the F gene was constructed using the available F gene sequences alongside others available in the GenBank (**Figure 4.36**).

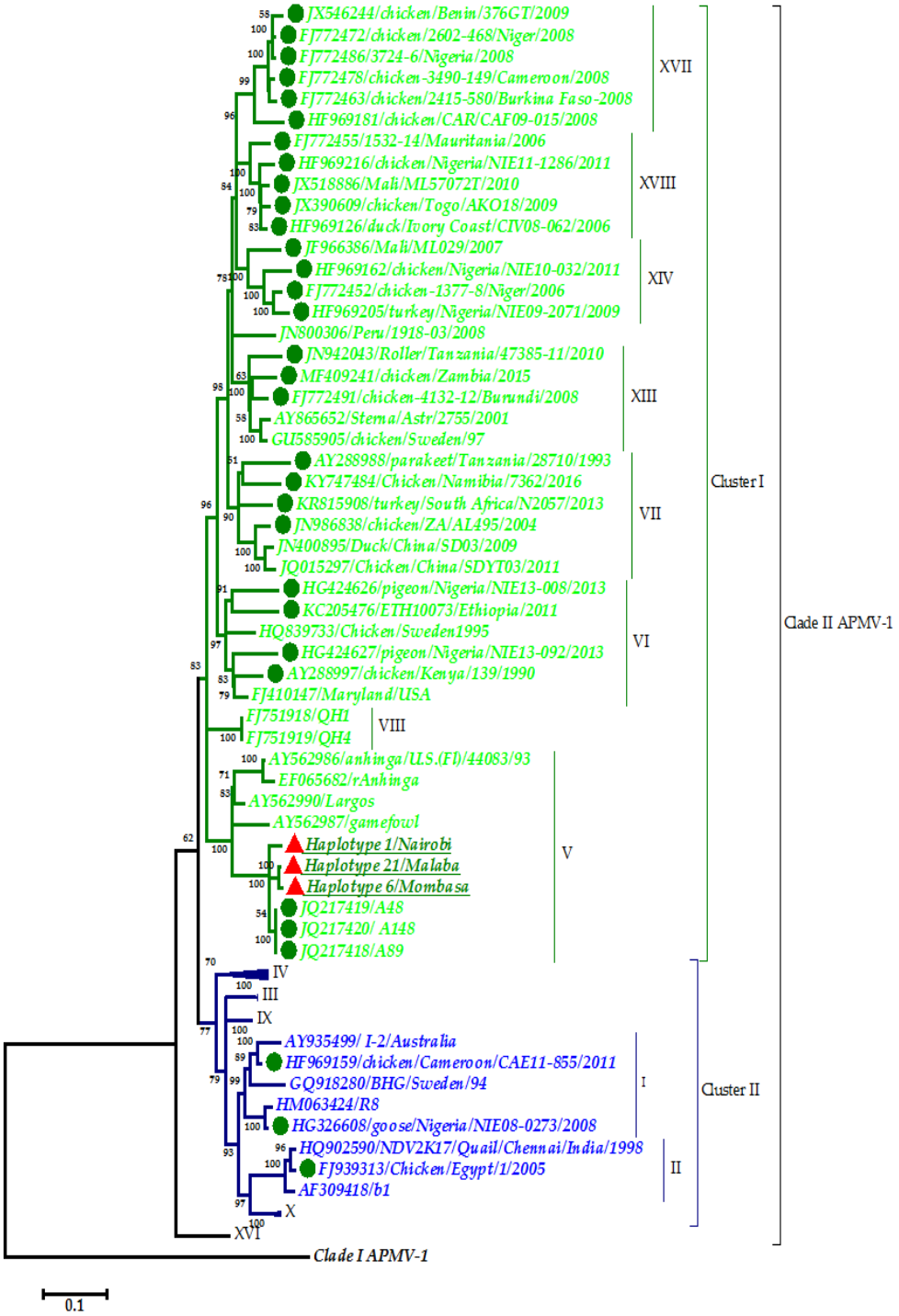

Figure 4.36: Phylogenetic tree of APMV-1 based on F gene coding sequences

The tree on **Figure 4.36** above was drawn with 60 APMV-1 F gene sequences of Class II each 1662bp. The tree was rooted on an APMV-1 strain of Class I. The tree shows the clustering of the APMV-1 strains of Clade II into two clusters. Cluster I consists of Genotypes V, VI, VII, VIII, XII, XIII, XIV and XVIII and Cluster II consists of Genotype I, II, III and IV as well as IX, X and XI.

The three study strains (underlined and marked with a red triangle in **Figure 4.36** above) were grouped together in Cluster I Genotype V. The Genotype V also consisted of APMV-1 strains from wild birds isolated in the US and Canada. In the Genotype V, the study strains and those previously isolated from Kenya formed a distinct group (branch value<90) separate from the others in the Genotype V. The tree also shows that most of the APMV-1 strains from Africa (marked with a green circle) were grouped in the Cluster I among the newer Genotypes VI, VII, XIII, XIV, XVII and XVIII.

The phylogenetic analysis was also constructed using HN coding sequences of study strains alongside 24 other APMV-1 strains of Class II from the GenBank. The tree (**Figure 4.37**) also clustered the APMV-1 strains into two clades as was observed with the F gene tree. However, the genotype XVI was also included in Cluster I for the HN sequences tree.

Most of the African isolates were grouped in the Cluster I Genotypes VII, XIII, XIV, XVII and XVIII. The tree grouped the study strains in Cluster I Genotype V. Within the Genotype V, the study strains were grouped separately (branch value> 90) together with a previous Ugandan and Kenyan strain.

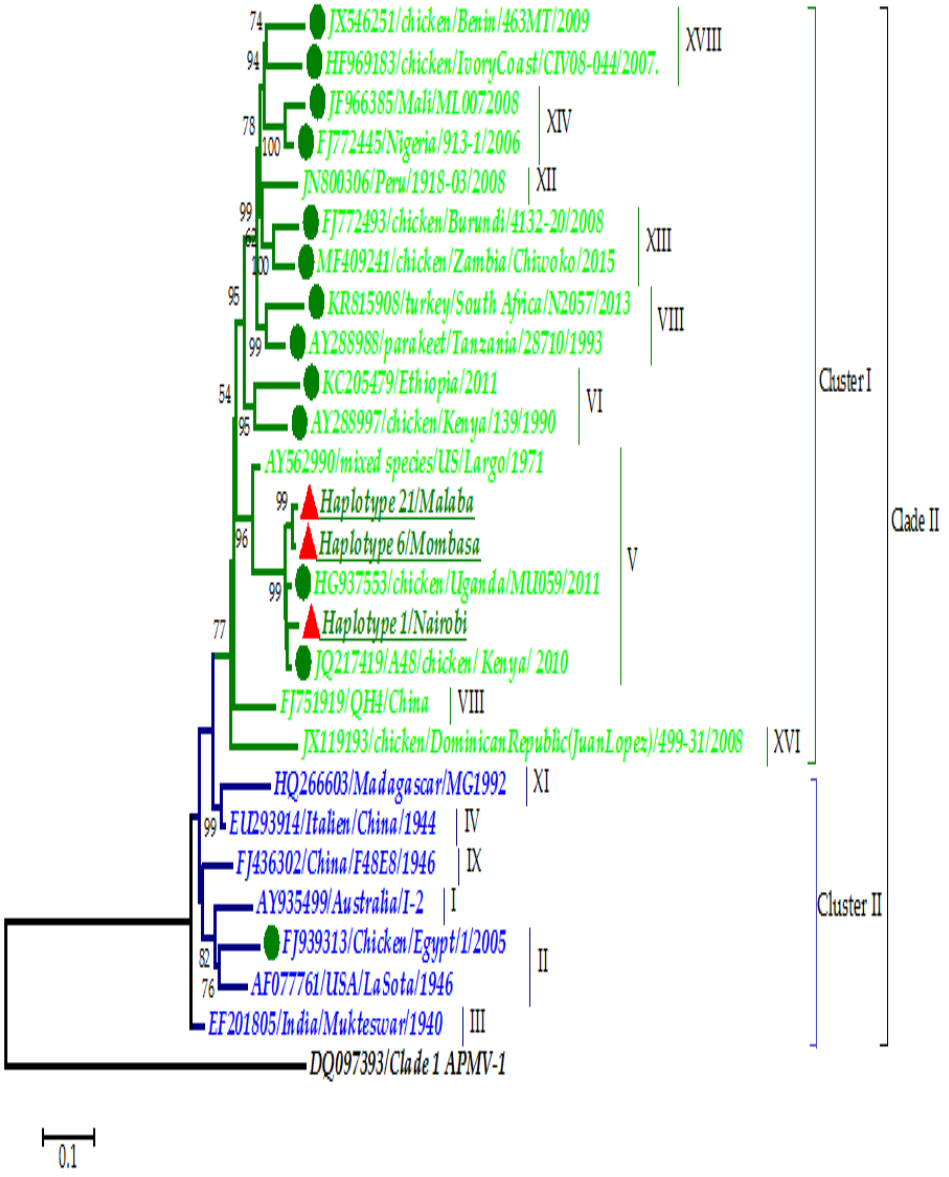

Figure 4.37: Phylogenetic tree of APMV-1 based on HN gene coding sequences

The phylogenetic tree based on the full coding sequence of the Matrix gene (**Figure 4.38**) also revealed that the APMV-1 strains from poultry in Kenya clustered with previous Kenyan isolates in Genotype V of class II. The tree was drawn with 36 APMV-1 genome sequences each 1094bp. The tree also reveals the close relatedness

of Genotypes IX, X and XI to older genotypes I, II, III and IV from which vaccines are derived.

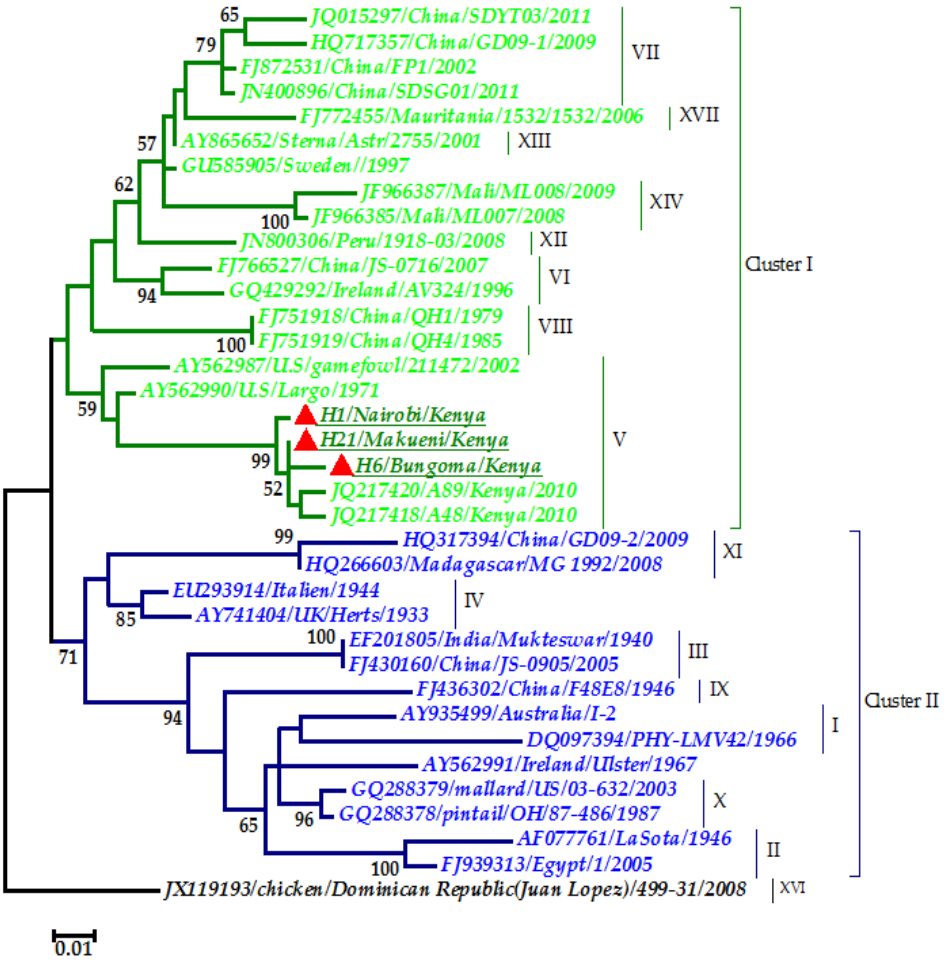

Figure 4.38: Phylogenetic tree of APMV-1 based on M gene coding sequences

The phylogenetic analysis of APMV-1 strains based on the full coding sequence of the L gene (**Figure 4.39**) revealed a similar clustering of APMV-1 strains of Class II to that observed with the F gene. For the Matrix gene phylogenetic analysis, APMV-1 strains of different sub-genotypes of Genotype V (Va, Vb and Vc) were included to observe their relationship with the study strains. Among the Sub-genotypes of Genotype V, the study strains seemed to be more closely related to strains of Sub-

genotype Vb than to Vc and Va. The tree was drawn with 38 APMV-1 genome sequences each 6614bp.

