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Molecular characterization of Newcastle disease viruses from live bird markets in Nigeria

By

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Submitted in partial fulfilment of the requirements for the degree

***Magister Scientiae* (Master of Science) in Veterinary Science**

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Date submitted: September, 2011

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ACKNOWLEDGEMENTS

I wish to express my gratitude and acknowledge the contributions of the following:

Dr S.P.R Bisschop for accepting to be my promoter and sourcing of fund for this work Also, his support and understanding throughout the duration of my studies

Dr Celia Abolnik for her kind disposition, encouragement, patience and tireless efforts in overseeing every step of this work

Management of ARC- OVI for granting me permission to use their facilities for this study

I am profoundly grateful to Bontsi Mochotlhoane for helping with the robot extraction and the PCR technique lessons. I thank Inel Burger for her concern and assisting with the real time PCR and sequencing.

My gratitude to the following staff of ARC-OVI: Mpho, Rachel, Amanda, Yolanda, Drs Romito and Christine for their encouragement.

I appreciate the Kind assistance and support of Drs Peter Smith and Thys Snyman. Antoinette van Wyk, Alida de Meillon and Hanli Moolman, all staff of the Poultry Reference Centre, Department of Production Animal Studies, University of Pretoria

To Antoinette and Johannes of Jotello Veterinary Library, University of Pretoria, I thank you for the library lessons and for assisting with the books and articles.

I am grateful to friends and colleagues at Onderstepoort: Bwala, Alex, Ularamu, Ben Mathew, Nana, Bagla, Ahmed, Calvin, Kimaro, Leo, Okechwuku and Habu, for good company and counsels.

The entire staff of Viral Research Division, NVRI, Vom together with all friends and relations for prayers and support.

Many thanks to Dr Pius Ekong and Jessini for helping with the GIS maps.

The staff of academic administration, I appreciate the efforts of Denise Marais and Magda Human for readily attending to my administrative needs and demands.

I appreciate the leadership and members of Glory land Christian Ministry, Pretoria, SA for providing the platform to meet my spiritual needs throughout my stay in Pretoria.

The management of NVRI, led by Dr Lami Lombin, Dr D. Shamaki (the Director, Research) and Dr T.M. Joannis (Head of Virology Research) for the permission to embark on this study.

I thank my wife Nannyi for her understanding and efforts in taking care of the home front in my absence. My children; Hanmak, Ponchang and Jinan for enduring the absence of a father

Above all, I thank God for seeing me through this work

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SUMMARY

Although Newcastle disease is reported to be endemic in Nigeria, little information exists on the molecular epidemiology and the lineage distribution of the Newcastle disease viruses (NDVs) in the country, especially in the live bird markets. Recent studies reported the identification of three distinct sub-lineages namely; 5f, 5g and 5h in West Africa, particularly sub-lineages 5f and 5g were identified in Nigeria.

In this study a total of 41 NDV isolates were analysed. Thirty six NDVs were recovered from a variety of poultry species from live bird markets in the six geo-political zones of Nigeria during active surveillance from 2007 to 2008. Five NDVs recovered from outbreaks in backyard and commercial poultry farms within the same period were also genetically characterized. A commonly used region of the virus genome that spans nucleotide 61 to nucleotide 374 of the Fusion protein, including the cleavage site was targeted. Based on sequence analysis, 39 of the isolates were classified as virulent. Of these, 20 were classified as sub-lineage 5g and 17 were classified as sub-lineage 5f. One isolate differ markedly from all other strains included in the phylogeny. Interestingly, 13 strains from the 5f group formed a distinct cluster that was not identified by other groups in similar studies.

Phylogenetic analysis, amino acid sequence determination of the F_0 cleavage site sequence analysis, pair wise distance analysis of the partial fusion protein gene sequences and Geographic Information System (GIS) mapping was done. Results showed close genetic similarities and provided evidence for the first time of the epidemiological link between the viruses circulating in the LBMs and those identified in outbreaks in backyard and commercial farms in Nigeria between 2007 and 2008. The emergence and identification of new sub-lineages gives an insight in to the high rate of genetic drift occurring in NDV strains in Nigeria, and raises concerns about the efficacy of current NDV control measures in the country. Thus there is need for continuous surveillance and characterization of NDV from Nigeria to monitor the emergence of new lineages and sub-lineages in the Nigerian poultry industry.

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LIST OF ABBREVIATIONS

%	: Percentage
^{o}C	: Degree centigrade
DOC	: Day old chicks
ELISA	: Enzyme-linked immune-sorbent assay
F	: Fusion gene
FAO	: Food and Agricultural Organization
FCT	: Federal Capital Territory
GIS	: Geographical Information System
HN	: Haemagglutinin- neuraminidase gene
ICPI	: Intracerebral Pathogenecity Index
IU	: International unit
LBM	: Live bird market
MDT	: Mean Death Time
MEGA	: Molecular Evolutionary Genetic Analysis
M-MLV RT	: Moloney Murine Leukaemia Virus Reverse Transcriptase
NVRI	: National Veterinary Research Institute
OIE	: World Organization for Animal Health
VRD	: Viral Research Department

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CHAPTER ONE: INTRODUCTION

1.1 Poultry

Poultry is the category of domesticated birds that people keep for the purpose of collecting their eggs, or for meat and/or feathers. They include members of the order *Galliformes* (which includes chickens, quails and turkeys) and the order *Anseriformes,* commonly known as "waterfowl" (e.g. domestic ducks and domestic geese). Poultry also include other birds which are killed for their meat, such as pigeons and doves or birds considered to be game, like pheasants (Encyclopedia Britannica, 2008; http://www.meriam-webster.com/dictionary/poultry)

1.2 Poultry population

The Nigerian poultry population is estimated at 150 million, of these, 98 million (65%) are rural and backyard poultry, mainly of indigenous stock. An estimated 6 million (4%) represent a mix of indigenous and exotic breeds that make up the semi-commercial sector. The remaining 46 million (31%) constitute the commercial sector (FAO, 2006).

About 65% of Nigerian commercial poultry is located in the South West with 25% based in the South South and South East zones. The remaining 10% is based in the North Central, North West and North Eastern zones (Adene and Oguntade, 2006; Obayelu, 2007).

1.3 Poultry structure and diversity

The Nigerian poultry industry has diversified over the years and can be broadly classified into four sectors based on FAO (2004) classification.

1.3.1 Sector 1: Industrial and integrated commercial system with clearly defined and implemented standard operating procedures for strict bio-security. Their operations include grandparent /parent stock farms. They are also involved in direct production of

poultry meat and eggs. Most of these operations are located in the South West and some parts of the South South zones.

1.3.2 Sector 2: Commercial poultry production system with low to moderate bio-security. The operations include a variety of poultry species with flock sizes ranging from 1 000 to 5 000. Poultry products (like eggs, broilers and cull-hens) are usually sold directly to retailers or vendors who transport them mostly on open top trucks to the retail market. This sector is spread all over the six zones but is predominant in the South West zone.

1.3.3 Sector 3: This is a semi-commercial poultry production system similar to the Sector 2, but with low bio-security. Operations include a variety of poultry species, with flock size ranging from 100 to 1 000. Poultry products (like eggs, broilers and cull-hens) are usually sold directly to retailers or vendors who transport them mostly on open top trucks to the retail market. This sector is spread over all six zones.

1.3.4 Sector 4: An extensive production system where, birds are kept under a low input-low-output management system. It is comprised of village/ indigenous poultry, and constitutes 65% (98 million) of the national flock. Most of these operations are in the northern part of the country. A large proportion of the domestic waterfowls kept in Nigeria fall under this sector, where they forage around ponds and wetlands, occasionally coming into contact with wild and migratory birds.

1.4 Live bird markets

Live bird markets (LBMs) are commonplace in most Nigerian cities, towns and villages. They range from moderately organised to very much disorganised operations depending on where they are located. The supply system is such that birds are gathered from different sources and are marketed live to people by the roadside or to people coming to a weekly or daily market.The quantity and type of poultry supplied and sold in the markets varies significantly with the months and season of the year, with high supplies, movement and sales during the months of March to April and November

to December. These coincide with the seasons of Muslim and Christian festivals of Eid-El-Fitre, Eid-El-Kabir, Christmas and Easter respectively (Mlozi *et al*., 2003). Most of the live bird markets in the North sell almost exclusively indigenous poultry with a few urban based markets dealing with a mix of exotic breeds sourced from backyard and commercial farms within the area supplied by cull- hen vendors.

In the South, the markets sell mostly spent commercial poultry from commercial farms within the vicinity and a mix of indigenous poultry brought from the north (Brandenburg, 2008)

1.4.1 Urban live bird markets

Mostly located within a larger market where other forms of buying and selling go on, usually in close proximity to poultry abattoirs. Also characterised by high turnover of multiple species of birds, which are confined in cane cages, wooden cages and seldom in plastic cages, the birds are usually kept in the market when not sold. Minimal to moderate sanitation is observed depending on where the market is located. The supply system is from different sources which may include spent poultry from commercial farms, village chickens, turkeys, guinea fowl, pigeons, ducks and occasionally wild-caught birds, most of the time sourced from other smaller village markets.

1.4.2 Village/ rural-based live bird markets

These are usually located in village markets. Bird types include village chickens, ducks and guinea fowl. The birds are usually few in number and are seldom housed but normally tethered and are displayed on bare ground for sale. The supply is usually from local households and neighbouring villages. In some instances birds are sourced from nomadic herdsmen in transit, where the birds are returned home when not sold. Sanitary measures are not applied.

1.4.3 Roadside live bird markets

These are usually found along major roads, especially at inter-state junctions. Birds are not housed and multi-species. The supply source ranges from spent hens vendors to village chickens and caged ornamental birds, and no sanitary measures are applied.

Figure1.1A and B: collection of multi-species of birds in cane cages on display in an urban LBM

Figure 1.2: Trucks loaded with a variety of bird types in cane cages in an urban LBM in Northern Nigeria ready to be transported to the southern part of the country

Figure 1.3: Indigenous birds displayed on the ground for sale in a village LBM in North-Central Nigeria

Figure 1.4: A road-side poultry vendor at a junction in a city in northern Nigeria

1.5 Movement of poultry and poultry products

Most of the rural poultry (based on indigenous breeds) is found predominantly in northern Nigeria. In contrast larger established commercial poultry farms (based on exotic commercial poultry) are concentrated mainly in the South especially the South

West. There are two movements of poultry across Nigeria; movement of live rural poultry bought from LBMs mainly from the Northern parts of the country to the South were the demand is high, and the movement of commercial Day Old Chicks and frozen poultry from the South to the North (Adene and Oguntade, 2006).

This inter-regional trade in poultry products provides a potential route for the transmission of Newcastle disease and other poultry diseases.

1.6 Family poultry and sustainable livelihood in Nigeria

Family poultry is defined as small flocks kept by households which use family labour in order to obtain food security, income and gainful employment for members of the family especially women and children (FAO, 2004; Sonaiya, 1990a).

In Nigeria, family poultry represents approximately 94% of total poultry keeping and accounts for about 4% of the total estimated value of the livestock resources in the country (FAO, 2004). This sector contributes to the improvement of the micro-economy of households, as the income from the sales is used for food, children's school fees and other unexpected expenses such as medicines. Family poultry generates 19-50% of rural family income (Sonaiya, 2007). Unlike cattle, sheep and goats which require large land for grazing and a long time span to reap from the investment, family poultry requires little land resources with a potentially quick return on investment. The flock size of family poultry in Nigeria ranges from 5 to 100 birds (Table 1.1).

Table 1.1: Flock size and family poultry farming objectives in Nigeria

Objective	flock size
Home consumption	1-10
Home consumption and cultural reasons	1-10
Income and home consumption	11-30
Income only	>50

Source: Sonaiya, 1990b

Poultry production, whether commercial or informal, remains a viable venture for sustainable livelihood to the average Nigerian family, both in terms of guaranteed supply of animal protein and economic returns (Table 1.1). The sector has the capacity to employ the teeming population of unemployed youths through the provision of jobs in the various sub-sectors like commercial feed and toll milling, commercial/backyard poultry farming, poultry product processing and marketing, hatchery and breeding operations, as well as marketing of veterinary implements and pharmaceuticals.