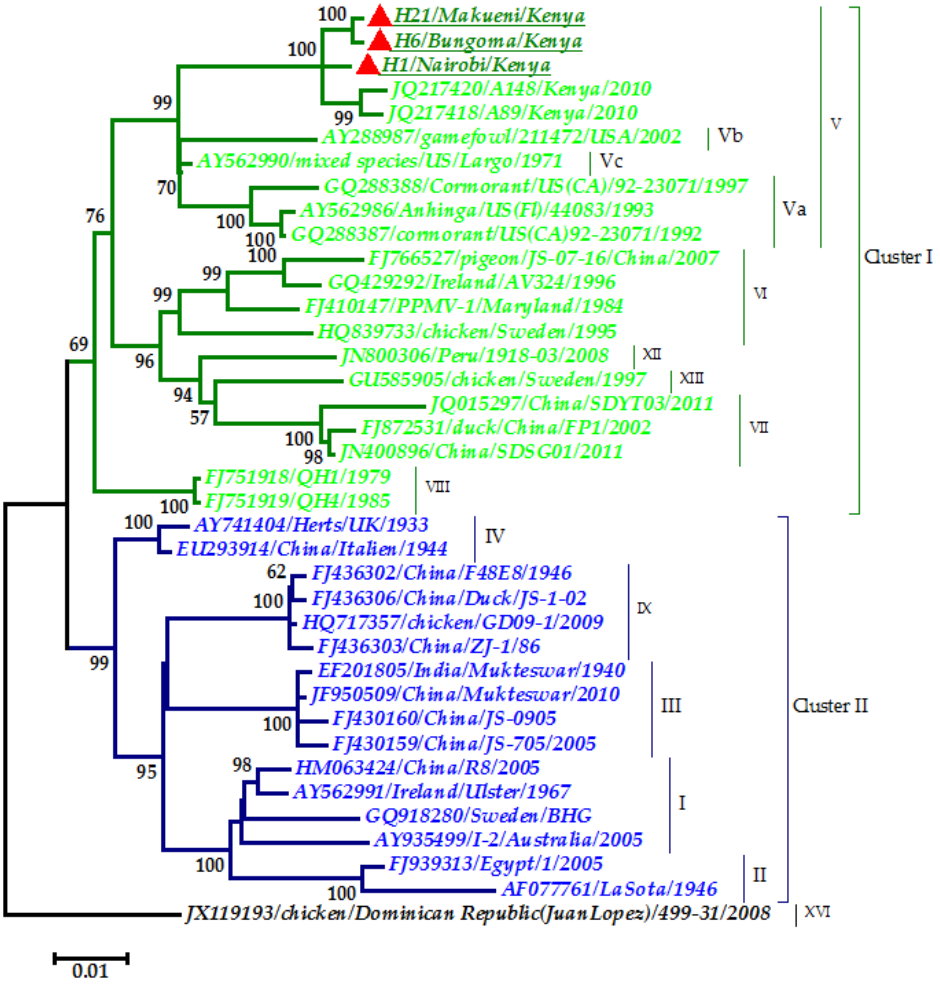

Figure 4.39: Phylogenetic tree of APMV-1 based on L gene coding sequences

Phylogenetic analysis of the full coding sequence of the P gene revealed similar classification of the study strains into Genotype V (**Figure 4.40**). However, the study strains seemed to cluster closer to APMV-1 strain *Largo/71 (AY562990)*, an APMV-1 strain of sub-genotype Vc.

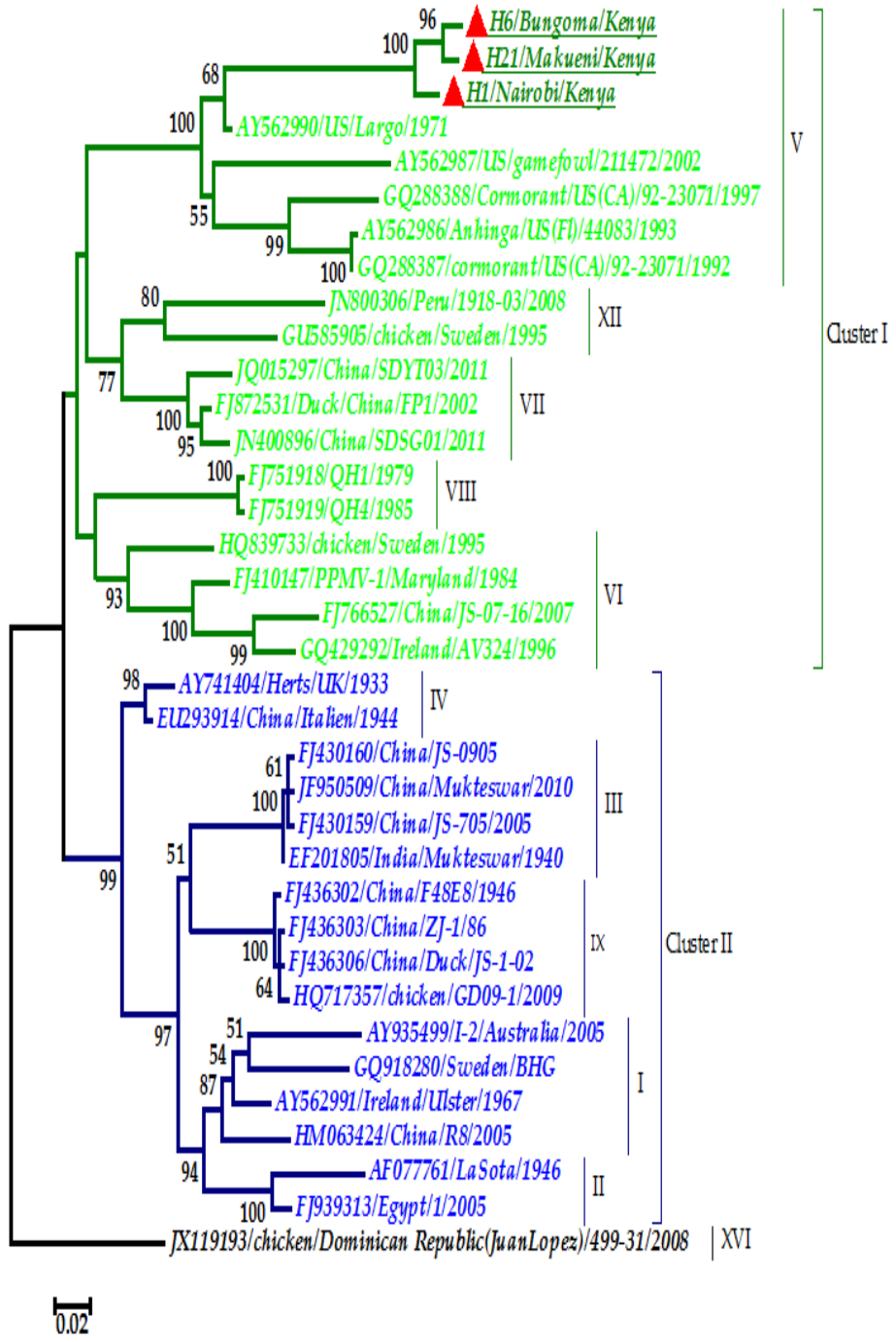

Figure 4.40: Phylogenetic tree of APMV-1 based on P gene coding sequences

4.4 Detection of Signatures of selection

4.4.1 Analysis of selective pressures on APMV-1 lineages

Model 3 fit significantly ($p<0.05$) for the Fusion gene as well as the HN, M and NP protein gene data (**Table 4.27**). This indicated that for these proteins there was heterogeneity in the selection pressures acting on the lineages. For P and L gene no lineage selection was detected and Model 0 was best fit for the P and L gene data This indicated that for the P and L proteins, the selective pressures acting on no lineages were homogenous. The overall dN/dS ratios for the selected models were indicative of negative selection for all the six proteins. The genes seemed to contribute most to lineage differentiation seemed to be the fusion and phosphoprotein genes (dN/dS of 0.289 and 0.272 respectively) (**Figure 4.27**).

Table 4.27: Model likelihood values and parameters estimates for detection of lineage selective pressures

Gene	**Model**	**lnL**[1]	**dN/dS**	**Df**[2]	**2Δ1**[3]
Fusion	M0 (one ratio)	-15550.976		50	
	M3 (discrete)	-15428.776	**0.289**		**122.199**($p<0.05$)
HN	M0 (one ratio)	-15193.897		98	
	M3 (discrete)	15065.054	**0.128**		**128.843**($p<0.05$)
M	M0 (one ratio)	-8639.108	**0.076**	69	**135.210**($p<0.05$)
	M3 (discrete)	-8503.897			
NP	M0 (one ratio)	-10956.530	**0.062**	73	**103.218**($p<0.05$)
	M3 (discrete)	-10853.312			
P	M0 (one ratio)	-9471.871	**0.272**	96	54.903($p>0.05$)
	M3 (discrete)	-9424.863			
L	M0 (one ratio)	-13859.470	**0.121**	73	83.359($p>0.05$)
	M3 (discrete)	-13776.111			

[1]Log likelihood, [2]Degrees of freedom. [3]Difference in log likelihood values between model 0 and 3

4.4.2 Identification of lineages under positive selection

To identify the APMV-1 lineages under selective pressures for the four proteins where the M3 model was selected as the best fit mode l (F, HN, M and NP), the computed dN/dS (ω) were compared across branches of phylogenetic trees. To determine the type of selective pressures on the tree branch ω >1 was indicative of positive selection, ω=1 indicated neutral selection, and ω<1 indicated negative or purifying selection.

The dN/dS ratios for tree branches of the Fusion gene phylogenetic trees are shown on **Figure 4.41**. The branch dN/dS ratios were computed by maximum likelihood estimated using a free ratio model (Model 3) which assumes an independent value for each branch. The dN/dS values are shown for each branch. The branch shown in red has a dN/dS>1 and is indicative of positive or adaptive selection. Positive selection was detected on eight branches (dN/dS>1) (**Figure 4.41**).

High dN/dS ratio was detected on the branch leading to the Cluster I (8.0). High dN/dS ratio was detected on branches separating Genotype XI from IV (5.99), also on the branch separating IX from III (5.66) and on the branch separating Genotype X from Genotypes I and II (3.65). High dN/dS ratio was also detected on the branch separating strain JX915243 from other strains of Genotype XVI (4.58). Other branches where the dN/dS ratio was >1 included the branch separating Genotype XII from XIII (1.15), the branch separating strains of sub-genotype Vb from others of Genotype V (1.48) and the branch separating previous Kenyan strains from study strains (1.98). None of the branches had a dN/dS ratio =1. The rest of the branches, some of which are shown had dN/dS<1 indicating the presence of negative or purifying selection.

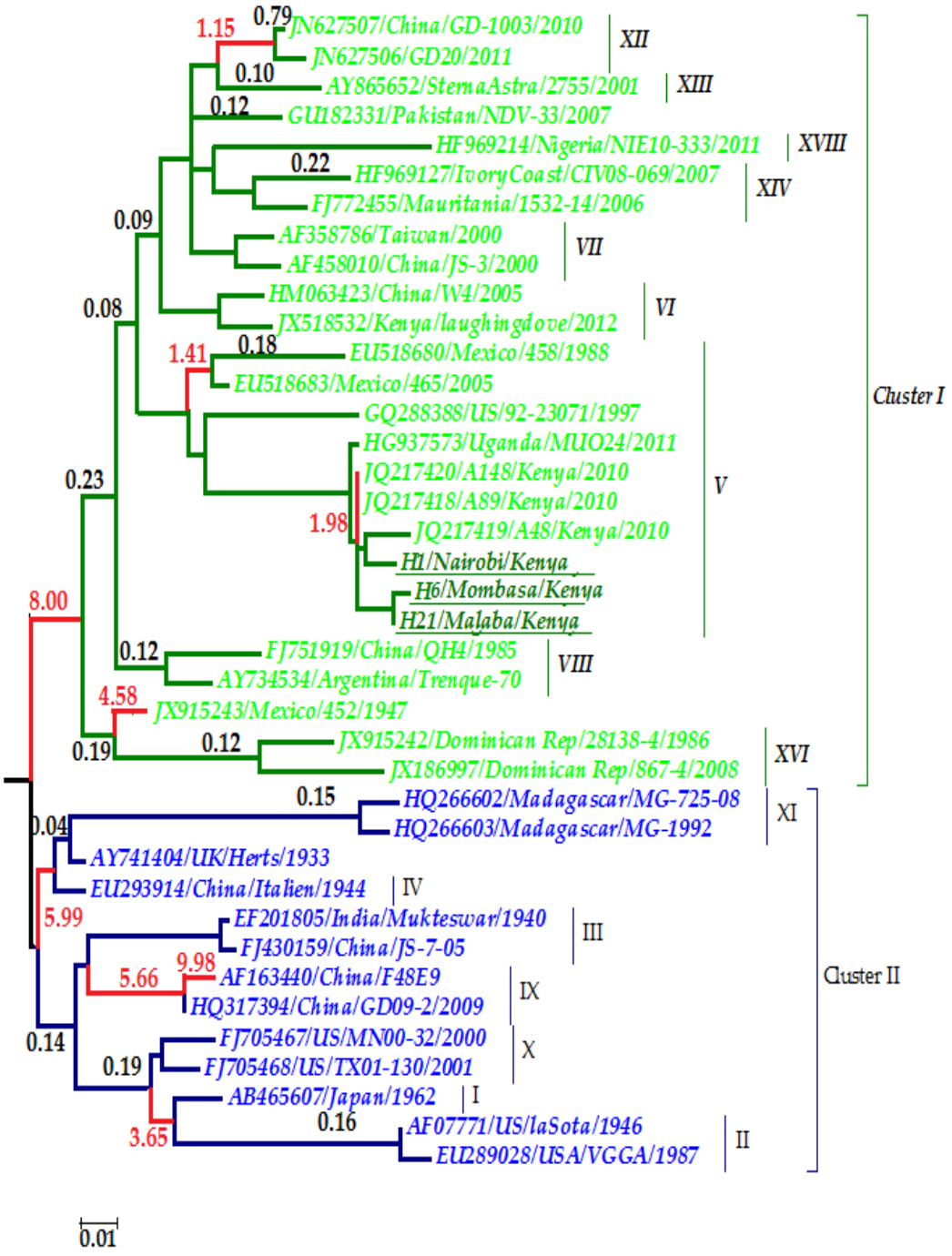

Figure 4.41: Phylogenetic tree of F gene coding sequence showing branch ω values

Figure 4.42 is a phylogenetic tree constructed using HN aa sequences showing the branch dN/dS values. Positive selection was detected on ten branches (dN/dS>1). High

dN/dS ratio (dN/dS =7.04) was detected on the branch separating Cluster I ('recent genotypes') from Cluster II. The dN/dS ratio on the branch separating Genotype VIII from others in Cluster I as well as dN/dS ratio on the branch separating Genotype XVI from others of Cluster I were both high (9.46 and 9.00 respectively). High dN/dS were also detected on the branch leading to Genotype XIII (8.64) and the branch leading to Genotype XII and XVIII (9.21) (**Figure 4.42**).

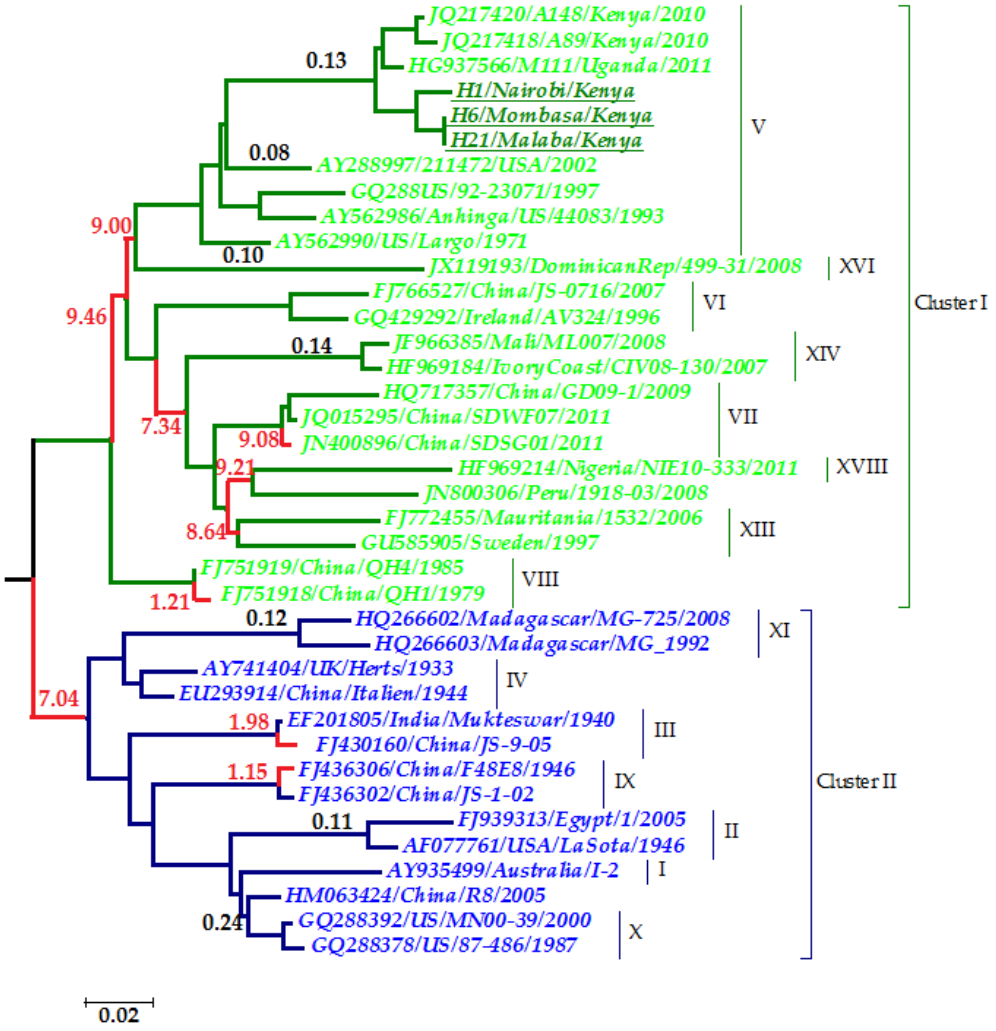

Figure 4.42: Phylogenetic tree of HN gene coding sequence with branch ω values

The branch separating strain *JN400896* from other strains of Genotype VII had high dN/dS values (9.08). Other branches that had dN/dS values indicative of positive

selection include: the branch separating strain *FJ751918* from the other strain of Genotype VIII (1.21), the branch separating strain *FJ430160*(1.98) and the branch separating strain FJ436306 from the other strain of Genotype IX (1.15). The rest of the branches had dN/dS ratio< 1 (**Figure 4.42** above).

Positive selection was detected on eight branches of the matrix protein (dN/dS>1)(**Figure 4.43).** High dN/dS ratio was detected on the branch separating Genotypes III, IX, II, X and I from strains of other Genotype of APMV-1 class II (9.0) as well as the branch separating Genotypes II, X and I from IX (8.63) and the branch separating Genotype II from Genotypes X and I (9.85).

High dN/dS ratio was also seen (7.59) on the branches separating strains *JQ015297* and *HQ717357* from others of Genotype VII. Similarly, high dN/dS ratio (9.26) was observed on the branch separating Genotype XIII from other Genotypes of Cluster I as well as the branch separating Genotype VIII (4.36) from other Genotypes of Cluster I. High dN/dS was also observed on the branch separating previous Kenyan isolates from the study strains (9.87). The rest of the branches had dN/dS ratio< 1.

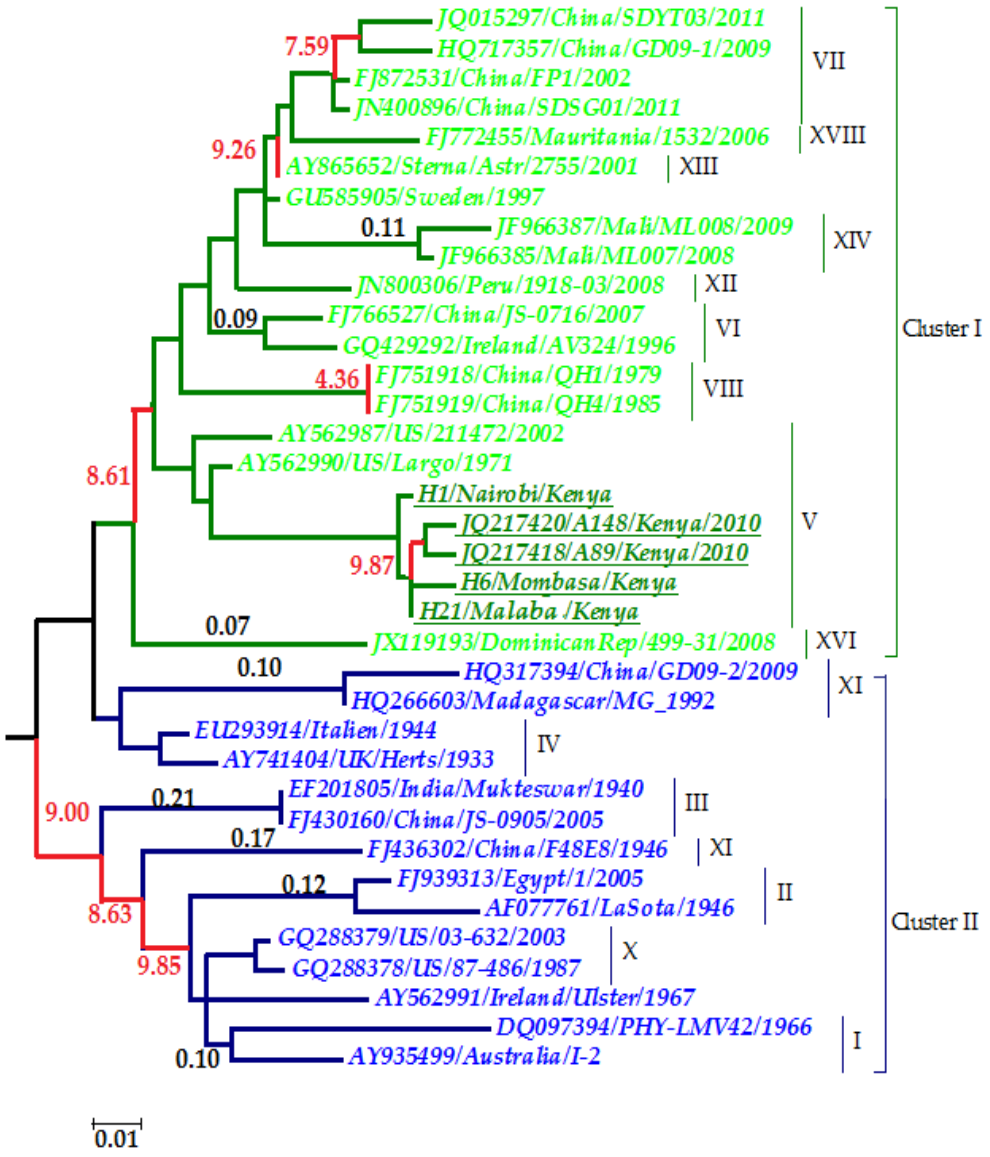

Figure 4.43: Phylogenetic tree of M gene coding sequence showing branch ω values

Positive selection ($d_N/d_S>1$) was detected on some branches of the NP gene phylogenetic tree (**Figure 4.44**). High d_N/d_S ratio was detected on the branch separating Genotypes I, II, and X (d_N/d_S =8.75) as well as the branch separating strain *FJ436302* from the other strain of Genotype IX (d_N/d_S =5.53) and the branch separating strain *EF201805* from other strains of Genotype III (8.99) (**Figure 4.44**). The value of the d_N/d_S ratio was also high on the branch separating Kenyan strains of

APMV from others of Genotype V (9.71) and on the branch leading to Genotype XIV (9.03). The branch lading to the strain *FJ751919* of Genotype VIII (d_N/d_S =3.67) and the branch leading to strain *FJ872531* of Genotype VII (d_N/d_S =1.20) were also indicative of positive selection.

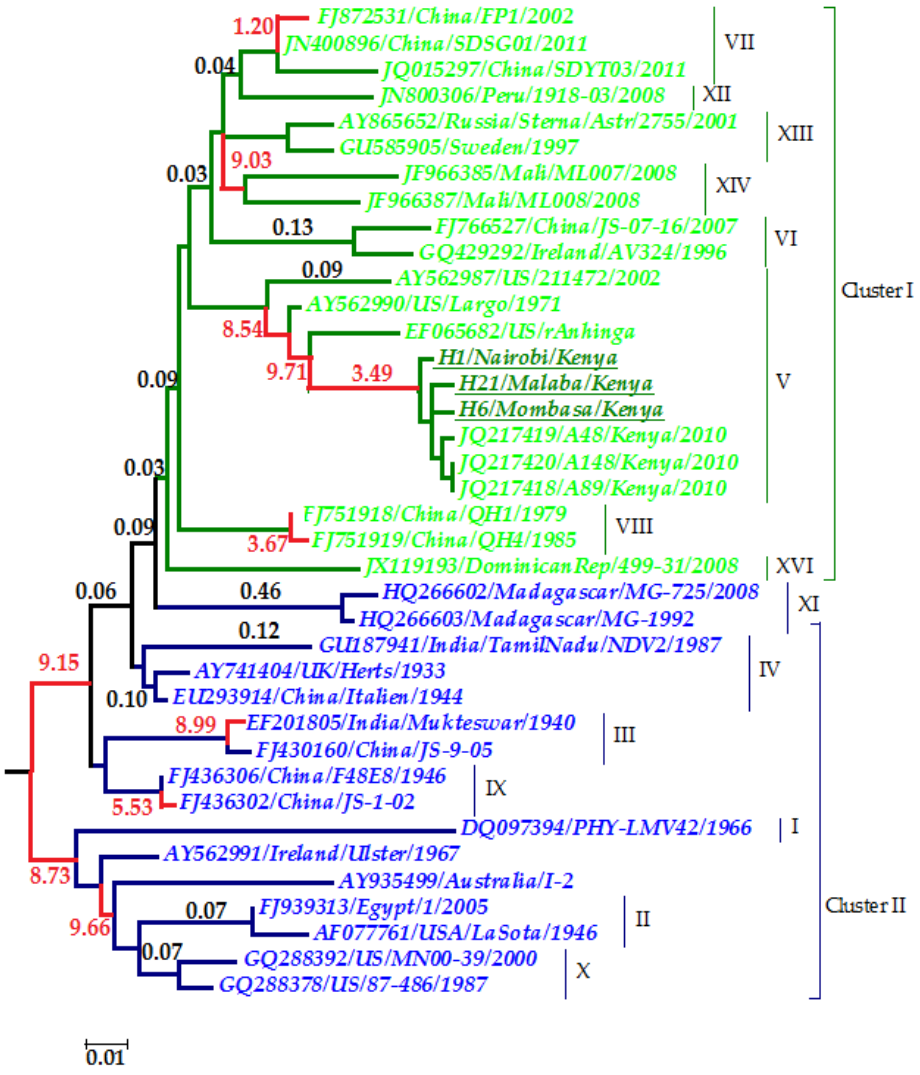

Figure 4.44: Phylogenetic tree of NP gene coding sequence showing branch ω values

4.4.3 Detection of positive selection on codon sites

Model M7fit the data for the P, M, HN and L protein genes. This was indicative of homogeneity in the selective pressures acting on the amino acid sites of these proteins. On the other hand, the model M8 fit the data for the F and NP protein genes *(p<0.05*) indicating the presence of variable selective pressures acting on the amino acid codons of these two proteins.(**Table 4.28**).