However the development of this important sub-sector is constrained by many factors which include poor government policies and disease burdens of various kinds, the most devastating being Newcastle disease (ND) which causes losses both in egg and meat production as a result of high morbidity and mortality.

Most of the Newcastle disease viruses from Nigeria have been characterized by the mean death time (MDT) and intracerebral pathogenecity index (ICPI) (Oladele, *et al.*, 2007) but little information exists on their molecular characteristics and epidemiology. Thus, the need to carry out a thorough investigation on the molecular epidemiology of ND in Nigeria, particularly the role of LBMs in order to understand the dynamics of the disease. This information will help in instituting effective control measures.

The aim of this study was to genetically characterize and phylogenetically group ND viruses obtained from LBMs in Nigeria from 2007 to 2008 and to compare them with isolates recovered from outbreaks in backyard and commercial farms within the same period.

The objectives were:

- To identify the strains that are prevalent in the LBMs and the species of birds that harbours them.
- To establish any existing epidemiological link between the viruses in live bird markets and the ones that caused outbreaks in backyard and commercial farms.
- To trace the possible origin of ND virus strains circulating in Nigeria

- To establish any relationship between ND virus strains in Nigeria and those reported for other West African countries

Geographic information systems were used to reveal the pattern of ND spread in Nigeria

CHAPTER TWO: LITERATURE REVIEW

2.1. Newcastle disease

2.1.1 Synonyms

Pseudo-fowl pest, Pseudovogel-pest, atypische geflugel pest, pseudo-poultry plaque, avian pest, avian distemper, Raniket disease, tetelo disease, Korean fowl plaque and avian pneumoencephalitis (Alexander and Senne, 2008)

2.1.2 Definition

Newcastle disease (ND) is a highly contagious and devastating viral disease of poultry of worldwide distribution with an enormous economic impact (Alexander, 1988; Peroulis-Kourtis, 2002). The disease remains one of the major problems affecting the poultry industry in both developed and developing countries (Alexander, 2000). Its recognition as a disease entity of viral origin is traced back to 1926 in Newcastle-Upon–Tyne (Alexander *et al.*, 2004).

According to the current OIE definition (OIE, 2009), Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater.

Or

b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F_2 protein and phenylalanine at residue 117, which is the N-terminus of the F_1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 to 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI) test.

2.1.3 Species affected and susceptibility

ND has been established in at least 241 species of birds representing 27of the 50 orders of the class (Kaleta and Baldauf, 1988). Most species of birds including chickens, turkeys, pheasants, pigeon, quails and guinea fowl are highly susceptible to virulent APMV-1. Waterfowl such as ducks and geese may be infected but show few or no clinical signs even to the strains virulent to chickens (Liu *et al*., 2007; Tsai *et al*., 2004). However, there have been reports of high mortality in double-crested cormorants due to Newcastle disease (Glaser *et al*., 1999). It has been suggested that wild birds and domestic waterfowl harbour avirulent strains of Newcastle disease virus. Also, the Columbidae family may be infected with APMV-1 adapted to those particular avian species designated pigeon paramyxovirus serotype- 1 PPMV-1 (Alexander *et al*., 1985; Pedersen *et al*., 2004).

2.1.4 Transmission

Transmission is primarily through direct contact between healthy birds and bodily discharges of infected birds. The disease is often mechanically spread by vaccination and debeaking crews, manure haulers, feed delivery personnel, poultry buyers, egg service people, and poultry farm owners and employees; where virus-bearing materials can be picked up on shoes, clothing or equipment and carried from an infected flock to a healthy one.

Experimental studies indicated that aerosol exposed chickens were able to seroconvert and shed the virus, an indication that airborne transmission occurred (Li *et al*., 2009). The respiratory route as a means of transmission depends on environmental factors like temperature, humidity and stocking density, especially where there is close bird-to-bird association. Egg- associated vertical transmission with virulent strains is possible but uncommon, as the embryo usually dies unless the viral titre in the egg is low (Chen *et al*., 2002). However, the major mode of transmission remains the faecal-oral route (Alexander, 1988; Alexander *et al*., 2004).

2.1.5 Symptoms

The disease affects the respiratory, nervous and digestive systems. The symptoms are variable depending on the pathotype, species of bird, concurrent disease and pre-existing immunity. The incubation period ranges from 2-15 days and mortality ranges from 0-100%, depending on the strain (Nanthakumar *et al.,* 2000).

2.1.6 Diagnosis

Clinical diagnosis is based on clinical signs as well as the high morbidity and mortality frequently characteristic of the disease. Confirmatory laboratory diagnosis is based on isolation of Newcastle disease virus (NDV) from tissues of dead birds, and swabs from live birds. Viral isolation is by inoculation of samples into 9 to11-day-old embryonated chicken eggs. Classically, the allantoic fluid from dead or dying embryos is tested for haemaglutinating (HA) activity and any agent that haemaglutinates is examined for haemaglutination inhibition (HI) activity with monospecific antiserum to APMV-1and molecular detection of NDV RNA from tissues or swabs by reverse transcription polymerase chain reaction that specifically targets NDV RNA (OIE, 2009).

2.1.7 Epidemiology

The global Newcastle disease situation is characterized by enzootic infection in many developing countries in East Asia, the Middle East, Africa and South America, where rural poultry from these regions are thought to serve as source of periodic ND epizootics that occur in disease-free countries around the world (Herczeg *et al.*, 1999; Wehmann *et al.,* 2003).

2.1.8 Aetiology

The etiologic agent of ND, Newcastle disease virus, belongs to the genus Avulavirus within the family *Paramyxoviridae* in the order *Mononegavirales* and is designated avian paramyxovirus 1 (APMV-1), one of the nine identified serotypes of the avian paramyxoviruses (Alexander, 1997; de Leeuw and Peeters, 1999; Mayo, 2002 and Pedersen *et al.*, 2004).

2.1.9 Zoonotic/public health significance

Newcastle disease infection in humans causes a transient conjunctivitis. There are reports of a more generalised infection resulting in chills, headache and fever without conjunctivitis. Infection is usually a result of direct contact with the virus (Alexander, 1988). Many of the documented cases involved laboratory workers who accidentally splashed high-titre NDV-infected allantoic fluids into their eyes, veterinary diagnosticians handling infectious tissues and vaccination crews (Swayne and King 2003).

2. 1.10 Newcastle disease in Nigeria

Newcastle disease is endemic in Nigeria with widespread epidemic outbreaks in both susceptible commercial and village poultry (Orajaka *et al*., 1999). Until the first report of highly pathogenic avian influenza (H5N1) in Nigeria in February 2006 (Joannis *et al.,* 2006; Benedictis *et al.,* 2007) ND was the leading cause of death in poultry. The first documented evidence of ND in Nigeria occurred in Ibadan South West Nigeria in 1952; however, the first laboratory confirmation was made in 1953 from outbreaks in the former Benue and Ibadan provinces (Hill *et al*., 1953). By 1954, NDV isolations were made from outbreaks in 11 other areas (Nawathe, 1975; West, 1972). All of the isolations were described as velogenic in chickens (Okoh, 1979). Serologic evidence of ND in captive Lanner's falcon (*Biamicus abyssinicus)* was reported in 1979 by Okoh. However, there was no virus isolation.

Earlier isolations and biological characterization by mean death time (MDT) and intracerebral pathogenicity index (ICPI) of ND virus isolates recovered from chickens and a parrot revealed that they were all velogenic strains (Adu *et al.,* 1985). In 1992, Ojech and Okoro reported the isolation and characterization of a lentogenic NDV strain from a feral dove (*Stigmatopelia senegalensis).* A recent study on feral birds from North Central Nigeria reported the isolation of lentogenic NDV strains (Ibu *et al*., 2009).

The major control measure of ND in Nigeria is vaccination. However, this is applicable only to commercial poultry. The use of ND vaccines in village and rural poultry is limited by cost, dose formats and lack of thermostability. As a result, most of the village and

rural poultry remain unvaccinated and highly susceptible to ND, with the associated economic losses (Adene, 1997).

The traditional vaccination programme in Nigeria typically involves the use of 3 types of live ND vaccines: (1) ND Hitchner B1 lentogenic strain-derived vaccine administered intra-occularly at day old for priming, followed by (2) ND-LaSota (a lentogenic strain), administered orally in drinking water at day 21. At six weeks of age, (3) ND-Komarov (a mesogenic strain) is administered intramuscularly and repeated at sixteen weeks (Okeke and Lamorde, 1988). All the abovementioned vaccines are produced by the National Veterinary Research Institute, Vom, Nigeria. This vaccine regime is based on reports of endemicity of ND (velogenic strains) in the country and studies which recommended that in order to build sufficient immunity to withstand the velogenic NDV strains, the regime of four successive vaccinations must be strictly adhered to (Adu *et al.*, 1990). In the last two decades, there has been an influx of foreign ND vaccines consequently, a wide range of live and inactivated oil based vaccines from Europe and other parts of the world are in use in the country. The current situation is that vaccination programmes are as varied as the vaccine types available. Despite vaccination, ND outbreaks in Nigeria have been frequent and widespread in both vaccinated and unvaccinated flocks (Adu *et al.*, 1990; Shoyinka, 1983).

2.2 Newcastle disease virus

NDV is an enveloped, single stranded non-segmented, negative sense RNA virus (Seal *et al.*, 2000). The genome consists of 15 186 nucleotides (nt) (de Leew and Peteers 1999), 15 192 nt (Huang *et al.,* 2004) or 15 198 nt (Czegledi *et al.*, 2006). The full length genome of NDV is an exact multiple of six, which is consistent with 'the rule of six' for paramyxovirus genomes (Kolakofsky *et al.*, 1998).

The NDV genome contains six genes in the order; 3'-NP-P-M-F-HN-L-5' that code for the six major polypeptides (nucleoprotein, phosphoprotein, matrix, fusion, haemaglutinin-neuraminidase and the large protein, respectively) (Lamb and Kolakofsky, 2002) as well as two non-structural proteins, W and V which may result

from differential initiation or transcriptional editing of the P gene mRNA (Qin *et al.*, 2008; Yusoff and Wen, 2001).

The nucleoprotein (N) appears as negative staining particles under electron microscope and flexible helical structures. It resembles the classical herringbone morphology with spikes protruding from a central channel (Yusoff and Wen, 2001). The NP is a conserved multifunctional protein which functions primarily to encapsidate the virus genome for the purpose of RNA transcription, replication and packaging. Also it is efficient in inducing NDV-specific antibodies in chickens (Mebatsion *et al.*, 2002).

The P gene is a multi-coding gene which can be edited in the editing site and transcribed into three different proteins, viz. P protein (unedited), V protein (+1 frame-shift), and W protein (+2 frame-shift) (Ding *et al.*, 2008). The three proteins are co-terminal at the N-terminus, but the C-terminus of each is unique (Steward *et al.*, 1993). Based on nucleotide sequence analysis, the P protein comprises 395 amino acids (aa) (McGinnes *et al.*, 1988). The precise role is not known but in association with L and NP proteins it forms an active complex, viz. the ribonucleoprotein (RNP) complex which is involved in genomic replication and transcription (Mebatsion *et al.*, 2002). In other paramyxoviruses, the phosphorylated P protein is a modulator component that plays a central role in all RNA synthesis (Lamb and Kolakofsky, 1996).

The L protein is the largest structural protein of NDV, comprising 2 204 aa with a calculated MW of 249 kilodalton (kDa). L and P are involved in the viral RNA synthesis (Mebatsion *et al.*, 2002).

The M protein is involved in intracellular transport and assembly of the NDV components (Coleman and Peeples, 1993). The M genes of many NDV strains have been sequenced and its translated product is 364 aa / MW 40kDa in size. It plays an important role in the assembly of the virus by interacting with the nucleocapsid, the lipid bilayer and also the regions of the surface of the membrane. The domains on the M proteins that are involved in the binding with the three macromolecules have yet to be delineated (Yusoff and Wen, 2001).

The ability to bind a sialic acid containing-receptor is one of the most important functions of the HN protein and plays a key role in the initial steps of the NDV infection cycle (Huang *et al.*, 2004). The HN protein is comprised of 2 000 nt that encode an open reading frame (ORF) of 571, 577, 581 or 616 aa. $HN_0$616, which could be observed in several lentogenic strains, can be converted to a biologically active HN protein by proteolytic cleavage of 45 residues from the C-terminus of the precursor HN_0. The other translations 571, 577 and 581 are already in their active form and are usually found in the virulent strains (Sagaguchi *et al.*, 1989).

The F protein contains a major host molecular marker for virulence (Otim *et al.*, 2004). It mediates fusion of the viral and cellular membranes. Synthesised as an inactive precursor, F_0 is a 553aa protein with a molecular weight of 55kDa. Usually the amino acid consensus is^{112}R/K-R-Q-R/K-R-F^{117} for velogenic strains and ^{112}G/E-K/R-Q-G/E-R-L^{117} for lentogenic strains (Collins *et al.*, 1993; Pritzer *et al.*, 1990). The amino acid sequence from aa 112 to 117 is proteolitically cleaved to generate two disulphide linked polypeptides, F_1 (48 to 54kDa) and F_2 (10 to 16kDa) by cellular proteases. The cleavability of F_0 is a major determinant for virulence *in vivo* (Aldous, 2001; Kim *et al.*, 2007; Liu *et al.*, 2003 Peroulis-Kourtis *et al.*, 2002; Singh *et al.*, 2005).

2.2.1 Physico-chemical properties

The infectivity of NDV is destroyed by heat, irradiation, pH and the action of several chemical agents. However, the rate at which the virus is destroyed depends on the virus strain, the duration of exposure, viral quantity and the medium of suspension (Alexander and Senne, 2008).

2.2.2 Biological properties

2.2.2.1 Haemaglutination activity (HA): The ability of NDV isolates to agglutinate red blood cells (RBCs) principally due to the binding of the HN protein on the virus to the receptors on the surface of the RBCs. This property is the principle behind HI test in the diagnosis of ND. Normally chicken RBCs are used in HA, but the virus agglutinates RBCs of all amphibians, reptiles and avian cells. Human, mouse and Guinea pig RBCs

were agglutinated by all NDV strains, whereas cattle, sheep, goat, swine and horse RBC agglutination varies with the strain of NDV (Alexander and Senne, 2008).

2.2.3 Virion structure

Under electron microscopic examination, NDV virions appear pleomorphic, most of which are roughly spherical with diameters around 100 to 500nm. Occasionally filamentous particles of approximately 100nm in length can be seen. The virion is enveloped with a lipid bilayer membrane (Yusoff and Wen, 2001). Embedded in the envelope are the glycoproteins HN and F which appear as tiny spike projections from the external surface of the membrane (Figure 2.1). The HN and F surface glycoproteins are the principal antigens that elicit a protective immune response (Seal *et al*., 2000).

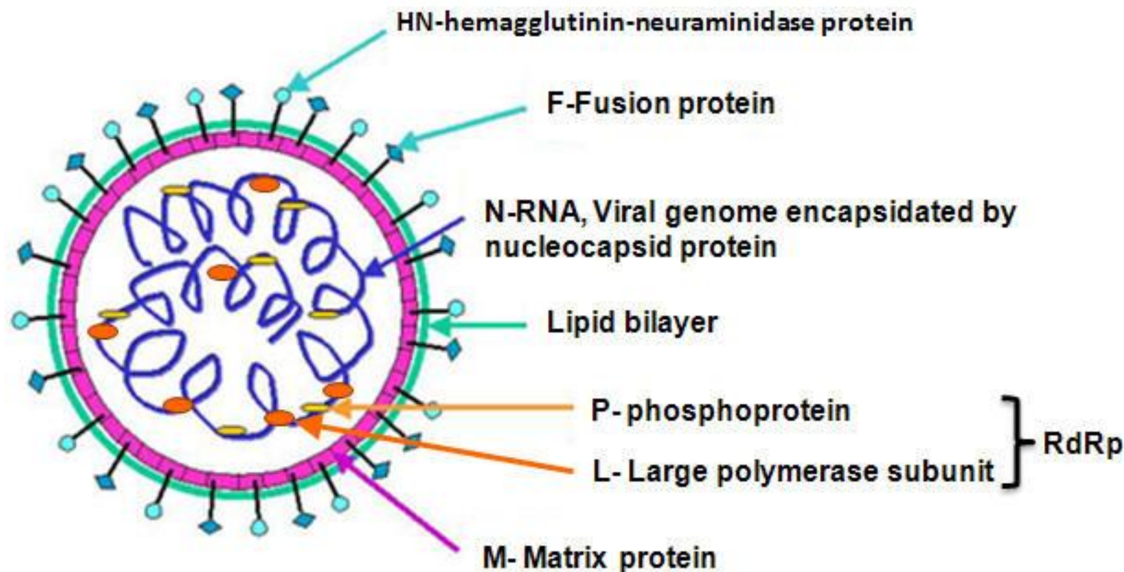

Figure 2.1: Newcastle disease virus structure

2.2.5 Association of Newcastle disease virus with cells

2.2.5.1 Newcastle disease virus adsorption and fusion

ND requires type specific HN-F interactions that must be present in the same bilayer to induce fusion (Lamb and Kolakofsky, 2002). HN mediates the attachment of the virus to the host cell receptor, whereas the F is responsible for virus penetration into the cell, virus-induced cell fusion and haemolysis (Toyoda *et al*., 1987). Gravel and Morrison

(2003) suggested that F and HN hold each other in a switched-off state and that the globular head region of HN from 124 to 151 binds to the membrane-proximal HRB region of F. When HN binds to sialic acid receptors, the association of the HN dimer is changed, which promotes F into its fusogenic state, sending its fusion peptide into the cell membrane.

The HA activity of the HN protein is caused by the adsorption of the virus specific receptors on the red blood cells to form a lattice network between the cells (Kimball, 1990). The HN hydrolyses the ketocidic bond between substituted neuraminic acids on the host receptors allowing the membranes to come closer together. This interaction of the HN with the cellular receptors induces conformational changes in the HN protein that activates the F protein which in turn makes contact with the host membrane, allowing the virus to penetrate the cell surface (Lamb and Kolakofsky, 1996; Stone and Morrison, 1997).

Following fusion, the M proteins underneath the membrane become dissociated from the nucleocapsid through an unknown mechanism, thereby releasing the nucleocapsid directly into the cytoplasm to begin viral transcription and genome replication, as well as the viral protein synthesis and processing (Yusoff and Wen, 2001)

2.2.5.2 Transcription and replication

The active transcriptase complex comprises of the NP, P and L proteins, as well as the encapsidated genomic RNA (Hamaguchi *et al.,* 1983). The P and L proteins act as the viral RNA-dependent RNA polymerase, transcribing the negative-encapsidated genomic RNA to produce the sub genomic mRNAs; 5' capped and 3' polyadenylated mRNAs that are required for the synthesis of the viral proteins (Figure 2.2). The viral RNAp begins all RNA synthesis at the 3' end of the genome. The genes are transcribed into mRNAs in a sequential manner. Genomic replication occurs by synthesis of a full length positive RNA molecule which in turn functions as a template for the production of negative genome RNA (Yusoff and Wen, 2001).

As infection progresses, and viral proteins accumulate, RNA synthesis switches from transcription to replication. The switch is mediated by binding of the NP monomers to

the growing RNA chain. The new NP monomers are recruited by the RNAp complex and added in sequence to the growing RNA chain that causes the polymerase to ignore the transcriptional start and stop codon signals, resulting in the production of full-length positive RNA.

Studies have shown that NDV replication follows the ''rule of six'' in which the length of the viral genome is an exact multiple of six and this relates to the fact that each NP monomer associates exactly with 6 nucleotides of the genomic RNA (Egelman *et al.*, 1989).

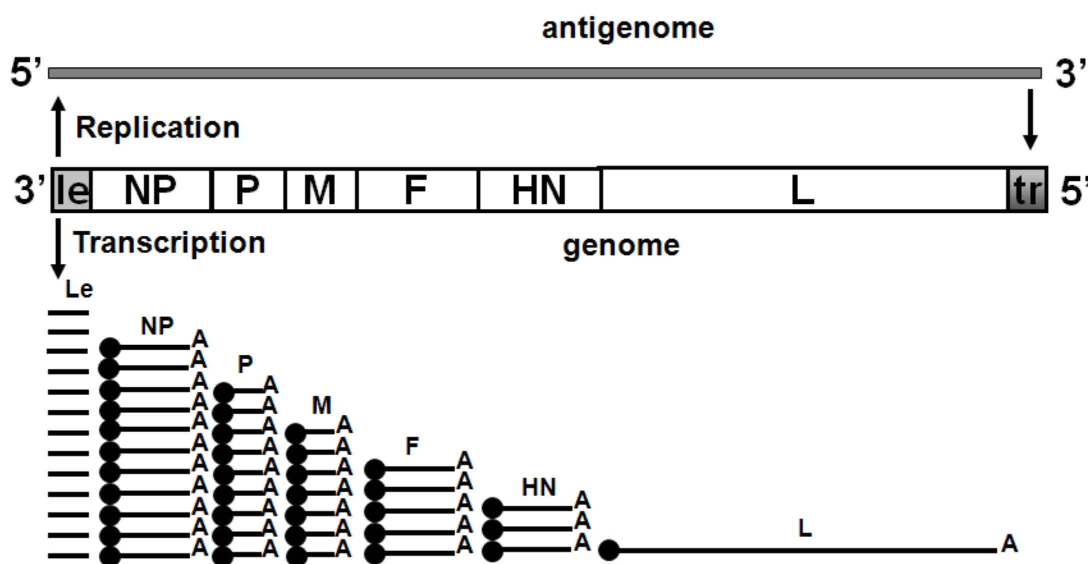

Figure 2.2: Newcastle disease virus transcription and replication.

Source: http://fst.osu.edu/li/research.htm

2.3 Virus characterization and strains

2.3.1 Biological characterization

Newcastle disease virus is categorized into lentogenic, mesogenic and velogenic strains on the basis of pathogenesis and virulence.The pathogenicity of newly isolated viruses can be assessed as follows:

2.3.1.1 Mean Death Time (MDT) in chicken embryos

This is an *in ovo* estimation of virulence, where the mean time for the minimum lethal dose to kill all the inoculated chicken embryos is calculated in hours. Based on the MDT test, NDVs have been classified into velogenic (taking under 60 hours to kill); mesogenic (taking 60 to 90 hours to kill); and lentogenic (taking more than 90 hours to kill) OIE, 2009.

2.3.1.2 Intracerebral Pathogenicity Index (ICPI)

Here, virus rich fresh allantoic fluid is inoculated into the brain of ten day-old chicks from a specific pathogen-free (SPF) flock. The birds are examined at 24-hour intervals for eight days and graded zero if normal, one if sick and two if dead. The index is the mean score per bird per observation over the ten day period. The most virulent viruses give ICPI values approaching the maximum score of 2.0, while lentogenic viruses give values of, or close to 0.0 (OIE, 2009).