Table 4.28: Model likelihood values for detection of codon selective pressures

Gene	**Model**	**lnL[1]**	**Df[2]**	**2Δ1[3]**
F	M7	-15364.411	2	
	M8	-15346.710		**17.701**(p<0.05)
HN	M7	-14922.857	2	
	M8	-14921.76		1.097(p>0.05)
M	M7	-8524.203	2	1.845(p>0.05)
	M8	-8522.358		
NP	M7	-10722.000	2	**6.675**(p<0.05)
	M8	-10715.324		
P	M7	-9296.091	2	1.668(p>0.05)
	M8	-9294.423		
L	M7	-13552.296	2	2.288(p>0.05)
	M8	-13550.008		

[1]Log likelihood, [2]Degrees of freedom. [3]Difference in log likelihood values between model 0 and 3. In bold are values where p-value<0.05 indicating presence of positively selected codon sites

Table 4.29 presents the LRT parameters for the selected models for each protein. The overall codon substitution rates on the different APMV-1 proteins varied. Fusion protein had the highest codon dN/dS ratios (ω) (1.632) followed by the nucleoprotein (dN/dS =1.341). The dN/dS ratios (>1) of the two proteins were indicative of positive

selection on their amino acids. The dN/dS ratios, for other proteins, P, M, HN and L were indicative of purifying selection (ω <1). The overall lowest rate of change was detected on the M and L genes (dN/dS ratios 0.378 and 0.614 respectively).(**Table 4.29**)

Table 4.29: Estimated parameters for detection of positive selection on amino acids sites

Gene	**Selected Model**	**dN/dS[2]**	**Estimated parameters**	**Positively selected aa[1]**
F	M8	**1.632**	p=0.476,q =3.929	20,28,514
HN	M7	0.725	p=0.256,q=1.730	
M	M7	0.378	p=0.290,q=3.153	
NP	M8	**1.341**	p=0.378,q =2.919	434
P	M7	0.915	p=0.341,q=0.831	
L	M7	0.614	p=0.238,q=1.550	

[1]Positively selected amino acid sites based on Bayes Empirical Bayes method with posterior probabilities >0.95; [2]Average dN/dS at codon sites (in bold are values depicting positive selection)

The dN/dS ratios for the HN and P genes tended towards 1 (0.725 and 0.915) indicative of neutralizing selection. For the F and NP genes; where the LRT was suggestive of positive selection on amino acid codon sites; the Bayes Empirical Bayes (BEB) method was used to calculate the posterior probabilities that each codon was under positive selection.

From the BEB analysis (presented on **Table 4.29** above), three aa sites were detected to be under positive selection on the Fusion protein. These included codons: 20, 28 and 514. Positive selection on amino acid lysine was detected at position 28 at posterior probability of 0.999. On the other hand, positive selection on amino acids methionine

and phenylalanine were detected at positions 20 and 514 at posterior probabilities 0.979 and 0.965 respectively.

Figure 4.45 shows the position of the sites predicted to be under positive selection on the Fusion glycoprotein. The Fusion protein structure was predicted by Chen *et al.,* (2001) showing the different domains. Model 8 (ß & ω) predicted the Fusion protein codon sites; 20, 28 and 514 to be under positive selection. The codon sites, 20 and 28 are located in the ß-barrel domain of the fusion peptide. This is found at the N-terminal of ß-barrel domain (Domain I). On the other hand, the codon site 514 is located in the transmembrane domain of the fusion peptide.(**Figure 4.45**).

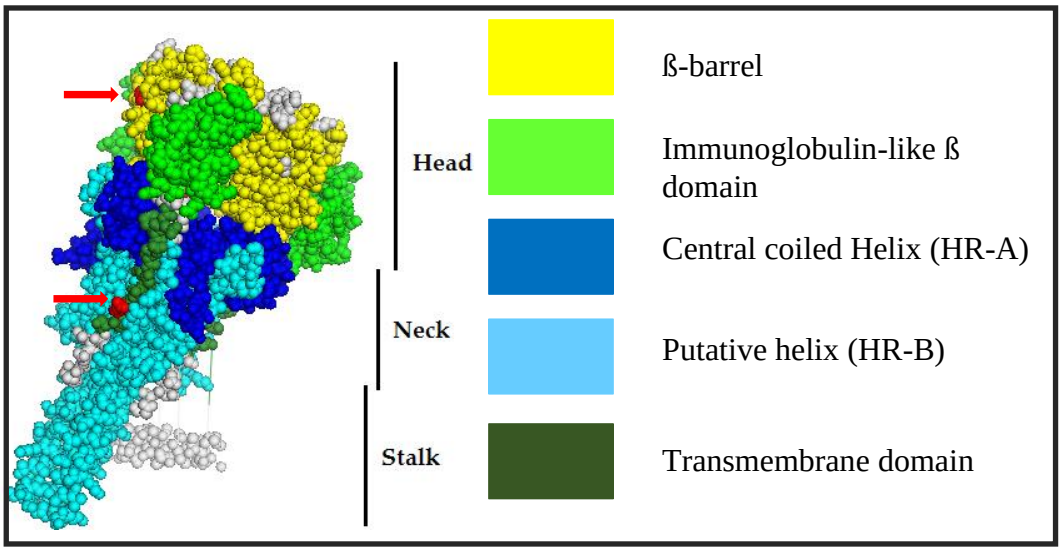

Figure 4.45: The 3-Dimensional structure of APMV-1 Fusion glycoprotein
The structure was adopted from Chen et *al.,* (2001); Red arrows show position amino acid codons under positively selected.

From the BEB analysis, positive selection on amino acid proline of the nucleoprotein was detected at position 434 at posterior probability of 0.975. **Figure 4.46** shows the position of the amino acid codon site predicted to be under positive selection on the Nucleoprotein. The structure was drawn as predicted by Alayyoubi *et al.,* (2015) showing the different domains. The site shown in red was predicted to be under

positive selection by Model 8 (beta & ω). It is located in the C-monomer region of NP, which is an extension of the C-terminal (N-Tail) domain that extends to neighboring protomers.

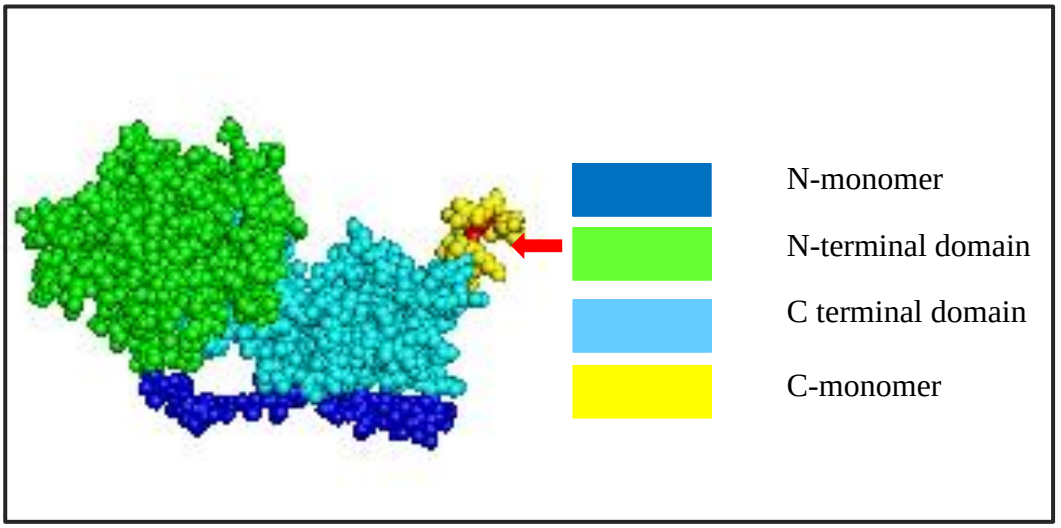

Figure 4.46: The 3-Dimensional structure of Paramyxovirus nucleoprotein
The structure was adopted from Alayyoubi *et al.*, (2015); Red arrow shows position amino acid codons under positively selected

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.0 Introduction

This Chapter discusses and interprets the findings of the study and highlights the significance of the study in the immediate context of genetic characteristics and risk factors of avian paramyxovirus in Kenya. The Chapter also presents Conclusions and Recommendations drawn from the study.

5.1. Discussion of Results

This was the first extensive study on genetic diversity and risk factors of avian paramyxovirus in Kenya. The study findings revealed the management practices and trade factors that contribute to occurrence of avian paramyxovirus in Kenya. The study also revealed differences in regional occurrence of avian paramyxovirus. Further, the study established that the Fusion gene and large RNA polymerase gene of APMV in Kenya are genetically diverse. Genomic characterization APMV-1 strains and that the Kenyan strains were distant from the vaccine strains. The study also detected presence of selection pressure on APMV-1 genes of global strains.

5.1.1. Risk factors associated with APMV infection in Kenya

This study is the first countrywide assessment of APMV in Kenya that utilized molecular assays to establish the APMV infection status of domestic poultry in between outbreaks. PCR assay using APMV universal primers based on a partial fragment of large polymerase gene showed higher APMV positivity in sampled poultry from LBMs than smallholder farms respectively. These detection rates on poultry farms reported in this study were similar to those reported in Tanzania using the F-gene assay (Yongolo *et al.,* 2002) as well as the detection rates reported in Ethiopia using an M gene assay (Mulisa *et al.,* 2014). The study also revealed high APMV infection rates of birds sold in live bird markets in Kenya. Studies in other countries of the Eastern Africa region have reported similarly high APMV infection rates in LBMs

(Byarugaba *et al.,* 2014; Mulisa *et al.*, 2014; Chaka *et al.*, 2013b). Reports from other region of Africa also corroborate the findings on the occurrence of APMV in LBMs (Omony *et al.,* 2016; Jibril *et al.,* 2014; Solomon *et al.*, 2012).Similar findings have also been reported in Asia (Barman *et al.,* 2016). LBMs are reported to contribute to the persistence and spread of APMV and serves as a source of infection to poultry flocks (Rapha & Fourni, 2015; Jibril *et al.,* 2014). This finding therefore indicates the important role played by LBMs in the epidemiology of APMV in Kenya.

The study identified risk factors for APMV infection of poultry in both LBMs and poultry farms. In the studied LBMs, various practices that could promote APMV transmission among birds were observed. Such practices included mixing of birds from different sources, mixing multiple poultry species and improper disposal of poultry wastes in the market. These practices promote viral exchanges between infected/infective material and healthy birds, thereby transmitting disease to a bigger population of poultry (Kirunda *et al.,* 2014b). These practices among traders have been identified as a risk factor for spread and transmission of viral poultry diseases in LBMs in Kenya (McCarron *et al.,* 2015). This study therefore highlights the need to involve traders in the implementing improved biosecurity measures in LBMs for the control of APMV in Kenya. This study established the presence of regional variation in APMV infection in both LBMs and poultry flocks. Geographical variation as seen in this study could also indicate the locality variation in epidemiology and ecology of APMV (Kemboi *et al.,* 2013; Miguel *et al.,* 2013), which results in some areas experiencing the inter-epidemic period with low virus activity while others experience high viral activity in early infection (Roy, 2012). In LBMs, however, instead of mere geographical variation, this study revealed higher risk of APMV infection of birds sold in major town and city markets than their rural counterparts did. It is highly likely that this is associated with the volume and diversities of poultry trading and patterns in city/town markets that favour the maintenance and circulation of APMV (Mulisa *et al.,* 2014). For instance, Nairobi metropolitan is a large urban area that attracts poultry centripetally from most parts of the country and as far as from Uganda. Poultry with

unknown disease status are mixed, and transported over long distances from various sub-regions and arrive at Nairobi city stressed and immune-compromised with increase susceptibility to infectious pathogens (McCarron *et al.*, 2015). This finding highlights the importance of the movement of poultry and poultry products in transmission of APMV (Abdisa & Tagesu, 2017) and emphasizes the need for controlling such movement in control of APMV in Kenya.

In poultry flocks, factors investigated included: feeding regime, breeding and restocking strategies, disease control, waste management and flock characteristics. The poultry farmers left the poultry to scavenge. Grain and kitchen leftovers were used to supplement birds in addition to free-range scavenging. The feeding regime did not directly pose as a significant risk factor for introduction of APMV infection into a poultry flock. However, scavenging contributed indirectly through increased possibility of contact with infective material or infected wild birds or poultry from neighboring farms. Regression analysis showed that confinement of poultry was associated with lower APMV infection. There were wild birds on sampled poultry farms in close proximity to poultry. Therefore, the role of wild birds in the epidemiology of APMV in Kenya is a possibility requiring further investigation. However, there is currently no evidence of APMV transmission between wild birds and poultry in Kenya. Restocking of poultry in smallholder farms was through gifts, purchasing chicks from neighboring farms or live bird markets. Movement of poultry and poultry products could contribute to transmission of APMV between farms through bird-to-bird contact. The risky behavior of eating sick and dead birds was common practice. Improper disposal of carcasses and poultry wastes with infective material being exposed and easily accessible to other poultry. Contact between poultry and infective material leads to increase transmission of APMV.