2.3.1.3 Intravenous Pathogenicity Index (IVPI)

Fresh infective allantoic fluid with HA titre of $>2^4$ is diluted 1:10 in sterile isotonic saline and 0.1ml of the diluted virus is injected intravenously into 10 6-week-old SPF chickens. Birds are examined at 24-hour interval for 10 days and scored at each observation; 0 if normal, 1 if sick, 2 if paralyzed or showing other nervous signs and 3 if dead. The mean IVPI is the score per bird per observation over 10-day period. Lentogenic strains and some mesogenic strains will have IVPI values of 0, whereas the indices for virulent strains will approach 3.0 (OIE, 2009)

2.3.2 Molecular basis/determinant of virulence

Sequence analysis of the F protein cleavage site can be used to predict potential pathogenicity of NDV in addition to conventional methods such as MDT and ICPI (Panda *et al.*, 2004). The primary molecular determinant for NDV pathogenicity is the amino acid sequence at the Fusion protein cleavage site (F_0) and the ease with which cellular proteases cleave the fusion protein (de Leeuw *et al.*, 2005; Gotoh *et al.*, 1992; Nagai *et al.*, 1976).

The F glycoprotein is synthesized as an inactive precursor F_0, this precursor is proteolytically cleaved at the peptide bond between residues 116 and 117, producing two active polypeptides F_1 and F_2, which are linked by disulphide bonds (Liu *et al.*, 2003). Virulent NDV isolates have dibasic amino acids flanking a glutamine residue (^{112}RRQR/KR^{116}) at the carboxyl terminus of the F_1 polypeptide. Avirulent isolates have a neutral amino acid in the place of basic arginine residues (^{112}GR/KQG/SR^{116}). Furthermore, the amino acid at position 117 of the F_2 polypeptide N-terminus is phenylalanine or leucine residue in virulent and avirulent NDV strains respectively (Peroulis-Kourtis *et al.,* 2002).

2.4 Phylogenetic studies

Molecular phylogenetics converts the information in sequence data into an evolutionary tree based on the similarities or differences between sequences. The main aim of phlylogenetic reconstruction is to describe evolutionary relationships in terms of relative recency of common ancestry, these relationships are represented as a branching tree with the branches joined by nodes and leading to terminals at the tips (Harrison and Langdale 2006). The techniques of analysis are based on distance, character (parsimony) and likelihood.

The distance method consists of two discrete steps; viz. conversion of aligned sequences into pair wise distance matrices, which are then used to generate a tree with the use of a clustering algorithm. These clustering algorithms include the unweighted pair-group method with Arithmetic mean (UPGMA) and neighbour joining (NJ) and minimum evolution. NJ is a bottom–up clustering method used constructing phylogenetic trees. The principle behind this method is to find pairs of operational taxonomic units OTUs (neighbours) that minimise the total branch length at each stage of the clustering of OTUs (Saitou and Neil 1987). It is fast, statistically consistent and suitable for data sets comprising of lineages of largely varying rates of evolution. It however, gives only one possible tree with reduced sequence information.

The character-based methods maximum parsimony is a non–parametric statistical method which operates by evaluating candidate phylogenetic trees according to an

explicit optimality criterion. The preferred tree is the one that requires the least (the most parsimonious) evolutionary change to explain some observed data.

Maximum likelihood calculates a likelihood value for each possible tree for a given set of sequences, and selects the tree with the highest likelihood based on a specified model of evolution (Felsentein, 1985). The method allows for an explicit examination of the assumptions made about sequence evolution and produces a number of trees with one as the most likely tree. It is the most computationally demanding phylogenetic analysis technique.

Bayesian inference is a recently introduced likelihood method that use Bayes' theorem to find the posterior probability of a tree given the data (Huelsenbeck *et al.*, 2001). The Bayesian method gives a tree together with the support for the tree.

Bootstrapping is a statistical test which displays the results as a tree and is used to test the reliability of a tree. Based on analyses of known phylogenies of laboratory phage populations, and computer simulations, a grouping is considered robust when it displays a bootstrap value of more than 70% (Hills and Bull 1993).

2.5 Classification of Newcastle disease virus into lineages

Analysis of nucleotide sequences has allowed differentiation of even extremely closely-related viruses resulting in epidemiological evidence of virus origin (Alexander *et al.*, 1999). By restriction fragment length polymorphism analysis and partial nucleotide sequence of the F gene, NDV strains were classified into nine genetic groups (I-IX), with genetic groups VI and VII divided into several subtypes (Ballargy-Pordany *et al.*, 1996; Tsai *et al.*, 2004). Recent genome size analysis as well as sequence of F and L genes revealed two distinct classes; I and II within NDV serotype 1. Classes I and II lentogenic viruses have been recovered primarily from waterfowl, shorebirds and live bird markets (LBMs) (Kim *et al.,* 2007; Seal *et al.*, 2005). Class II velogenic viruses have been isolated mostly from domestic fowl and some wild birds (Aldous *et al.,* 2003; Lee *et al.*, 2009) class II is further characterized into genotype I-IX (Kim *et al.,* 2007).

Aldous *et al.,* (2003) broadly re-classified NDV isolates into 6 (1-6) lineages - these correspond with the existing geno-groups (I-IX) already reported namely: lineage 1(I), 2(II), 4(VI) 5(VII). The study considered genotypes III, IV, V and VIII as sub lineages of lineage 3. Where, 3a, 3b, 3c and 3d correspond to III, IV, V and VIII respectively. (Kim *et al.,* 2007).

Table 2.1: Genetic classification of NDV strains and their corresponding groupings as reported by different authors

Genotypes	**Lineages**	**Classes**
(Ballargy-Pordany *et al.,* 1996; Liu *et al.,* 2003 ; Tsai *et al.*, 2004)	(Aldous *et al.,* 2003: Snoeck *et al.*, 2009)	(Kim *et al.,* 2007)
I	1	II
II	2	II
III	3a	II
IV	3b	II
V	3c	II
VIII	3d	II
VI a – VI d	4a – 4d	II
VII a – VII e	5a – 5e	II
	5f – 5h	II
IX		II
	6	I

2.6 Newcastle disease panzootics

Virulent strains of NDV spread throughout the world via different panzootics. The first panzootic appears to have arisen from South East Asia, starting in the mid-1920s and took 30 years to spread throughout the world. Genotypes II (lineage 2) III (sub-lineage 3a) and IV (sub-lineage 3b) were responsible for this panzootic. The viruses responsible for the second panzootic belonged to genotype V (sub-lineage 3c) and VI (sub-lineage 4a). The pandemic started in the Middle East in the late 1960's; and it spread to most countries by 1973. This panzootic was associated with importation of caged psittacine species (Alexander, 1988; Ballargy-Pordany *et al.,* 1996; Tsai *et al.,* 2004).

The third panzootic, caused by genotype VI (lineage 4), a pigeon paramyxovirus serotype 1 (PPMV-1) is a pigeon-adapted variant of avian paramyxovirus serotype 1. It affects pigeons and doves (*Columbiformes*) and is known to infect poultry (Alexander *et al*., 1985; Pedersen, 2004). It originated from the Middle East and was detected during the 1980s and continues around the world (Alexander, 2001). Racing pigeons and doves kept by fanciers were the primary species involved, with outbreaks in chickens. This outbreak spread through contact between birds at pigeon races, bird shows and through international trade in these species. In Japan, while outbreaks in domestic fowl have been limited and sporadic, outbreaks in pigeons have been on going from 1984 to the present (Mase *et al*., 2002). The NDVs isolated from pigeons from 1984 to 1995 fell into a subgroup of NDV genotype VI. A novel NDV subgroup of genotype VI emerged and has been circulating in pigeons since 1995. Many isolates obtained from pigeons before 1995 have ^{112}GRQKRF^{117} sequences at the cleavage site in the F protein gene, but all isolates obtained from pigeons after 1995 have the ^{112}RRKKRF^{117} sequence, which is typical of virulent strains (Mase *et al*., 2002). Characterization of the pigeon paramyxovirus (PPMV-1) isolated in chickens in South Africa, indicated that the isolate is most closely related to the Japanese strains (Abolnik *et al.,* 2004b).

The fourth panzootic started the early 1990's and continues to date involving Asia, Europe and Africa. Genotype VII (lineage 5) is responsible for this panzootic (Abolnik *et al*., 2004a; Alexander, 2001; Liu *et al*., 2007).

Molecular characterization and phylogenetic analysis of 79 recent NDV isolates from mainland China in 2008 showed that all isolates had the motif ^{112}R-R-Q/R-R-F^{117} at the cleavage site of the fusion protein. For genotyping, a phylogenetic tree based on nucleotides 47-435 of the F gene was constructed and all 79 isolates were divided into genotypes VIId (sub-lineage 5d) and III, most of which were genotype VIId (Liu *et al.*, 2008).

2.7 Newcastle disease virus in Africa

In Africa, ND outbreaks are frequent, widespread and mostly underreported, because the system of animal disease surveillance and reporting is inefficient. However, a study on isolates from the Southern African countries of Zambia, Zimbabwe (1995) and Mozambique (1994-95) revealed the presence of genotypes II, II and VIIb respectively (Aldous *et al.,* 2003). Phylogenetic studies on isolates from South Africa between 1990 and 2002 revealed that velogenic field viruses belonging to genotypes VIII, VIId and VIIb with a striking similarity with viruses from the Far East (Abolnik *et al.,* 2004a).

In a survey of over 300 Newcastle disease viruses from across the world, isolates from Sudan and Ethiopia were assigned into lineages 3b and 1 which correspond to genotypes IV and I respectively (Aldous *et al.,* 2003). In the same study, analysis of a partial nucleotide sequence of the F gene of isolates recovered from ducks and a chicken in Tanzania were found to belong to sub lineages 3c and 4a; corresponding to genotypes V and VIa respectively.

A recent study reported that all strains isolated from the Sudan between 2003 and 2006 were of lineage 5d, which were phylogenetically more related to lineage 5d (genotype VII) viruses from the Middle East than those reported for West and Southern Africa (Hassan *et al.*, 2010).

ND isolates from Eastern Uganda in 2001 were found to be velogenic by biological characterization and fusion protein cleavage site sequence analysis. The ICPI for all 16 isolates was 1.8. Analysis of their hemagglutinin-neuraminidase protein gene sequences revealed a novel genotype (VIa) unrelated to those that caused previous outbreaks in

Uganda. The F protein sequence (^{112}RRQKRF^{117}) around the F_2-F_1 cleavage site of all 16 isolates suggested a high level of virulence for the Ugandan NDV isolates (Otim *et al.,* 2004).

Sequence analysis of the partial F gene sequences of NDV detected from commercial and non commercial farms in the countries of Nigeria, Niger and Burkina-Faso in West Africa revealed that some of the NDVs causing outbreaks in this region were phylogenetically different from the NDVs circulating in the Southern and other parts of Africa (Snoeck *et al*., 2009). A comparative analysis of NDV sequences from West Africa with the representatives of the genetic lineages reported by Aldous *et al*.,(2003) and Ballangi-Pordany *et al.* (1996) indicated that the viruses clustered with representatives of lineages 1, 2, 3, 4 and 5 which correspond to genotypes I, II, III, VI and VII respectively. Results of the study further showed that lineages 1, 2, 3, 4 and 5 were detected in Nigeria (North-West and South-West regions). The deduced amino acid sequences of the F protein cleavage of all lineage 1(GKQGR*L) and lineage 2(GRQGR*L) were of low virulence. While Lineage 3 (RRQKR*F) lineage 4 (RRKKR*F) and lineage 5 (RRQKR*F or RRRKR*F) were of high virulence. Lineages 2 and 3 were found to be vaccine strains while lineages 4 and 5 were reportedly wild- type strains.

The phylogenetically distinct strains were grouped as lineages 5f, 5g and 5h (Snoeck *et al.,* 2009). Another study by Cattoli *et al.* (2009) on the ND viruses causing outbreaks in the Western and Central African countries of Nigeria, Niger, Burkina-Faso, Ivory Coast, Mauritania, Cameroon and Burundi reported that apart from the isolate from Burundi, the majority of NDV isolates circulating in West and Central Africa belonged to a novel lineage which was tentatively named as lineage 7. The finding revealed that the new lineage included those from West Africa reported as sub lineages 5f, 5g and 5h previously by Almeida *et al.* (2009) and Snoeck *et al.* (2009).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Viruses

Forty-one (41) NDV isolates were used for this study (Table 3.1). Of these, 36 were obtained from different bird species from live bird markets (LBMs) in the six geopolitical zones of Nigeria from 2007 to 2008. These samples were taken from apparently healthy, clinically ill and dead birds during active surveillance. The remaining five isolates were obtained from outbreaks in backyard and commercial poultry during routine diagnosis of poultry samples at the Virology department of the National Veterinary Research Institute (NVRI), Vom. Isolation was done by passage in 9 to 11 day old embryonated chicken eggs, and NDV was identified by HA and HI tests, using standard procedures (OIE, 2009).