The study established that the introduction of ‘new’ birds into the flock from neighboring farms or markets was a major risk factor to APMV infection. This is done to replace or increase birds in a flock and is a factor related to movement of live birds

into the flock. Introduction of birds that harbor APMV into poultry flocks from LBMs or other farms could result in disease outbreaks (Snoeck *et al.,* 2009). Previous studies have reported the presence of birds that become carriers of APMV (Byarugaba *et al.,* 2014; Yongolo *et al.,* 2011). Such birds survive outbreaks and are asymptomatic but shed the virus which is infectious to other birds (Chaka *et al.,* 2013a; Otim *et al.,* 2007). Such birds are sold to markets and other farms. This study also established that a good percentage of farms sold birds during an outbreak of ND. Such birds sold to neighbors and LBMs though healthy looking birds could be in the incubation period before any evident clinical signs of disease (Chukwudi, *et al.,* 2011). This perpetuates the infection to healthy flocks and regions that take in such birds. However, this study only included management factors; however, there are ecological, demographical or behavioural factors that could also influence the transmission of APMV. Therefore, more studies that include the other factors are needed in order to develop more inclusive control strategies against APMV infection in domestic poultry in Kenya. Poultry flocks that had mixed species of birds had higher APMV infection than those who reared one species of poultry. It has been reported that poultry species such as ducks, guinea fowls, geese among other species though asymptomatic to infection serve as reservoirs for APMV (Alexander & Senne, 2008) thus infecting chicken which are more susceptible to APMV infection (Abdisa & Tagesu, 2017).

This study found an association between large chicken flock size and increased APMV infection. In large chicken flocks, different ages of chicken from chicks to adults are present (Abdisa & Tagesu, 2017; Okeno *et al.,* 2012). This indicates the presence of very young birds with low immunity and much higher susceptibility to APMV infection thereby increasing the risk of APMV transmission within the flock (Chaka *et al.,* 2013b). Contrary to another study in Kenya, this study, (Njagi et al., 2010a), found lower APMV infection in poultry flocks that were confined. The extensive management of poultry exposes them to contact with poultry from other homesteads some of which could be shedding the virus (Chaka *et al.,* 2013a). This allows easy spread of the virus to other flocks. On the contrary, decreasing contact between flocks

through confinement reduces chances of APMV being introduced into the flock. However, this implies the need to supplement poultry. Supplementation of poultry with grain and locally available feeds is more likely to confine the birds closer home (Ndegwa *et al.,* 2015; Alfred *et al.,* 2012). This may be incorporated into the management of smallholder flocks to reduce their movement and contact with other birds. Generally, this study found low adoption of disease control and biosecurity measures in smallholder poultry flocks in Kenya. Similar results have been reported for smallholder poultry in developing countries (Conan *et al.,* 2012) and also in Kenya (Ndegwa *et al.,* 2015). Vaccination is the most effective control method for APMV (ACIAR, 2014) , however various socioeconomic factors are thought to limit its uptake in smallholder poultry flocks (Copland & Alders, 2013). This highlights the need for intervention to improve uptake and sustainability of vaccination in smallholder poultry flocks in Kenya.

5.1.2. Genetic diversity of APMV strains in Kenya

This study presents the first countrywide genetic study of prevailing avian paramyxovirus strains circulating in domestic poultry in Kenya. The study characterised APMV-1 strains from poultry populations in different regions of Kenya Analysis of the sequences of the partial large polymerase protein and the Fusion protein gene as well as the genome sequences revealed the presence of genetically diverse but closely related APMV strains belonging to serotype one circulating on poultry farms and in live bird market in Kenya.

Analysis of evolutionary distance between the Genomic and Gene sequences revealed that slight differences between obtained Kenyan APMV-1 strains. Diel *et al.*, (2012a) while developing new criteria for characterization of APMV-1, suggested evolutionary distances of 3% as sufficient for assigning strains into different sub-genotypes. The sequence differences between obtained Kenyan viruses was not sufficient to consider each of them as a different sub-genotype of APMV-1. However, there was sufficient

variation in the sequences of APMV-1 strains from Kenya to allow for genetic differentiation into haplotypes.

There was good corroboration between strain assignment of haplotypes using both L and F gene sequence analysis. However, F gene analysis of samples from the Coastal and Eastern region seems to have clustered the strains into fewer haplotypes than L sequence analysis. This could be explained by the lower number of F gene positive than L gene positive samples in these two regions. The L gene assay was able to detect low viral loads of APMV. The sampled birds in Coast and Eastern might have had low viral loads detectable by the L gene assay and not by F gene assay.

Generally, no regional clustering was observed in the assignment of APMV-1 strains into haplotypes. Rather, most regions shared the identified haplotypes. This implies cross-regional circulation in Kenya of APMV-1 strains with a common ancestry. The study showed high haplotype diversity in each sampled region using both the F and L genes. This was indicative of the diversity within the Kenyan APMV-1 strains. There was higher APMV-1 haplotype diversity in LBMs than on poultry farms. Specifically Nairobi markets had high haplotype diversity compared to other markets. Live bird markets are focal areas for collection of birds with different viral strains possibly from different regions (McCarron *et al.*, 2015). This is especially so for city markets such as Nairobi. This allows for mixing and spread of viral strains from different regions (Solomon *e*t *al.*, 2012).

Haplotype diversity was similarly high in all sampled regions However; the nucleotide diversity between regions was low and indicated the low divergence and close relatedness of APMV-1 strains circulating in the different regions. This suggest intermixing of APMV strains from different regions of Kenya. This could occur through uncontrolled movement of poultry and poultry products.

Analysis of the partial L gene sequence revealed that one haplotype had great divergence from the other haplotypes and was identical to *LaSota*, an APMV-1 vaccine

strain that is commonly used in Kenya to vaccinate chicken against Newcastle disease. The haplotype was obtained from a chicken sample in Chwele market in Bungoma. Traders did not have all details such as vaccination history of their poultry stock. It was likely that the sampled bird had recently been vaccinated on the farm before being sold to the trader. The isolated strain could therefore have been the LaSota vaccine strain that is administered as an attenuated vaccine. Circulation of lentogenic strains in poultry and other birds is thought to contribute to evolution and further divergence of APMV-1 viruses (Chong et al., 2010). Therefore, presence of vaccine strain circulating together with the wild APMV-1 strains in our domestic poultry may influence the evolutionary dynamics of APMV in Kenya.

The study identified sites in the coding proteins of the Kenyan APMV-1 strains which could be used as genetic markers. Specifically such markers could be used to differentiate the field strains from vaccine strains. This is because the Kenyan APMV-1 strains differed greatly from the commonly used vaccine strains *(LaSota, B1-Hitchner and I-2*). Among the proteins, the highest variability was observed in the phosphoprotein, specifically in the region spanning in the positions 134 to 239 of the P gene amino acid sequence. This corresponds to the RNA editing site of the P gene. This site is where stuttering of the polymerase complex occurs over the transcription template leading to insertion of non-templated nucleotide(s) guanine and formation of non-structural protein V and W (Hausmann, & Kolakofsky, 1994). The insertion of one G gives rise to the V while insertion of two Gs leads to expression of W protein (Locke *et al.,* 2000; Hausmann *et al.,* 1999; Steward *et al.,* 1993). The V protein has been shown to be a key determinant host specificity of APMV-1 strains due to its ability to antagonize hosts interferon (IFN) activities (Alamares *et al.,* 2010). The variability in the protein between genotypes explains the ability of different genotypes to adopt to new hosts by shutting off the hosts' immunity (Gainey *et al.,* 2008; Dillon & Parks, 2007).

On the NP protein, the study strains had a six nt insertion at the 5' end that is common for the 'recent' genotypes of APMV-1 Class II. This insertion however, this insertion lacked in the vaccine strains. However, there were other regions common between the vaccine strains and study strains. For example, the NP protein had a conserved region spanning nucleotides 322-336 observed in both strains and this region has been reported in all viruses of the family *Paramyxovirinae* and is thought to be responsible for the N-N assembly during RNA binding (Longhi, 2009; Kho *et al.*, 2003).

The study, through analysis of the amino acid motif at cleavage site of the F gene, established that all APMV-1 strains obtained from poultry in Kenya were virulent. The cleavage site of the F gene is responsible for the interaction with the host enzymes to allow for viral entry into the host cells (Kim *et al.*, 2011). The amino acid motif therefore determines the type of cells that the virus can infect (Jardetzky & Lamb, 2014). The motif seen in the Kenyan strains of APMV-1 is such that the virus can bind to proteases present in all cell types and therefore infect all systems in the host including respiratory, enteric and nervous system and cause fatal disease (Anderson & Wang, 2015). This indicates that the strains of APMV-1 characterized in this study may be responsible for the current ND outbreaks experienced in poultry in the country.

5.1.3. Phylogenetic relationship of APMV strains

The study used different APMV genes to infer the phylogenetic and evolutionary relationship of Kenyan APMV strains with other strains available in the GenBank in order to contribute to the understanding of the diversity and evolutionary dynamics of APMV strains in Kenya. In this study, all the obtained strains of APMV were identified as APMV serotype 1(APMV-1). Currently, the fusion gene is used to classify APMV-1 into two clades: Class I which is less variable and Class II which is highly diverse and contains eighteen known genotypes (I-XVIII) (Courtney *et al.*, 2013; Almeida *et al.*, 2013; Diel *et al.*, 2012a).

Phylogenetic analysis of the six APMV-1 genes including the fusion gene consistently grouped all the APMV strains from different regions in Kenya into one genotype together with APMV-1 strains from North and South America belonging to Class II Genotype V (Diel et al., 2012b) distant from the vaccine strains in Genotype II.

Genotype V viruses have been associated with ND outbreaks that occur in poultry in various parts of the world from the 1970s to 1980s and is associated with *Psittacine* species of birds and is reported to spread rapidly (Dimitrov *et al.,* 2016a). Genotype V strains emerged in South and Central America in the 1970s (Susta *et al.,* 2014) and were linked to outbreaks in Europe in that same period (Rue *et al.,* 2010). These viruses also caused outbreaks in North America between the 1970s and 1980s (Diel *et al.*, 2012b). Genotype V virus might have been introduced in Kenya during this period and continues to survive in domestic poultry. However, the study findings indicate that the APMV-1 in Kenyan poultry formed a separate cluster that is genetically unique from other viruses of Genotype V. This point to possibility of independent evolution of APMV-1 in the local bird population in Kenya. Byarugaba *et al.,* (2014) reports robustness of Genotype V with low genetic distance of between strains of the Genotype V.

Previously, Genotype V strains were classified into three sub-genotypes (Susta *et al.,* 2014; Garcia *et al.,* 2013). However, this study revealed that APMV-1 strains obtained from this study alongside previous strains from Uganda and Kenya seemed to form a distinct cluster separated from other sub-genotypes of genotype V. To date, this strain of viruses unique to Kenya and Uganda have not been found in any other region in Africa. While forming a new classification system based on the F gene, Diel *et al*., (2012a) gave criteria under which a new sub-genotype is to be assigned. One of the criteria was to have a high bootstrap value (>60%) at the defining node on the phylogenetic tree. On the phylogenetic trees of various genes, a high bootstrap value was observed at the node clustering the study APMV-1 strains, the Ugandan and previous Kenyan strains. This is a good indication for probable inclusion of the study

APMV-1 strains into a new sub-genotype Vd within genotype V. Clustering of Kenyan APMV-1 strains and Ugandan strains indicates exchange of viruses between the two countries. This is not surprising since the Ugandan and Kenyan poultry sectors and trade are interlinked due to uncontrolled cross border movement of poultry between the two countries. Movement of poultry and poultry products has been implicated in the introduction and spread of APMV strains (Mulisa *et al.,* 2014) and could therefore be responsible for movement of strains across the Uganda-Kenya border and also from region to region within Kenya.

In the study, phylogenetic trees of the six APMV-1 genes revealed clustering to APMV-1 strains into two groups. The first group is known as the 'early' genotypes and their genomes contain 15,186 nucleotides. These appeared between 1930 and 1960 and include genotypes; I, II, III, IV, IX, X and XI (Miller *et al.,* 2010). The second group comprise 'recent' genotypes, which appeared after 1960, and have 15192 nt. These include genotypes V, VI, VII, VIII and X-XVIII. For the recent genotypes, a six nucleotide insertion in the 5' non-coding region of the NP gene. This insertion was observed for the strains obtained from Kenyan poultry. The importance of the insertions for the pathogenicity of the virus is not well understood. However, 'recent' genotypes, which appeared two decades after introduction of APMV-1 vaccination, are thought to be more efficient in spreading even among immunized birds than the earlier genotypes (Czeglédi *et al.,* 2006). It remains to be elucidated if this insertion was a result of immunological selection following introduction of vaccines against ND.

5.1.4. Signatures of selection

This is the first study involving Kenyan APMV-1 strains and other African strains of newer genotypes that investigated the role of selective pressure on the evolution of the six genes encoding functional proteins of APMV-1 strains of class II. Findings of this study improved the understanding of evolutionary dynamics of APMV-1 and helped to predict the pattern of future outbreaks and prevent their occurrence.

The study findings revealed that of all APMV-1 genes, the fusion was the most variable protein. The fusion gene encodes an outer membrane glycoprotein, which interacts with the host immunity and is thus prone to adaptive evolution. The most conserved genes seemed to be the M and L genes. Previous studies report sequence conservation of the Matrix and L genes of the paramyxoviruses (Battisti *et al.,* 2012; Govindarajan & Samal, 2004). All APMV-1 genes seemed to be under negative selective pressures with the exception of a few localized regions where positive selection was detected. This was evidence of the conserved structure and function of the APMV-1 genes. This is in agreement to previous findings by Miller *et al.,* (2009a) who reported negative selection on the APMV-1 genes. Positive selection is an evolutionary process that enhances the fixation of emerging advantageous mutations in the population with a much higher frequency compared to the wild-type allele (Suzuki & Gojobori, 1999). On the other hand, negative selection often removes deleterious mutations in biological functional protein to maintain the long-term stability during evolution (Meiklejohn *et al.,* 2007).

This study detected positive selection on the gene encoding the fusion glycoprotein. The fusion glycoprotein forms the outer viral envelop and mediates viral fusion to the host cell membrane. The protein undergoes a large, irreversible conformational change or refolding, which promotes the fusion of the viral and cellular bilayers, opening a pore to transfer the viral genome into the cytoplasm (Jardetzky & Lamb, 2014; Lamb & Jardetzky, 2007).

The Fusion protein is produced as a precursor protein (F0) which undergoes cleavage by the host enzymes. The crystal structures of the uncleaved fusion glycoprotein has been demonstrated to be organized into head, neck and stalk regions (Morrison, 2003; Chen *et al.,* 2001;). The domains of the fusion protein are important in folding while others influence the fusion activity of the protein. The head is comprised of a twisted *ß* domain and an immunoglobulin-like domain. The neck is formed by the C-terminal extension of the heptad repeat region HR-A encased by the helix HR-C and a 4-

stranded ß sheet. The stalk on the other hand is formed by the HR-A, the heptad repeat HR-B the transmembrane (TM) and cytoplasmic domains (Russell *et al.,* 2004; Morrison, 2003; Chen *et al.,* 2001). The sites detected to be under positive selection (aa 20 and 28) were found in N-terminal of the twisted 7-stranded ß barrel–like domain of the head region. The N-terminal of this domain joins the immunoglobulin-like domain of the head region and together they are postulated to play a role in stabilization of the helical bundle stalk. They are also important in activation of the conformational change that results in the opening of the trimer head domain and exposure of target membranes onto which host receptors attach in order to activate the fusion activity (Morrison, 2003).

Mutations in this region of the F protein has been reported to cause destabilization of the native state and thus resulting in hyperactive fusion and increase viral attachment and invasion of host cells (Russell *et al.,* 2004). Miller *et al.,* (2009a) also detected positive selection in the region spanning aa 1-28 of the fusion protein. This region has been demonstrated to be highly entropic and heterogeneous and is located in the ß barrel–like domain of the N-terminal end in the F glycoprotein (Miller et al., 2009a). Positive selection is likely to occur in the N-terminal end of the Fusion glycoprotein given that this region is highly variable and diverse in its structure.

The study also detected positive selection on another site within the TM domain of the F protein. Previous studies have indicated that the specific sequence of the TM domain of the NDV F protein is important for the conformation of the pre-activation form of the ecto-domain, the interactions of the protein with HN protein, and fusion activity (Gravel *et al.,* 2011). Amino acid substitutions in this domain are may play a critical role in enhancing fusion activity (Bissonnette *et al.,* 2009).

Positive selection was also detected on the gene encoding the Nucleoprotein (NP). A previous study also detected positively selected sites in the NP gene of APMV-1 (Fan *et al.,* 2017). The NP gene is involved in viral infection and replication (Habchi & Longhi, 2012). The NP is involved in the formation of complexes with the

phosphoprotein and Large protein that are essential in the function of the viral polymerase (Dochow *et al.,* 2012). The structure of NP has not been properly defined in APMV-1. However, like other *paramyxoviruses*, the NP of APMV-1 has two main domains, a conserved N-terminal region and a highly variable C-terminal which are flanked by monomers that extend to neighboring protomers to form complexes (Blanchard *et al.,* 2004). The site (aa 434) detected to be under positive selection was located in the monomer adjacent to the C-terminal domain. The C-terminal domain and its adjacent monomer are postulated to be involved in binding the polymerase complex (Krumm *et al.,* 2013; Buchholz *et al.,* 1994). In the *Sendai* virus, the NP region spanning amino acid 400-439 were postulated to be involved in binding of an additional factor or positioning of the NP for binding with other proteins (Buchholz *et al.,* 1994).

Analysis of the genes encoding the Fusion, Nucleocapsid, matrix and hemagglutinin-neuraminidase proteins revealed heterogeneity in selective pressures in different genotypes of class II of APMV-1. Of interest was detection of positive selection on branches leading to genotypes IX and XI an indication that the two genotypes are under high selective pressure for adaptive selection. This was detected in the analysis of the F, HN, M and NP genes. Genotype IX strains are virulent viruses that have been recovered mainly from China and are thought have common ancestry with genotype III (Dimitrov *et al.,* 2016a; Diel *et al.,* 2012a). On the other hand, genotype XI strains are virulent strains that have mainly been recovered from Madagascar between 2008 and 2011 and share common ancestry with genotype IV (Dimitrov *et al.,* 2016a; de Almeida *et al.,* 2013; Diel, *et al.,* 2012a). This may imply presence of evolution in the two genotypes with common ancestry to earlier genotypes, which may no longer be circulating in avian species. The two genotypes though sharing ancestry have evident amino acid sequence divergence that renders them virulent. Positive selection may play a role in such differentiation and induce variations that are evident phenotypically.

5.2. Conclusion

From the study findings, avian paramyxoviruses circulating in domestic poultry in Kenya are genetically diverse with distinguishable molecular variation. However, these strains related. Further, the study concludes that avian paramyxovirus occurrence in domestic poultry in Kenya is associated with risky management and trade practices.

5.2.1. Risk factors associated with APMV occurrence in poultry

The status of poultry in Kenya in terms of avian paramyxovirus infection has previously been characterized using serological based methods (Njagi *et al*., 2010b). Using this method, Njagi *et al.,* (2010b) found an APMV prevalence of 14% in poultry in Kenya and identified factors that significantly influenced APMV infection on poultry farms including restocking methods and season among others. The limitations in the use of serological methods however, is that they estimate the level of exposure to the virus rather than measure the level of current infection. The current study utilized molecular methods to characterize the APMV infection status of poultry in Kenya and to test the hypothesis that factors present in LBMs and poultry farms are not associated with APMV infection in poultry. The study was not only a build-up of the work by Njagi *et a.,l* (2010a) on the risk factors of APMV on poultry farms but was also the first study to demonstrate the presence of APMV and their risk factors in live bird markets in Kenya.

Results of the study supported the alternative hypothesis that factors predisposing poultry to APMV infection were indeed present on both poultry farms and LBMs in Kenya. The study revealed restocking from other farms and LBMs and flock size as risk factors associated with APMV infection on poultry farms. On the other hand, location in cities or major towns as well as number of trade networks were identified as risk factors associated with APMV infection of poultry in LBMs. Generally, the identified risk factors indicated the low awareness and use of biosecurity measures among poultry keepers and traders. These factors perpetuate transmission and spread of APMV between flocks and regions.

5.2.2. Genetic diversity of APMV strains in Kenya

Newcastle disease outbreaks are common occurrence in the poultry industry in Kenya. The outbreaks result in significant economic losses annually. The study collected and characterized the diversity of APMV from poultry in different regions of Kenya. This was the first countrywide genetic study of APMV in poultry in Kenya.

The Kenyan APMV strains studied displayed high genetic diversity. However, from the results shown, the genetic distances and similarities observed in these populations signify close relatedness of APMV circulating in poultry in Kenya. This provides some advantages in the control of the diseases caused by this viral agent. High similarity between APMV strains collected from different regions at different points in time between 2014 and 2016 indicates possibly low evolution and high rate of inter-regional mixing of Kenyan APMV-1 strains in poultry. Poultry movement and trade could be largely responsible for homogenous populations observed in different geographical regions of Kenya. The close relatedness of Kenyan APMV strains was observed using both the F gene, which is considered the most variable APMV gene, and the L gene, which is considered one of the most conserved genes. This observation was also consistent with high similarity observed in the sequenced genomes of study strains. However, single nucleotide polymorphisms (SNPs), specifically substitutions, were commonly observed in the Kenyan APMV strains. SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein produced (Jia-Rui & Zeng, 2012). Such SNPs are conserved during evolution and can be used as genetic markers (Soler *et al.,* 2017). Variations in the amino acid sequence may result in functional changes of proteins. Such changes have been reported even for APMV-1 (Xu *et al.,* 2016).

The study established that the amino acid sequences of the different strains were highly similar. Variations in amino acid sequence in the Kenyan strains of APMV-1 was minimal. This was an indication that substitutions in different strains did not result in differentiating in the protein sequences. Analyzing genetic diversity of disease causing

agents provides a theoretical framework and basis for research in appropriate control tools. The implication of this finding is that control programs should be aimed at reducing further diversification of APMV in Kenya. That includes reduction in the introduction of new APMV strains in the Kenyan poultry sector. This study contributes to understanding of APMV strains in Kenya and their possible spread and diversity within the country and in the East African region. The study also contributed to the database of APMV-1 partial gene and whole genome sequences from Africa for further research on appropriate diagnosis and control of APMV-1.

5.2.3. Phylogenetic relationship of APMV strains in Kenya to global strains

The APMV strains detected in Kenya all belonged to serotype 1. APMV-1 strains isolated worldwide have been characterized into two clades: Class II strains are characterized by high genetic diversity and are classified into 18 known genotypes (Diel *et al.,* 2012a). APMV-1 strains sharing temporal, geographical and epidemiological parameters have been classified in similar clades and genotypes (Diel *et al.,* 2012a). However, strains of different genotypes have been isolated from the same geographical locations (Qin *et al.,* 2008b).

Analysis of the complete genomes of three APMV-1 strains from Kenyan poultry confirmed that they were of 'recent' genotypes. The genome analysis and genetic profile of APMV in Kenya indicated the close relationship between strains from different regions and revealed high genetic distance and variability between the Kenyan APMV-1 strains and commonly used vaccine strains such as *Lasota, B1-Hitchner* and *I-2*. Use of vaccines that are homologous to the field strains has been suggested as a way to reduce viral shedding following vaccination and possibilities of recombination of vaccine and field strains (Miller *et al.,* 2013; Miller *et al.,* 2009b; Miller *et al.,* 2007). Although the Kenyan strains seemed genetically distant from vaccine strains of APMV-1, it is not an indication of lack of protection by current vaccines against the Kenyan strains. There is need for efficacy studies to evaluate the protection levels conferred by the current vaccines on Kenyan APMV-1 strains.

Furthermore, the study identified some unique amino acid substitutions in the sequences of Kenyan and Ugandan sequences that could be used as genetic markers to differentiate them from vaccine strains and that could also be explored in formulation of genotype-matched vaccines.

Phylogenetic analysis of the gene and genome sequences of Kenyan strains showed that they all clustered in Class II genotype V alongside strains from Uganda and others from Europe and America. The clustering of the Kenyan strains and Uganda strains in a separate distinct group within the Genotype V suggests a similar ancestry of Kenyan and Ugandan strains. Other than Europe and America, APMV-1 strains of genotype V had not been reported on other continents (Garcia *et al.,* 2013) until the report of strains of the genotype in Uganda (Byarugaba *et al.,* 2014) and the confirmation of presence of strains of genotype V in Kenya found in this study. The route of introduction of strains of genotype V into the country still needs further investigation. However, two possible routes could be movement of poultry and poultry products from neighboring Uganda or through wild bird reservoirs.

Genotype V viruses are likely to have emerged in Central and/or South America in 1970s and subsequently spread into Europe (Susta *et al.,* 2014). Strains of the genotype V have been recovered from recurrent outbreaks in double-crested cormorants and from caged birds, peri-domestic bird species, and its spread has been associated with birds of *Psittacine* species. These migratory birds could have introduced the virus in the country given that Kenya is along the wild bird migratory path. However, this phenomenon needs further investigation. In Uganda, there are indications that the strains of genotype V were present from as early as 2001 (Byarugaba *et al.,* 2014). In Kenya, it is not known whether the strains were present as early as in Uganda or whether they were introduced much later. However, isolates available in GenBank (Benson *et al.,* 2017) from Kenyan poultry isolated and sequenced in 2010 belong to the same genotype as the Ugandan isolates (Byarugaba *et al.,* 2014). The close relatedness of Kenyan and Ugandan strains implies the need for controlled cross-

border movements of poultry and poultry products to avoid introduction of further diverse strains of APMV-1. Since APMV-1 in Kenya are genetically similar to those in Uganda, regional effort in controlling of the disease would be beneficial.

5.2.4. Signatures of selection on APMV-1 proteins

A dataset of APMV-1 genomes that included novel genotypes and strains from Kenya was evaluated to characterize the evolutionary forces affecting genes encoding structural proteins on the APMV-1 genome. The study findings indicated an important role played by selective pressures in the diversification of lineages and amino acid sites of APMV-1. Overall, APMV-1 functional proteins were conserved with variations only seen in localized regions.

The study findings confirmed that the fusion protein displayed the highest genetic variability and .was under adaptive evolution. This is expected of the fusion protein, an envelope protein that is exposed to the host's immune system. The protein could therefore be under adaptive selection to overcome the host's immune system. It was interesting that none of the amino acids in the cleavage site of the fusion protein was under positive selection and instead the amino acids at the site were under negative selection. This implies that the site is highly conserved, and may not favor any mutations that change the virulence of the virus.