Table 3.1: list of NDV isolates hosts, regions, lineages and accession numbers (results of this study)

Vom laboratory code	OVI-ARC laboratory code	Virus strain Abbreviation	Host	Year	Location	Lineage	Accession number
KB/KMB/C22-26	NG/CH/01	NGCK0108	Chicken	2008	Kebbi	5	HQ456833
KT/CH/C18-21	NG/CH/02	NGCK0208	Chicken	2008	Katsina	5	HQ456834
VRD/07/676WK	NG/CH/03	NGCK0307	Chicken	2007	Taraba	5	HQ456835
VRD/08/302[A]	NG/CH/04	NGCK0408	Chicken	2008	Akwa-Ibom	5	HQ456836
VRD/08/514[A]	NG/CH/05	NGCK0508	Chicken	2008	Cross-River	5	HQ456837
VRD/08/255	NG/CH/06	NGCK0608	Chicken	2008	Plateau	5	HQ456838
VRD/08/166	NG/CH/07	NGCK0708	Chicken	2008	Plateau	5	HQ456839
VRD/08/201	NG/CH/08	NGCK0808	Chicken	2008	Plateau	5	HQ456840
VRD/08/149	NG/CH/09	NGCK0908	Chicken	2008	Plateau	5	HQ456841
VRD/08/196	NG/CH/10	NGCK1008	Chicken	2008	Plateau	2	HQ456842
VRD/08/316[A]	NG/CH/11	NGCK1108	Chicken	2008	Plateau	5	HQ456843
VRD/08/378[B]	NG/CH/12	NGCK1208	Chicken	2008	Bayelsa	4	HQ456844
KB/KMB/C22-26B	NG/CH/13	NGCK1308	Chicken	2008	Kebbi	5	HQ456845
VRD/07/625	NG/CH/15	NGCK1507	Chicken	2007	Kano	5	HQ456846
VRD/07/640[A]	NG/CH/16	NGCK1607	Chicken	2007	Abuja (FCT)	5	HQ456847
VRD/07/624C	NG/CH/17	NGCK1707	Chicken	200 7	Sokoto	5	HQ456848
VRD/07/644	NG/CH/18	NGCK1807	Chicken	2007	Edo	5	HQ456849
VRD/07/609	NG/CH/19	NGCK1907	Chicken	2007	Ekiti	1	HQ456850
VRD/07/590	NG/CH/20	NGCK2007	Chicken	2007	Zamfara	5	HQ456851
VRD/07/687K-D	NG/CH/21	NGCK2107	Chicken	2007	Nassarawa	5	HQ456852

Vom laboratory code	OVI-ARC laboratory code	Virus strain Abbreviation	Host	Year	Location	Lineage	Accession number
VRD/07/625K-4	NG/CH/22	NGCK2207	Chicken	2007	Kano	5	HQ456853
VRD/07/648A	NG/CH/23	NGCK2307	Chicken	2007	Oyo	5	HQ456854
VRD/07/595	NG/CH/25	NGCK2507	Chicken	2007	Edo	5	HQ456855
VRD/07/97D	NG/CH/28	NGCK2807	Chicken	2007	Kano	5	HQ456856
VRD/07/675FGC	NG/CH/29	NGCK2907	Chicken	2007	Kano	5	HQ456857
VRD/07/675DBC	NG/CH/32	NGCK3207	Chicken	2007	Kano	5	HQ456858
VRD/07/648 AD4	NG/CH/33	NGCK3307	Chicken	2007	Oyo	5	HQ456859
VRD/07/734A	NG/CH/34	NGCK3407	Chicken	2007	Jigawa	5	HQ456860
VRD/07/691A	NG/CH/35	NGCK3507	Chicken	2007	Edo	5	HQ456861
VRD/07/736C	NG/CH/38	NGCK3807	Chicken	2007	Abuja (FCT)	5	HQ456862
VRD/07/649 Y2	NG/CH/40	NGCK4007	Chicken	2007	Yobe	5	HQ456863
VRD/07/687K-C	NG/CH/42	NGCK4207	Chicken	2007	Nassarawa	5	HQ456864
VRD/07/722C	NG/CH/43	NGCK4307	Chicken	2007	Kano	5	HQ456865
VRD/07/732A	NG/CH/44	NGCK4407	Chicken	2007	Lagos	5	HQ456866
VRD/07/649 Y1	NG/CH/45	NGCK4507	Chicken	2007	Yobe	5	HQ456867
VRD/07/697	NG/CH/48	NGCK4807	Chicken	2007	Ebonyi	5	HQ456868
VRD/07/597D3	NG/DK/26	NGDK2607	Duck	2007	Yobe	5	HQ456869
VRD/07/597D1	NG/DK/27	NGDK2707	Duck	2007	Yobe	5	HQ456870
VRD/07/673GR-GF	NG/GF/49	NGGF4907	Guinea-fowl	2007	Niger	5	HQ456871
VRD/07/660A	NG/GF/53	NGGF5307	Guinea-fowl	2007	Zamfara	5	HQ456872
VRD/687K-PG	NG/PG/52	NGPG5207	Pigeon	2007	Nassarawa	5	HQ456873

3.2 Viral RNA extraction

samples were imported to South Africa under the permit no: 13/1/1/30/2/9/6-137(APPENDIX B) and transported in their inactivated form in a MagNaPure lysis buffer (Roche, Mannheim, Germany) to the Ondersteeport Veterinary Institute where the analyses were performed

3.2.1 Robotic extraction

High-throughput RNA extraction was achieved using a MagNA Pure LC total nucleic Acid isolation kit (Roche, Mannheim, Germany) on a MagNA Pure robotic extraction device (Roche, Germany).

3.2.2 Manual extraction

Repeat RNA extractions on some of the inactivated allantoic fluid was done manually using Trizol(R) LS Reagent (Invitrogen Cat No. 10296-028) according to the manufacturer's recommendations.

Pellets were air dried and 40µl of RNase free water was used to reconstitute RNA pellets that were stored at +4°C until used.

3.3 Real-time Reverse Polymerase chain Reaction for influenza A

The VLA TaqMan(R) Influenza A/H5/H7 Detection Kit (850 Lincoln Centre Drive, Foster City, California 94404, USA) was used according to the protocol recommended by the manufacturer on a Step One Plus TM Real-Time PCR thermal cycler (Applied biosystems, 850 Lincoln Centre Drive, Foster City, USA).

3.4 Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

One-step RT-PCR was performed for the amplification of a 1180 base pair (bp) fragment spanning the region between nucleotide 610 of the matrix gene through nucleotide 581 of the fusion gene, including the F_0 cleavage site.

Using a 30µl reaction volume which contained the following components: 200 u/µl M-MLV Reverse Transcriptase (Promega, Madison WI, USA), 5 units TaKaRa Ex Taq,

10X Ex Taq Buffer, 2.5Mm dNTP mixture(TAKARA BIO INC Shiga, Japan), 40 U Ribonuclease Inhibitor (Amersham Biosciences, Uppsala Sweden), distilled water, 20 pmoles of the following oligonucleotides, and 5ul of extracted RNA:

M610 5’-CTGTACAATCTTGCGCTCAATGTC-3’ (forward primer)

NDVF581 5’-CTGCCACTGCTAAGTTGTGATAATCC-3’ (reverse primer)

Reaction mixtures were cycled in a Verity ™ 96-well thermal cycler (Applied Biosystems) as follows:

42 °C for 20 min Reverse transcription

94 °C for 5 minutes Initial denaturation

35 cycles of:

94°C for 30 sec Denaturation

53 °C for 30 sec Annealing

72°C for 1 min Elongation

Products were electrophoresed at 120V for 35 minutes in 1% agarose gel stained with ethidium bromide. Bands were visualised using a gel documentation system (SYNGENE GBOX, United Kingdom) and amplicons of the correct size were excised using a scalpel blade. Gel extraction was performed using a QIAquick Gel extraction kit (Qiagen, Hilden, Germany) and DNA was quantified using a NanoDrop spectrophotometer (Wilmington, DE 19810 USA)

3.5 DNA Sequencing

The quantity of each template used in the sequencing reaction was calculated based on a 40ng total concentration per reaction according to the recommended protocol for Big Dye Terminator sequencing (Applied Biosystems, 52 Rocco Drive Scoresby Australia) of PCR amplicons as follows:

2µl of sequencing buffer to each tube

4µl of sequencing mix each

1µl of reverse primer F581 (3.2 pmol)

40ng of NDV amplicon

Water to a total volume of 20µl

Standard Big Dye thermal cycling conditions were used:

96°C for 1minute	initial denaturation
25 cycles of;	
96°C for 10 seconds	denaturation
50°C for 5 seconds	annealing
60°C for 4 minutes	extension

3.5.1 Sequencing reaction precipitation

50µl of 100% ethanol and 2µl of 3M sodium acetate (NaOAc) were combined in 0.5ml tubes and the total volume of each sequencing reaction was transferred into this. Reactions were incubated at room temperature, in the dark for 10 minutes. The tubes were centrifuged at 13 000rpm for 15 minutes. Using a micropipette, all the fluid was removed; care was taken not to disturb the pellet. 250µl of 70% ethanol was added and centrifuged at maximum speed of 13 000rpm for 15 minutes. All the fluid was removed, and tubes were air- dried for 10 minutes at room temperature.

3.5.2. DNA sequencing reaction

A 10µl of HiDi Formamide (Applied Biosystems) was added to each tube and vortexed briefly. The mixture was re-suspended with a pipette and transferred to the appropriate well in the sequencing plate using the plate layout. The plate was loaded on the Verity ™ 96-well thermal cycler (Applied Biosystems) and denatured at 96°C for 2 minutes then cooled at +4°C. Reactions were analysed on a 3130 Genetic Analyser (Applied Biosystems).

3.5.3 Sequence Editing

Nucleotide sequence editing was conducted with BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) and Chromas Lite software (http://www.technelysium.com.au). Briefly, sequences were checked for basecalling, and edited for length prior to export in FASTA format.

3.5.4 Alignment

Blast homology searches (http://www.ncbi.nlm.nih.gov/blast) of the 314 bp region were done to identify closely related sequences, to be included in multiple sequence alignments of the 314-nucleotide sequence from this study. Representatives of lineages 1 to 6 (Aldous *et al.,* 2003) and sub lineages of lineage 5 (Almeida *et al*., 2009; Cattoli *et al*., 2009; Snoeck *et al*., 2009) were also retrieved for comparison.

Multiple sequence alignments were prepared using ClustalW software (http://www.ebi.ac.uk/clustalw/index.html).

The preliminary phylogenetic trees were reconstructed using the Unweighted-pair group method with arithmetic mean algorithm (UPGMA), Minimum Evolution (ME) and

Maximum Parsimony (MEGA v4.1 software, Tamura *et al*., 2007). The Neighbour joining tree was selected as the most suitable of these for use as the final tree. The robustness of the groupings was assessed by bootstrap resampling of 1 000 replicates. A distance matrix was generated using the maximum composite likelihood method.

CHAPTER FOUR: RESULTS

All NDV isolates (n=41) were tested by real time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) for the presence of influenza A RNA. Results revealed that all the NDV isolates used in this study were free of co-infection with influenza A strains.