However, mutations in other sites other than the cleavage site are likely to influence the virulence of APMV (Seth *et al.,* 2004). Other than the fusion and nucleoprotein, that had localized amino acids under positive selection, amino acid codons across the other proteins seemed to be highly conserved. Since the first occurrence of Newcastle disease, the population of APMV-1 has expanded over the years. This may indicate increased or more severe outbreaks in the host. The heterogeneity demonstrated in the selection pressures acting on various genotypes could further signal possible future expansion of APMV-1 strains globally. Genotype IX and XI seemed to display the greatest rate of evolutionary changes. These are virulent viruses genetically closely

related to strains of the early genotypes III and IV, which were of lesser virulence and are now extant (Dimitrov *et al.*, 2016a). These genotypes may be under higher pressure to survive where their closer related strains did not survive. Findings of this study signify different evolutionary dynamics acting on different APMV-1 proteins and lineages. The results indicate that selective pressures play a significant role in the evolutionary dynamics of APMV-1. This is useful in predicting future genetic diversity and survivability of global APMV-1 strains hand may help establishment of relevant control and mitigation measures.

5.3. Recommendations

Findings of this study revealed the high genetic diversity, close relatedness of avian paramyxovirus strains from poultry in different region of Kenya, and enhanced the understanding of the epidemiology of avian paramyxovirus-1 in Kenya.

5.3.1 Suggestions for improvement

From the study findings, the following intervention strategies are recommended for the control of APMV-1 in Kenya:

a) Strict implementation of quarantine policy in areas affected with APMV-1 infection.

b) Enhanced processing of poultry into safe products to restrict live bird trade and chances of virus transmission and introduction into new areas.

c) Enhances control of cross-border movement of poultry and poultry products.

d) Improvement of farmer extension services on poultry management to enhance biosecurity practices and reduce the risk of APMV.

e) Training of live bird traders on biosecurity and safe trade practices that reduce the risk of APMV in LBMs.

5.3.2 Suggestions for further studies

To gain even more insightful information on the epidemiology of APMV in Kenya, the study recommends the following:

1. A more detailed understanding of the risk factors of avian paramyxovirus requires a longitudinal study that examines the factors in relation to different time points. A cross sectional study as in this study may identify risk factors applicable at the time of study and miss others.
2. A more extensive sampling and characterization of viruses from wild birds is required to generate information for improved understanding of the role of wild birds in the epidemiology of avian paramyxovirus-1 in Kenya.
3. Further studies should be undertaken to determine the antigenic difference and efficacy of vaccine strains against Kenyan strains of avian paramyxovirus 1.

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APPENDICES

Appendix 1: Questionnaire used in characterization of poultry farms

Number of questionnaire …..... Date ……
Village……. Ward……
County…… Region……
GPS coordinates ……

1. **Household information**

Name of farmer…… Sex ……
Farm size (ha)…… Number in HH……

2. **Flock Information**

Species of birds kept

Species	Number	source
Chickens		
Ducks		
Geese		
Guinea fowls		
Turkeys		
Others- what?		

How many chickens do you have?

category	Number
Adult males	
Adult females	
Growers	
Chicks	

From which source do you replace your birds?

a) Own breeding ……..
b) Purchase from other farms ……..
c) Purchase from market …….
d) Given as gifts …….
e) Others (Specify) …….

3. Feeding Information

How do you keep your birds? (Tick)

a) Free range ……..
b) Partial confinement ……..
c) Total Confinement …….
d) Others (specify) …….

Do you give feed to your birds? Yes ☐ No ☐

If yes, list the type of feed and source

Feed	Source

4. Housing information (Tick)

Do you house your birds? Yes ☐ No ☐

If yes, where do you house your birds?

a) Chicken coup ……..
b) Kitchen ……
c) Others (specify) ……..……

How often do you clean your poultry house? (Specify the number of times)

a) Every day ……..
b) Weekly ……..
c) Monthly ……..
d) Others (specify) …….

5. Health information

Do you vaccinate your chicken? Yes ☐ No ☐

If yes, which month did you lastly vaccinate? ……..

Which vaccines did you use?

Number /species of birds	ND vaccine	Other vaccines (Specify)

Which other vaccines do you use regularly? …….

Has any bird suffered from any disease condition in the last three months?

Yes ☐ No ☐

If yes, which birds were affected?

Species	Ages	Number sick	Number dead

Did they show any of the following symptoms? (Tick)

Gastrointestinal signs	Respiratory signs	Nervous signs	Other signs
1. greenish, watery diarrhea	2. Sneezing,	6. Nervousness,	13. Swelling of the tissues around the eyes and in the neck
	3. gasping for air,	7. depression,	14. Sudden death
	4. nasal discharge,	8. muscular tremors,	
	5. coughing	9. drooping wings,	
		10. twisting of head and neck,	
		11. circling,	
		12. complete paralysis	

Do you treat your birds regularly? Yes ☐ No ☐

If yes, when was the last time you treated your birds? .....

What was the treatment? (Specify name of product)

a) Herbal (Specify) ……..
b) Drug from agrovet (Specify) ……..
c) Drug given by Vet/AHA (specify……..
d) Others (specify) …….

How do you dispose the sick and dead birds?

Eat them	
Sell them	
Treat them	

Other-what?	

6. Bird level health data (Each bird selected for sampling)

Bird ID…… Sex M……. F…….

Breed…….. Species…… Age……..

Source of bird

Bred on farm……. From neighbor…….. From market……

Has the bird shown signs of disease within the last two weeks?

Yes☐ No☐

If yes, which symptoms?

……

……

……

Was it treated? Yes ☐ No ☐

Which treatment was used?

……

……

……

Appendix 2: Questionnaire used in characterization of live bird markets

Number of questionnaire ….....Date ……

Village…….Ward…….……

County……..Region……..

GPS coordinates ……..

1. **Market information**

Name of market……Number of traders……

Number of birds traded per market day……

Type of market enclosure (tick)	**Location of market (tick)**	**Frequency of trade**	**Facilities available**
Enclosed with: Concrete wall structures ……	Within City/ ……	Daily……	Poultry Slaughter slab….
Wooden/mud/iron-sheet structures…	Within Major town ……	Once weekly…… …	Birdcages ……
Open air ……	Rural/trading center ……	Twice weekly…… …	Garbage disposal containers
	Others (Specify)……	Others (specify)…… …	

2. **Trader information**

Name of trader……..… Sex: M ……F……..

Species of birds stocked by trader at sampling time

Species	Number	Breed	source
Chickens			
Ducks			
Geese			

Guinea fowls			
Turkeys			
Others- Specify?			

3. Trading practices

In which markets do you sell your poultry? (Name of markets)…… ……	Where do you buy your birds? a. Live bird markets, b. Directly from farmers c. Others (specify ----------	What is your role in the poultry trade a. Sells live birds to other dealers b. Sells live birds to final consumers c. Slaughter the poultry and sells meat d. Other……	How often do you clean your birdcages? a. Daily b. Weekly c. Monthly d. never
What is the distance between your source of birds and the market (km) ……	How do you transport your birds to the market? a. Bicycle b. Motorbike c. Public vehicle d. Private car	Do you examine the poultry for presence of disease symptoms before buying? Yes/ no	12. If yes, how often? a. Always b. Sometimes c. Rarely d. never
Do you buy poultry with	If yes, how many birds have you bought in the last	How do you dispose of birds that have disease symptoms?	Who are your main customers?.....

disease symptoms? Yes/ no	one month that have disease symptoms? ……..	a. Sell as live birds b. Slaughter and sell to hotels c. Consume d. Throw away e. Bury f. Others(Specify)	…… …… ……..…… ……

4. Housing information (Tick)

Where do you house your stock in the night?

a) In the market ……..

b) At home (specify location) ……..

c) Others (specify) ……

5. Health information

In your current stock are there birds showing signs of illness? Yes N☐ ☐

If yes, which birds are affected?

Species	Ages	Number sick	Number dead

What signs have you observed in the sick birds?

Gastrointestinal signs	Respiratory signs	Nervous signs	Other signs
6. greenish, watery diarrhea	7. Sneezing,	6. Nervousness,	13. Swelling of the tissues around the eyes and in the neck
	3.gasping for air,	7. depression,	14. Sudden death

	9. coughing	9. drooping wings,	
		10. twisting of head and neck,	
		11. circling,	
		12. complete paralysis	

Does the disease also affect poultry from other traders in this market?

Yes ☐ No ☐

6. Bird level health data (Each bird selected for sampling)

Bird ID…… Sex M……. F…….

Breed…….. Species…… Age……..

Source of bird

Farm/Market…… Village……..

Ward…….. County……

Region……

Has the bird shown signs of disease within the last two weeks? Yes N☐ ☐

If yes, which symptoms?

……

……

Appendix 3: Description of APMV-1 strains used in the phylogenetic analyses

Accession No	Species	Country of origin	Isolate name	Year of collection	Genotype
AB465607	Chicken	Japan	Japan/Ishi/62	1962	I
Z12110	Chicken	Ireland	Ulster Mutant	1967	I
AY935492	Chicken	Australia	98-1249	1998	I
AY935499	Chicken	Australia	I-2vaccine	2005	I
AY935500	Chicken	Australia	I-2progenitor	2005	I
FJ600539	Mule duck	China	FJ0801	2008	I
HM063424	Water rail	China	R8/Guang-dong	2005	I
AF077761	Chicken	USA	Lasota	1946	II
AF309418		USA	B1	1947	II
AY225110		China	HB92/V4		II
EU289028	Turkey	USA	VG/GA	1987	II
EF201805		India	Mukteswar	1940	III
EF211808	Goose	China	JS/2/05/Go	1940	III
EF430159	Chicken	China	JS/7/05Ch	1940	III
EF589136	Fowl	China	Guizhou	1940	III
FJ480786	Mallard	China	NDV/Mallard/HLJ383/06	1940	III
AY741404		UK	Herts/33	1940	IV
EU293914		China	Italien	1940	IV
GU187941	Chicken	India	NDV-2//Namakkal/ Tamil Nadu	1940	IV
M24702		UK	Herts33	1940	IV
AF163440		China	F48E9		IX
AY341061	Chicken	China	LuoY		IX
FJ436302	Chicken	China	F48E8	1946	IX
FJ436304	Chicken	China	FJ01/85	1985	IX
FJ436306	Duck	China	JS/1/02	2002	IX
FJ705459	Cormorant	Canada	Cormorant/98CNN3-V1125/1998	1998	V a
FJ705460	Cormorant	Canada	Cormorant/95DC02150/1995	1995	V a
FJ705461	Cormorant	Canada	Cormorant/95DC2345/1995	1995	V a
JN872161	Cormorant	USA	Cormorant/Wisconsin/498260-2/07	1997	V a
AY288993	Chicken	Honduras	Chicken/Honduras/15/00	2000	V b
AY562987	Fowl	USA	Gamefowl/U.S.(CA)/211472/02	2002	V b
AY562990	Psittacine	USA	Largo/71	1971	V b
EF520718	Gamefowl	USA	Gamefowl/US (CA)/212676/2002	2002	V b
JN872181	Chicken	Honduras	Chicken/Honduras/44813/2000	2000	V b
JN942027	Chicken	Nicaragua	Fighting Cock/Nicaragua/95066-9/2001	2001	V b
JN942039	Amazon parrot	USA	Amazon/California/28936/1988	1988	V b
EU518683	Chicken	Mexico	Chicken/Mexico (Estado de Mexico)/465/2005	2005	V c
KJ577136	Chicken	Mexico	Chimalhuacan	1973	V c
HM117720	Chicken	Mexico	NDV-P05	2005	V c
JQ697743	Chicken	Mexico	Chicken/MX/NC02-634/2010	2010	V c
JQ697744	Chicken	Mexico	Chicken/MX/NC04-635/2010	2010	V c
JX974435	Chicken	Mexico	NDV1/10	2010	V c
KC808508	Gamefowl	Mexico	Gamefowl/Mex/616/2008	2008	V c
KC808509	Gamefowl	Mexico	Gamefowl/Mex(DF)/619/2008	2008	V c
KC808510	Scarlet macaw	Mexico	Scarlet macaw/Mex(Chiapas)/672-ZM12/2009	2009	V c
AF218134	Pheasant	Italy	IT-47	1974	Va
AF218136	Turkey	Italy	IT-51B	1972	Va
AF525370	Chicken	Germany	DE-173	1970	Va
AF525375	Chicken	Germany	DE-183	1974	Va
AF525377	Amazon	Germany	DE-190	1970	Va