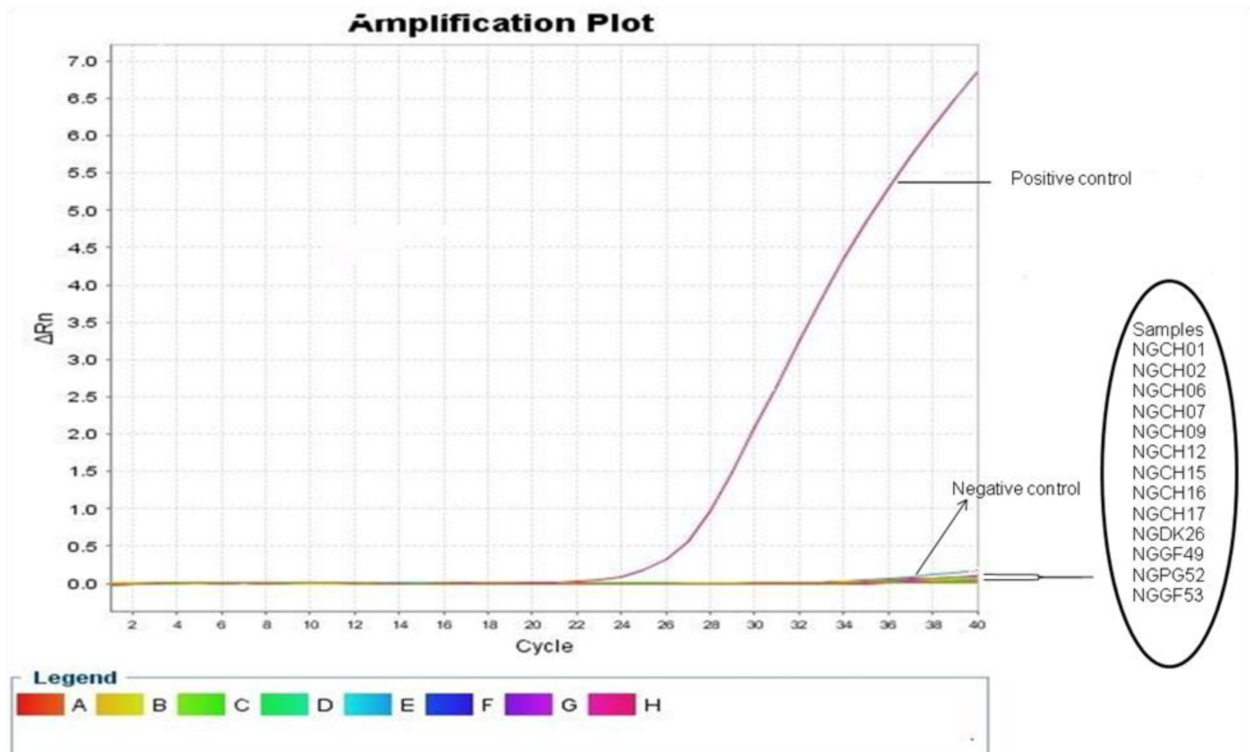

Figure 4.1: Example of a fluorescent amplification plot of AIV rRT-PCR performed on samples used in this study. The legend represents the multicomponent dyes for the detection of the target samples and the controls of the assay

The test was successful as indicated by the positive control that had a Ct value of 22. The negative control (water) and all the Nigerian samples yielded no amplification, indicating a negative result. Evaluation of the internal positive control (IPC) curves (data not shown) provided confirmation that no inhibitors were present in the extracted RNA samples.

A 1 181 base pair (bp) DNA fragment spanning position 610 of the matrix gene to position of 581 of the fusion gene, including the F_0 cleavage site, was amplified for each of the 41 isolates. The PCR gel photo of selected isolates is shown in Figure 4. 2

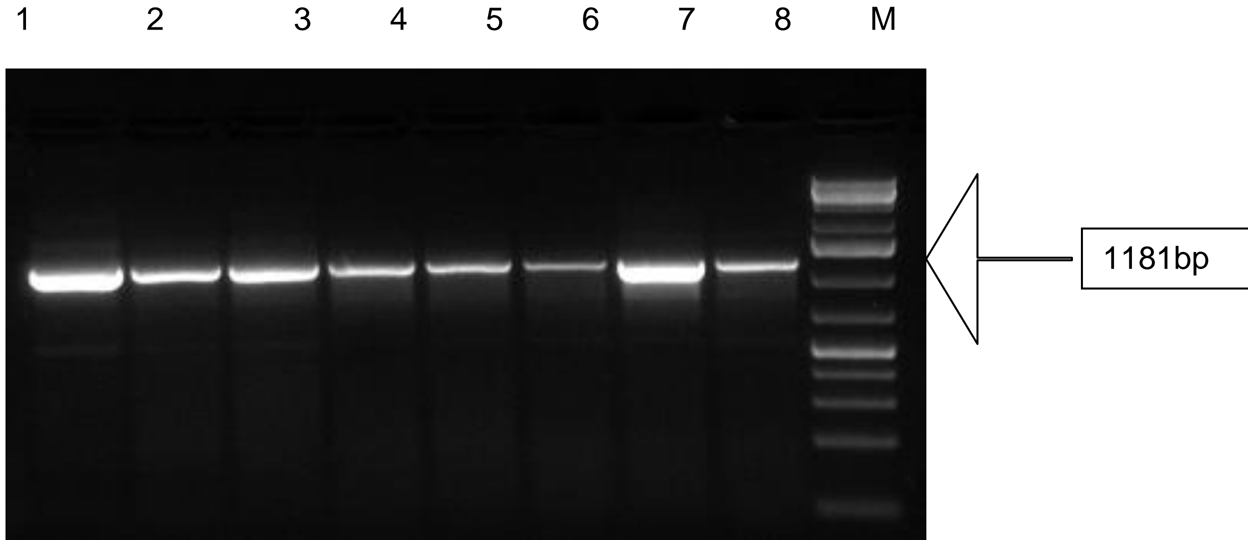

Figure 4.2: Agarose gel electrophoresis of the 1181bp targeted PCR product of NDV isolates (lanes 1-8) from Nigeria characterized in this study. “M” indicates a 500bp DNA molecular weight marker.

4.1 Analysis of the amino acid sequence at the F_o proteolytic cleavage site

DNA was sequenced and translated to protein sequences. Analysis of the amino acid residues (21 to 124) of the 41 NDV strains together with those of reference strains was done (Figures 4.13, 4.14, 4.15 and 4.16). The deduced amino acid motif at the F gene cleavage site was analysed to determine the pathotypes, revealing that, 39 of the viruses contained a pair of dibasic amino acid at the cleavage site, characteristic of virulent strains. Two isolates (NGCK1907 and NGCK1008) had monobasic amino acids sequences, characteristic of lentogenic strains. Of the 39 viruses characterised as virulent, 26 exhibited the sequence motif ^{112}RRQKR^{116}-F and 13 exhibited the motif ^{112}RRRKR^{116}- F (Table 4.1). Of the 2 lentogenic strains, NGCK1907 exhibited the sequence motif ^{112}GRQGR^{116}-L and NGCK1008 contained the sequence motif ^{112}GKQGR^{116}- L (Table 4.1).

Table 4.1: Proteolytic cleavage sequence, strain and lineage designation of viruses analysed in the study

Isolate name	Fusion gene cleavage motif	Virus strain	Lineage
NGCK1507	RRRKRF	Virulent	5
NGCK1607	RRQKRF	Virulent	5
NGCK1707	RRQKRF	Virulent	5
NGCK1807	RRRKRF	Virulent	5
NGCK1907	GKQGRL	Avirulent	1
NGCK2007	RRRKRF	Virulent	5
NGCK2107	RRQKRF	Virulent	5
NGCK2207	RRQKRF	Virulent	5
NGCK2307	RRQKRF	Virulent	5
NGCK2507	RRRKRF	Virulent	5
NGDK2607	RRQKRF	Virulent	5
NGDK2707	RRQKRF	Virulent	5
NGCK2807	RRQKRF	Virulent	5
NGCK2907	RRQKRF	Virulent	5
NGCK3207	RRRKRF	Virulent	5
NGCK3307	RRRKRF	Virulent	5
NGCK3407	RRRKRF	Virulent	5
NGCK3807	RRQKRF	Virulent	5
NGCK4007	RRQKRF	Virulent	5
NGCK4207	RRQKRF	Virulent	5
NGCK4307	RRQKRF	Virulent	5
NGCK4407	RRQKRF	Virulent	5

NGCK4507	RRRKRF	Virulent	5
NGCK4807	RRRKRF	Virulent	5
NGGF4907	RRQKRF	Virulent	5
NGPG5207	RRQKRF	Virulent	5
NGGF5307	RRQKRF	Virulent	5
NGCK0108	RRQKRF	Virulent	5
NGCK0208	RRRKRF	Virulent	5
NGCK0308	RRRKRF	Virulent	5
NGCK0408	RRRKRF	Virulent	5
NGCK0508	RRQKRF	Virulent	5
NGCK0608	RRQKRF	Virulent	5
NGCK0708	RRQKRF	Virulent	5
NGCK0808	RRQKRF	Virulent	5
NGCK1008	GRQGRL	A virulent	2
NGCK1108	RRQKRF	Virulent	5
NGCK1208	RRQKRF	Virulent	4
NGCK1308	RRQKRF	Virulent	5

4.2 Phylogenetic analysis

Phylogenetic analysis of the partial F gene nucleotide sequence (nt 61 to 374) including the F_0 cleavage site of 41 isolates was done to assess genetic relatedness. They were compared with the corresponding sequences of reference strains using Mega v4.1 software (Tamura *et al.*, 2007). Based on the topology of the tree, the 41 isolates in this study were grouped with representatives of lineages 1, 2, 4, and 5 (Figure not shown).

To relate the origin and allow for inferences about each isolate, separate Neighbour Joining trees were generated for all the lineages identified in this study with corresponding reference strains.

Lineage 1

One virus' sequence (NGCK1907), recovered from a chicken, clustered with representatives of lineage 1 including the avirulent I-2 vaccine strain, strain FM200800 isolated from a chicken in Nigeria in 2005 and strain AF503639 recovered from a Shelduck in France in 1976 (Figure 4.3). Three other strains from the republic of South Africa, viz AF532741ZA341/P/99, AF532147 ZA348/B/00 and AF532743 ZA37401/X01 recovered in 1999, 2000 and 2001 respectively also clustered with the aforementioned.

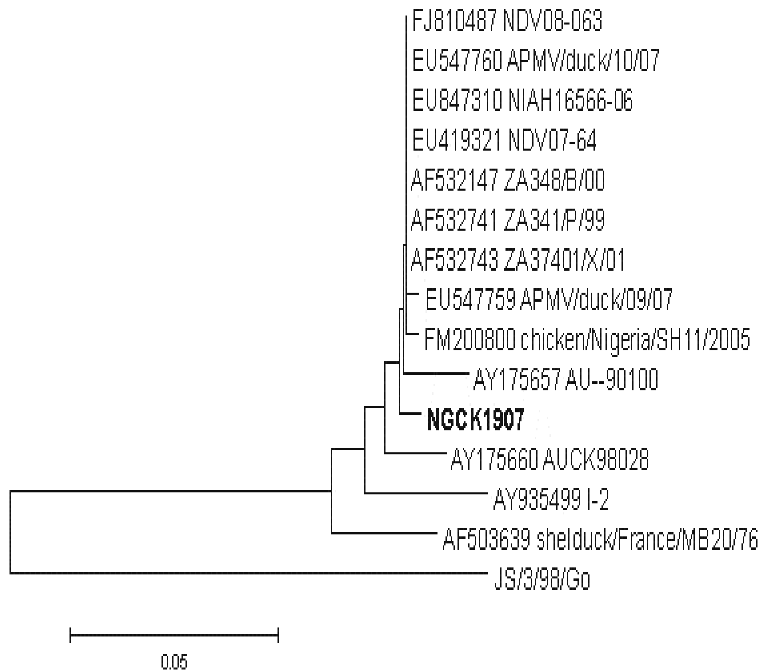

Figure 4.3: Neighbour joining tree based on a 314-nt region of the fusion gene for lineage 1 strains with the Nigerian strain from this study indicated in boldface.

Lineage 2

NGCK1008, isolated from a commercial farm in Jos, clustered with representatives of lineage 2 that includes the widely-used LaSota vaccine strain (Figure 4.4). Sequence identity 100% across the region analysed.

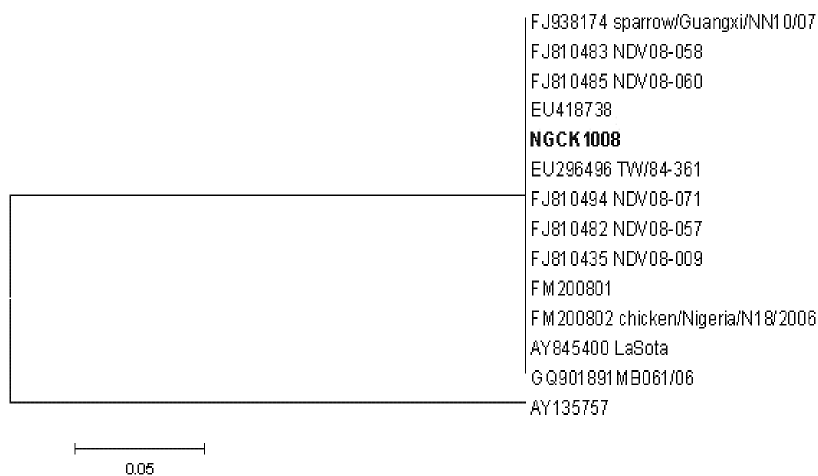

Figure 4.4: Neighbour joining tree based on a 314-nt region of the fusion gene for lineage 2 strains, with the Nigerian strains indicated in boldface

Lineage 4

NGCK1208, isolated from a local chicken in Bayelsa state, clustered with representatives of lineage 4 (Figure 4.5). This sequence differs by only 6nt across the region analysed from strain DQ202263, isolated from a grouse in Russia in 2004, compared to a 12nt difference from strain FM200798, recovered from a parrot in Nigeria in 2007 (nucleotide alignments in Figure 4.11)

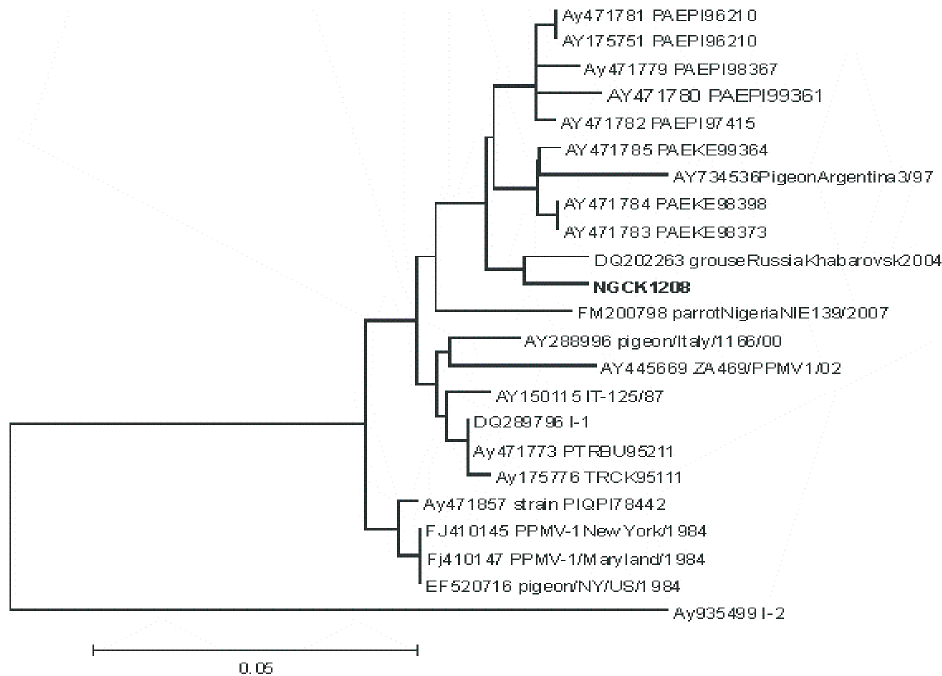

Figure 4.5: Neighbour joining tree based on a 314-nt region of the fusion gene of lineage 4 strains with the Nigerian strain indicated in boldface.

Lineage 5

Lineage 5 sequences displayed marked heterogeneity with clusters that correspond to sub-lineages as defined by (Cattoli *et al*., 2009 and Snoeck *et al*., 2009)

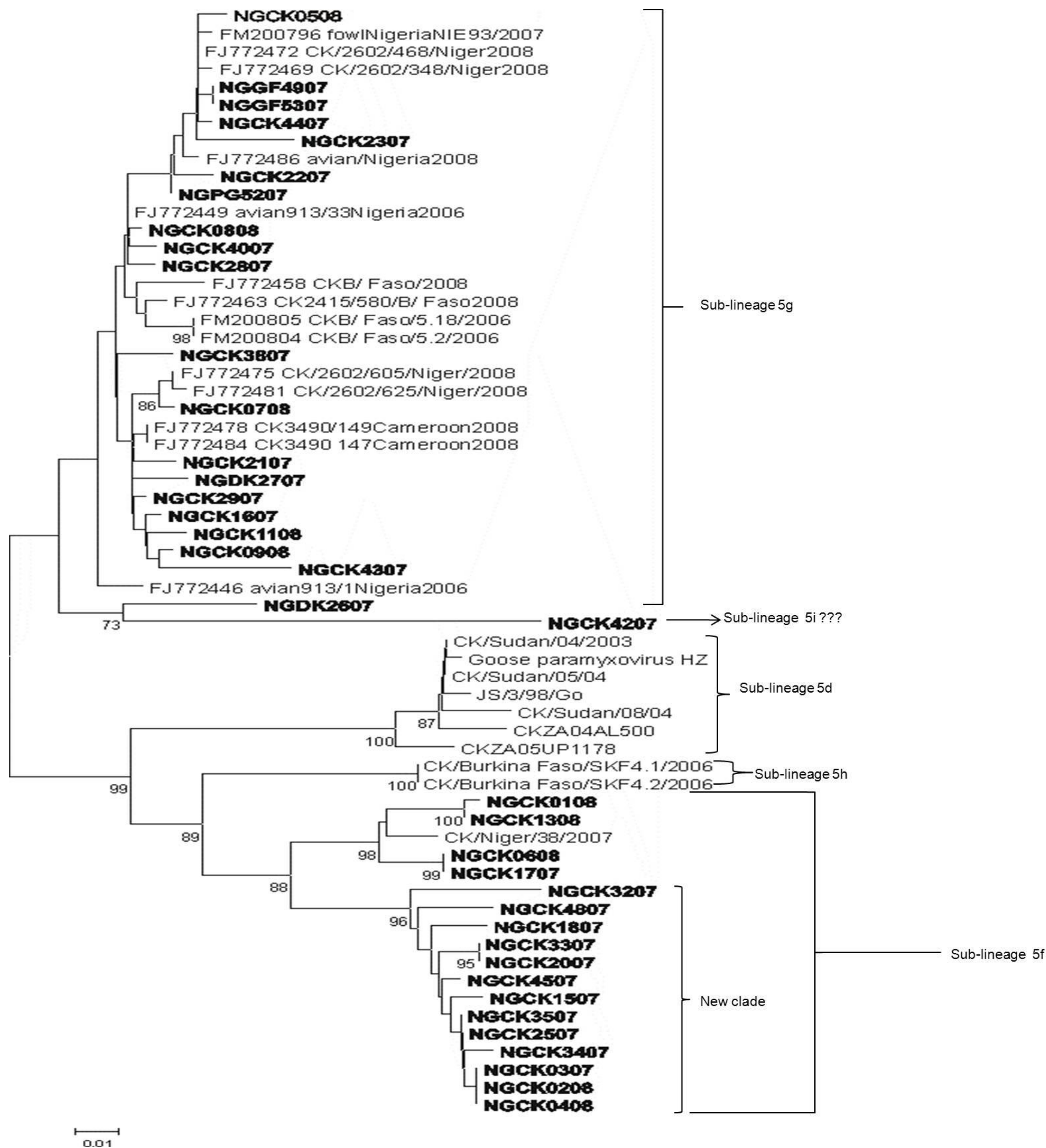

Figure 4.6: Neighbour joining tree based on a 314-nt region of the fusion gene for lineage 5 strains, with Nigerian strains from this study indicated in boldface.

Thirty-eight sequences, viz. NGCK0108, NGCK0608, NGCK1308, NGCK1707, NGCK0208, NGCK0308, NGCK0408, NGCK1507, NGCK2507, NGCK1807, NGCK2007, NGCK3207, NGCK3307, NGCK3407, NGCK3507, NGCK4507, NGCK4807 NGDK2907, NGCK4207, NGCK0508, NGCK4407, NGGF4907, NGGF5307, NGCK2207, NGPG5207, NGCK2307, NGCK2807, NGCK4007, NGCK0808, NGCK0908, NGCK4307, NGCK1607, NGCK1108, NGCK2107, NGDK2707, NGCK2907, NGCK0708 and NGCK3807 clustered with representatives of lineage 5, previously reported by Snoeck *et al*. (2009), and representatives of those recently grouped tentatively as lineage 7 by Cattoli *et al.* (2009). These Nigerian lineage 5 strains were distinctively different from other lineage 5 strains reported on the African continent (e.g. sub-lineage 5d reported in Southern Africa and Sudan) and other parts of the world (Figure. 4.6).

Seventeen strains, viz. NGCK0108, NGCK0608, NGCK1308 NGCK1707 NGCK0208, NGCK0308, NGCK0408, NGCK1507, NGCK2507, NGCK1807, NGCK2007, NGCK3207, NGCK3307, NGCK3407, NGCK3507, NGCK4507 and NGCK4807 grouped with a Niger Republic strain (chicken/Niger/38/2007) reported by Snoeck *et al* as lineage 5f. Four viruses from this group clustered with the Niger strain with a high bootstrap value of 98%. The second major cluster in this sub-lineage was composed of 13 strains from the current study that cluster apart. (Figure 4.6)

The rest of the virus sequences in this study, namely NGCK0508, NGCK4407, NGGF4907, NGGF5307, NGCK2207, NGPG5207, NGCK2307, NGCK2807, NGCK4007, NGCK0808, NGCK0908, NGCK4307, NGCK1607, NGCK1108, NGCK2107, NGDK2707, NGCK2907, NGCK0708, NGCK3807 and NGDK2607 grouped with strains previously reported from Nigeria, Niger, Burkina-Faso and Cameroon as sub-lineage 5g.

Sequence NGCK4207 which clustered with NGDK2607 did not show as close homology with the majority of the viruses in this study nor the ones earlier reported for the sub-region (Figure 4.15)

None of the sequences in this study clustered with the lineage 5h strains from Burkina-Faso.

4.3 Genetic distance similarities and divergence

Based on the nucleotide sequence alignment, genetic distance analysis, that included pairwise distance comparison of the nucleotide sequence using the maximum composite likelihood method, was computed for lineages, sub-lineages and clusters within sub-lineages (APPENDIX C).

Lineage 1

The mean divergence in this group was 1.8% with a maximum divergence of 4.3% between NGCK1907 and a strain recovered from a Shelduck in France in 1976 (AF503639). The lineage 1 strain identified in this study had 96% similarity with the avirulent I-2 vaccine strain (AY935499 I-2) and a 99% sequence homology, differing only by three nucleotides, with strain FM200800 that was recovered from a Nigerian chicken in 2005.

Lineage 2

Pairwise distance comparison revealed 100% homology between NGCK1008 and the reference strains, including LaSota/46 strain (AY84500).