Appendix 3: Description of APMV-1 strains used in the phylogenetic analyses

Accession No	Species	Country of origin	Isolate name	Year of collection	Genotype
AF525396		Yugoslovia	Yu(Vo)-1744	2002	Va
AY116971		Slovonija	HR-448	1979	Va
AY444496	Yellow nape parrot	USA	U.S(MA)/19120/87	1987	Va
EF065682	Chicken	USA	rAnhinga		Va
GQ288382	Cormorant	Canada	Cormorant/98 CNN3-V1125DC02150/1998	1998	Va
GQ288383	Cormorant	Canada	Cormorant/Canada/95DC02150/1995	1995	Va
GQ288387	Cormorant	USA (MN)	Cormorant/US (MN) 92-40140/1992	1992	Va
GQ288388	Cormorant	USA (CA)	Cormorant/US(CA)/92-23071/1992	1992	Va
AF402118		Bulgaria	BG-55	1975	Va
HG937573	Chicken	Uganda	NDV/chicken/Uganda/MU024/2011	2011	Vd
HG937580	Chicken	Uganda	NDV/chicken/Uganda/MU040/2011	2011	Vd
JQ217418	Chicken	Kenya	A89	2010	Vd
JQ217419	Chicken	Kenya	A48	2010	Vd
JQ217420	Chicken	Kenya	A148	2010	Vd
AY288995	Dove	Italy	Dove/Italy/2736/00	2000	VI
AY288996	Pigeon	Italy	Pigeon/Italy/1166/00	2000	VI
AY288997	Chicken	Kenya	Chicken/Kenya/139/90	1990	VI
FJ865434	Pigeon	China	S-1	2002	VI
GQ507801	Chicken	South Korea	Kr-102/89	1989	VI
JX518532	Dove	Kenya	Laughing Dove/B2/Kenya/Isiolo	2012	VI
AY444497		Indonesia	Moluccan/Indonesia/904/87	1987	VII
GQ288381	Cormorant	USA (CA)	Cormorant/US(CA)/D9704285/1997	1997	VII
AF358786	Chicken	Taiwan	TW/2000	2000	VII
AF458010	Chicken	China	JS-3/00	2000	VII
GQ245818	Ostrich	China	YZ-22-07-Os	2007	VII
GU227738	Dove	Serbia	NDV/Serbia/749/2007	2007	VII
AY734534	Chicken	Argentina	Trenque Lauquen	1970	VIII
FJ751918	Chicken	China	QH1	1979	VIII
FJ751919	Chicken	China	QH4	1985	VIII
FJ705464	Mallard	USA	Mallard/US(OH)/04-411/2004	2004	X
FJ705467	Mallard	USA	Mallard/US(MN)/MN00-32/2000	2000	X
FJ705468	Mottled duck	USA	Mottled duck/US(TX)/TX01-130/2001	2001	X
HQ266602	Chicken	Madagascar	MG/725/08	2008	XI
HQ266603	Chicken	Madagascar	MG/1992/08	2008	XI
HQ266604	Chicken	Madagascar	MG/Meola/08	2008	XI
JN627504	goose	China	Goose/China-GD/12/2011	2011	XII
JN627506	Goose	China	Goose/China-GD/20/2011	2011	XII
JN627507	goose	China	Goose/China-GD/1003/2010	2010	XII
JN627508	goose	China	Goose/China-GD/450/2011	2011	XII
JN800306	Chicken	Peru	Poultry/Peru/1918-03/2008	2008	XII
AY865652	*Sterna albifrons*	Russia	Sterna/Astr/2755/2001	2001	XIII
GU182323	Chicken	Pakistan	SPVC/Karachi/NDV/43/2008	2008	XIII
GU182331	Chicken	Pakistan	SPVC/Karachi/NDV/33/2007	2007	XIII
JN682190	Chicken	Pakistan	Chicken/CP/Islamabad2/2010	2010	XIII
HF969214	Chicken	Nigeria	Chicken/Nigeria/NIE10-333/2011	2011	XIV
JX119193	Chicken	Dominican Republic	DominicanRepublic//499-31/2008	2008	XVI

Appendix 3: Description of APMV-1 strains used in the phylogenetic analyses

Accession No	Species	Country of origin	Isolate name	Year of collection	Genotype
JX186997	Chicken	Dominican Republic	Dominican/Republic/867/2008	2008	XVI
JX915242	Chicken	Dominican Republic	DominicanRepublic/28138-4/1986	1986	XVI
JX915243	Chicken	Mexico	Mexico/Queretaro/452/1947	1947	XVI
HF969184	Chicken	Ivory Coast	CIV08-103	2007	XVII
FJ772446		Nigeria	Avian/913-1/Nigeria/2006	2006	XVII
FJ772449		Nigeria	Avian/913-33/Nigeria/2006	2006	XVII
JF966385		Mali		2008	XVII
FJ772455		Mauritania	Avian/1532/14/Mauritania/2006	2006	XVIII
FJ772466	Chicken	IvoryCoast	Chicken/2601/Ivory Coast/2008	2008	XVIII
HF969127	Chicken	Ivory Coast	CIV08-069	2007	XVIII
EF612277		USA	AK/196	1998	Class I

Appendix 4: Partial L gene sequences of APMV obtained from poultry in Kenya

#	Location	Haplotype 1D	Sample ID
1	LB (Busia)	Haplotype 1	PFC0200730
2	Coast (Kilifi)	Haplotype 1	PFC0300836
3	WH (Bungoma)	Haplotype 1	LBC0100700
4	WH (Cheptais)	Haplotype 1	PFC0100703
5	WH (Chwele)	Haplotype 1	LBC0100707
6	LB (Bunyala)	Haplotype 1	PFC0200712
7	LB (Chakol)	Haplotype 1	PFC0200715
8	LB (Chakol)	Haplotype 1	PFD0200719
9	LB (Migori)	Haplotype 1	LBC0200722
10	LB (Homabay)	Haplotype 1	LBC0200723
11	LB (Kisumu)	Haplotype 1	LBC0200726
12	Eastern (Makueni)	Haplotype 1	LBC0400678
13	N (Kawangware)	Haplotype 1	LBC0500733
14	WH (Kimilili)	Haplotype 1	PFC0100697
15	WH (Kimilili)	Haplotype 1	PFD0100698
16	LB (Kisumu)	Haplotype 1	PFC0200786
17	Coast (Marikiti)	Haplotype 1	LBC0300808
18	Coast (Marikiti)	Haplotype 1	LBC0300811
19	Coast (Marikiti)	Haplotype 1	LBC0300812
20	Coast (Likoni)	Haplotype 1	PFC0300820
21	Coast (Mtepeni)	Haplotype 1	PFC0300825
22	Coast (Mtepeni)	Haplotype 1	PFC0300822
23	Coast (Dabaso)	Haplotype 1	PFC0300850
24	Coast (Dabaso)	Haplotype 1	PFC0300856
25	Coast (Dabaso)	Haplotype 1	PFC0300858
26	N (Machakos)	Haplotype 1	LBC0500646
27	WH (Kabuchai)	Haplotype 1	PFC0100661
28	N (Kibra)	Haplotype 1	LBC0500586
29	N (Machakos)	Haplotype 2	LBC0500649
30	Eastern (Makueni)	Haplotype 2	LBC0400671
31	Eastern (Makueni)	Haplotype 3	LBC0400668
32	Eastern (Makueni)	Haplotype 4	LBC0400676
33	LB (Bunyala)	Haplotype 4	PFC0200685
34	LB (Amukura)	Haplotype 4	PFC02002055
35	Coast (Dabaso)	Haplotype 5	PFC0300839
36	Coast (Dabaso)	Haplotype 5	PFC0300855
37	Coast (Marikiti)	Haplotype 5	LBC030080
38	Coast (Majengo)	Haplotype 5	LBC0300828
39	LB (Ageng'a)	Haplotype 5	PFC02002080
40	LB (Bunyala)	Haplotype 5	PFC0200687

Appendix 4: Partial L gene sequences of APMV obtained from poultry in Kenya

#	Location	Haplotype 1D	Sample ID
41	Coast (Majengo)	Haplotype 5	LBC0300815
42	LB (Homabay)	Haplotype 5	PFC0200695
43	LB (Ageng'a)	Haplotype 6	PFC0200689
44	WH (Bomet)	Haplotype 7	LBC0100752
45	WH (Kakamega)	Haplotype 8	LBC0100753
46	Eastern (Meru)	Haplotype 8	LBC0400873
47	WH (Kericho)	Haplotype 9	LBC0100782
48	Coast (Likoni)	Haplotype 9	LBC0300810
49	WH (Chwele)	Haplotype 10	LBD0100783
50	Coast(Majengo)	Haplotype 11	LBC0300829
51	Coast(Mkomani)	Haplotype 12	LBC0300849
52	Eastern(Makueni)	Haplotype 13	LBC0400601
53	WH(Kericho)	Haplotype 13	LBC0100618
54	N(Burma)	Haplotype 13	LBC0500633
55	N(Burma)	Haplotype 13	LBC0500634
56	WH(Bomet)	Haplotype 13	LBC0100674
57	N(Machakos)	Haplotype 13	LBC0500608
58	WH(Kimilili)	Haplotype 13	PFC0100100
59	N(Kibra)	Haplotype 13	LBC0500587
60	Coast (Kilifi)	Haplotype 13	PFC03003098
61	WH(Bomet)	Haplotype 13	LBC0100988
62	Coast(Mkomani)	Haplotype 13	PFC0300851
63	N(Kitengela)	Haplotype 13	LBC0500894
64	Eastern(Meru)	Haplotype 13	LBC0400904
65	Coast(Kilifi)	Haplotype 13	LBC0300852
66	Coast(Majengo)	Haplotype 14	LBC0300857
67	Coast(Kilifi)	Haplotype 15	LBC0300864
68	Coast(Kilifi)	Haplotype 16	LBC0300869
69	Eastern(Makueni)	Haplotype 17	LBC0400872
70	Eastern(Meru)	Haplotype 18	LBC0400903
71	Eastern(Makueni)	Haplotype 18	LBC0400679

Appendix 5: Partial F gene sequences of APMV-1 obtained from poultry in Kenya

#	**Location**	Haplotype ID	Sample ID
1	Eastern (Makueni)	Haplotype 1	LBC0400679
2	WH (Kabuchai)	Haplotype 1	PFC0100661
3	N (Machakos)	Haplotype 1	LBC0500646
4	N (Burma)	Haplotype 1	LBC0500633
5	WH (Kericho)	Haplotype 1	LBC0100631
6	WH (Kericho)	Haplotype 1	LBC0100618
7	N (Kibra)	Haplotype 1	LBC0500583
8	N (Kibra)	Haplotype 1	LBC0500587
9	N (Kibra)	Haplotype 1	LBC0500586
10	N (Kitengela)	Haplotype 1	LBC0500522
11	N (Kibra)	Haplotype 2	LBC0500576
12	N (Kawangware)	Haplotype 3	LBC0500733
13	WH (Bomet)	Haplotype 4	LBC0100674
14	WH (Bomet)	Haplotype 4	LBC0100673
15	N (Kitengela)	Haplotype 4	LBC0500894
16	Eastern (Makueni)	Haplotype 5	LBC0400601
17	WH (Bomet)	Haplotype 5	LBC0100671
18	WH (Kimilili)	Haplotype 6	PFC01001002
19	Coast (Kilifi)	Haplotype 6	PFC03003098
20	Coast (Marikiti)	Haplotype 6	LBC0300811
21	LB(Bunyala)	Haplotype 7	PFC0200687
22	LB(Ageng'a)	Haplotype 7	PFC02002080
23	WH(Bungoma)	Haplotype 7	LBC0100699
24	N(Machakos)	Haplotype 7	LBC0500608
25	Eastern(Makueni)	Haplotype 7	LBC0500600
26	WH(Bungoma)	Haplotype 7	LBC0100700
27	WH(Cheptais)	Haplotype 7	PFC0100703
28	WH (Chwele)	Haplotype 7	LBC0100707
29	LB(Homabay)	Haplotype 7	LBC0200692
30	N (Machakos)	Haplotype 7	LBC0500597
31	N (Burma)	Haplotype 7	LBC0500634
32	Coast (Dabaso)	Haplotype 7	PFC0300858
33	Eastern (Makueni)	Haplotype 8	LBC0400678
34	WH(Kimilili)	Haplotype 9	PFC0100697
35	LB (Bunyala)	Haplotype 10	PFC0200685
36	LB (Homabay)	Haplotype 10	PFC0200695
37	LB (Ageng'a)	Haplotype 10	PFC0200689
38	Eastern (Makueni)	Haplotype 11	LBC0400676
39	WH (Kimilili)	Haplotype 11	PFD0100698
40	LB (Amukura)	Haplotype 12	PFC02002055

Appendix 5: Partial F gene sequences of APMV-1 obtained from poultry in Kenya

#	**Location**	Haplotype ID	Sample ID
41	LB (Chakol)	Haplotype 12	PFC02002063
42	LB (Amukura)	Haplotype 13	PFC02002054
43	LB (Chakol)	Haplotype 14	PFC0200715
44	WH (Chwele)	Haplotype 15	PFC01001002
45	N (Kitengela)	Haplotype 16	LBC0500525
46	Coast (Likoni)	Haplotype 17	PFC0300410
47	Coast (Dabaso)	Haplotype 17	PFC0300855
48	N (Machakos)	Haplotype 18	LBC0500649
49	N (Kitengela)	Haplotype 19	LBC0500523
50	Eastern (Meru)	Haplotype 20	LBC0400903
51	WH (Kabuchai)	Haplotype 21	PFC0100660
52	LB (Bunyala)	Haplotype 22	LBC01001007

Appendix 6: Publications from this research work

1. **Ogali, N. I**., Wamuyu, L.W., Lichoti,J.K., Mungube,E.O., Agwanda, B. R. and Ommeh, S.C.(2018) Molecular Characterization of Newcastle Disease Virus from Backyard Poultry Farms and Live Bird Markets in Kenya. *International Journal of Microbiology* Volume 2018, Article ID 2368597

2. **Ogali, N. I**., Mungube, E. O., Lichoti, J.K., Ogugo, M.W, and Ommeh, S.C.(2018) A study of Newcastle disease virus in poultry from live bird markets and backyard flocks in Kenya *Journal of Veterinary Medicine and Animal Health* Vol. 10(8), pp. 208-216