Lineage 4

The mean distance similarity of NGCK1208's sequence with the reference strains was 96.9%, with mean difference of 3.1%. NGCK1208 shared 98.1% similarity with strain DQ202263, isolated from a grouse in Russia in 2004; however it had a divergence of 4.0% with strain FM200798 that was recovered from a parrot in Nigeria in 2007. The aforementioned strain furthermore shared a 95% similarity with AY288996, isolated from a pigeon in Italy in 2000. The highest divergence with the reference strains was 4.3%, viz. between NGCK1208 and two pigeon isolates from New York, USA (FJ410145 and EF520716), isolated in 1984.

Lineage 5

The mean sequence similarity within the strains that grouped with sub lineage 5f was 93.7%. The first cluster within this sub-lineage which included NGCK0108, NGCK0608, NGCK1308, NGCK1707 and chicken/Niger/38/2007 had a mean genetic similarity of 98.5%. The mean similarity within the second cluster, which included 13 strains from this study, was 97%. The strains NGCKO108 and NGCK1308, recovered from local chickens within the same epidemiological unit in Kamba, Kebbi state had 99.7% similarity, whereas NGCK1707, isolated from a chicken in Sokoto state (North West), and NGCK0608, recovered from an outbreak in a commercial farm in Jos, Plateau state (North Central) had a sequence homology of 100%. Three sequences, viz. NGCK0208, NGCK0308 and NGCK0408, collected from different geopolitical zones, had a sequence homology of 100%. Similarity between virus strain NGCK1707 from Sokoto and NGCK0108 from Kebbi was 96%. Both NGCK1707 and NCK0108 were 97.4% similar when compared to the strain (chicken/Niger/38/2007), from the Republic of Niger.

The sequences belonging to sub-lineage 5g had mean similarity of 97.1%. The highest divergence in this group was 7.8% between NGCK4307 and NGCK2307. Three of the isolates recovered from outbreaks in backyard and commercial farms grouped with this sub-lineage. NGCK1607, isolated from a local chicken in a live bird market in Abuja, the Federal Capital Territory (FCT), shared 99% sequence homology with NGCK0908 that was recovered from an outbreak in a commercial farm in Bukuru; an urban settlement in Jos, Plateau, central Nigeria. NGCK2807, isolated from a chicken in Kano State (North-West), differed by 1% from NGCK0808, recovered from an outbreak in a backyard farm in Jos city, central Nigeria. Another strain, NGCK2907, isolated from a chicken in Kano shared sequence homology with strains from other northern zones; having 98.7% similarity to NGCK0708 from a commercial in Jos, 98.4% similarity with NGCK3807 and NGCK1108 from Abuja (FCT) and Gombe respectively.

Strain NGCK0508, isolated from a chicken in Calabar, Cross river state in the South South zone shared 99% homology with two strains (NGGF5307 and NGGF4907)

recovered from Guinea fowls in the Northern states of Zamfara and Nassarawa respectively (Figure 4.9).

4.4 Deduced amino acid residue substitutions

The deduced amino acid sequences (residue 21 to 124) of the F gene for the 41 strains analyzed in this study, including those of the reference strains were compared and several characteristic amino acids substitutions were identified.

Lineage 1 strain NGCK1907 had an $A \rightarrow T^{29}$ and $R \rightarrow G^{112}$ substitution when compared to the I-2 vaccine strain (AY93599) and an $E \rightarrow G^{105}$ substitution compared to Nigerian strain FM200800 (Figure 4.17).

Lineage 2 strain NGCK1008 had 100% homology with all reference strains across the region examined, including the LaSota vaccine strain. (Figure 4.16)

The lineage 4 strain (NGCK1208) identified in this study (Figure 4.15) had a $T \rightarrow I^{26}$ substitution in comparison with strain DQ202263 isolated from a grouse in Russia (as did all other sequences in the multiple alignment), but contained a common mutation with the latter virus ($N \rightarrow S^{30}$) that was not shared with other strains.

All the lineage 5 strains in this study (n=38, Fig 4.14) shared unique $N \rightarrow K^{51}$ and $I \rightarrow V^{44}$ substitutions with sub lineages 5g, 5f and 5h reported for West Africa when compared with lineage 5d reported to be circulating in South Africa, Sudan and other parts of the world. NGCK0508 had a unique $D \rightarrow E^{47}$ substitution, NGCK1807 had a unique $L \rightarrow F^{37}$ substitution, and NGCK1607 a $V \rightarrow I^{43}$ substitution.

NGCK3207 had a unique $M \rightarrow A^{26}$ substitution and shared an $A \rightarrow G^{39}$ substitution with a Sudanese strain (chicken/Sudan/08/04). Virus strains NGCK0208, NGCK0308 and NGCK0408 shared a unique $L \rightarrow P^{23}$ substitution. NGCK2107 had a unique $A \rightarrow Y^{120}$ substitution, and NGDK2707 had a unique $G \rightarrow E^{104}$ substitution (Figure 4.14). Strain NGCK4807, which grouped with sub-lineage 5f, shared a unique $T \rightarrow A^{106}$ residue with two Cameroonian chicken strains in sub-lineage 5g.

Nineteen of the strains analysed in this study that grouped within sub-lineage 5g shared a unique R→H^{100} substitution with representatives of sub-lineage 5g isolated in other West African countries, which distinguished them from sub-lineages 5f and 5h.

All the lineage 5 strains in this study, together with the reference strains, differed at the amino acid level from the two representatives of lineage 5h from Burkina-Faso by a P→S^{36} residue, except NGCK3207 and NGCK4207 which had a P→L^{36} substitution. Strain NGCK1707 that was isolated from a live bird market in Sokoto State shared a unique I→V^{52} substitution with NGCK0608, recovered from a commercial farm in Jos, central Nigeria. NGCK1108, NGCK2107, NGCK1607, NGCK2907, NGDK2707 and NGCK4307 all isolated from LBMs shared a T→A^{93} substitution with strains NGCK0708 and NGCK0908 that were recovered from commercial farms in Jos. NGCK2807 had a unique T→A^{89} substitution.

The virus strains NGCK0108, NGCKO608, NGCK1308 and NGCK1707 that clustered with chicken/Niger/38/2007 shared a unique M→L^{22} substitution with the Niger strain. NGCK0608 and NGCK1707 shared a I→V^{52} substitution with lineage 5d from South Africa, Sudan and the strain, chicken Burkina-Faso FJ777458. Residues 80 to 90 appear to be conserved for all the lineage 5 strains in this study (including the reference strains) except for strain NGCK2807 which has a unique T^{89}→A substitution.

Although Sequence NGCK4207 is basal to sub lineage 5g in the phylogeny, it had some unique amino acid substitutions, particularly in the stretch of amino acids at positions 58 to 66 which is completely conserved across all the other strains used in the alignment.
In addition NGCK4207 did not share the R→H^{100} substitution that distinguished the sub-lineage 5g from sub lineage 5f and 5h. Furthermore, its genetic distance (20%) from the other sub- lineages included in the phylogeny suggests it is a distinct sub-lineage.

4.5 Geographic information system

Using the latitude and longitude coordinates obtained for each isolate, the GIS software Arc View 3.1(Environmental Systems Research Institute Inc. Redlands, CA, USA) was used to overlay the locations of each isolate used in the study, and maps were generated to visualise the spread, distribution and relationships of the strains that were sequenced in this study.

The North and South distribution identified 30 isolates for the North and 11 for the South. Distribution by geopolitical zones indicated the following: n=14 for the North West, n= 5 for the North East, n= 9 for the North Central, n= 6 for the South South, n= 4 for South West and n=1 for the South East (Figure 4.7). Sixteen of the isolates from the North are located along major roads in settlements close to the state capitals, except for NGCK0108 from Kebbi state and NGCK1707 from Sokoto state, which lies along international borders between Nigeria and Niger republic to the North (Figure 4.9). Also, NGCK0308 collected from Wukari a town 150km away from the state capital in Taraba state. All the isolates from the South are located around urban centres and close to state capitals (Figure 4.7). Five isolates from Kano state clustered around a network of major roads from other northern states in to Kano city- the state capital (Figure 4.8).

The five isolates recovered from outbreaks in backyard and commercial farms in Plateau state, in North Central Nigeria, were concentrated around Jos, the state capital, which serves as a link between most states of the northern and the southern parts of the country.

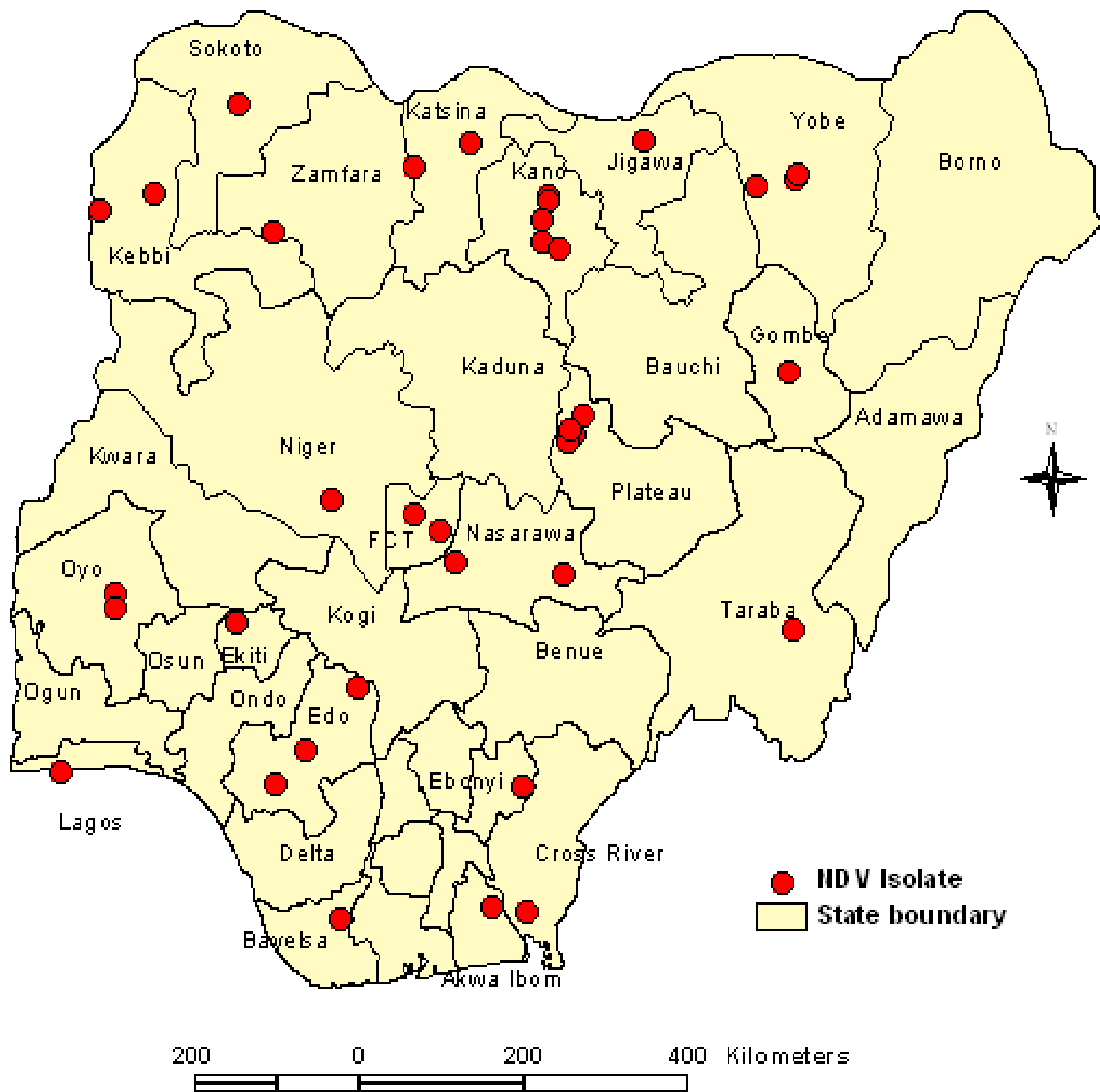

Figure 4.7: Map of Nigeria showing NDV isolate distribution by states

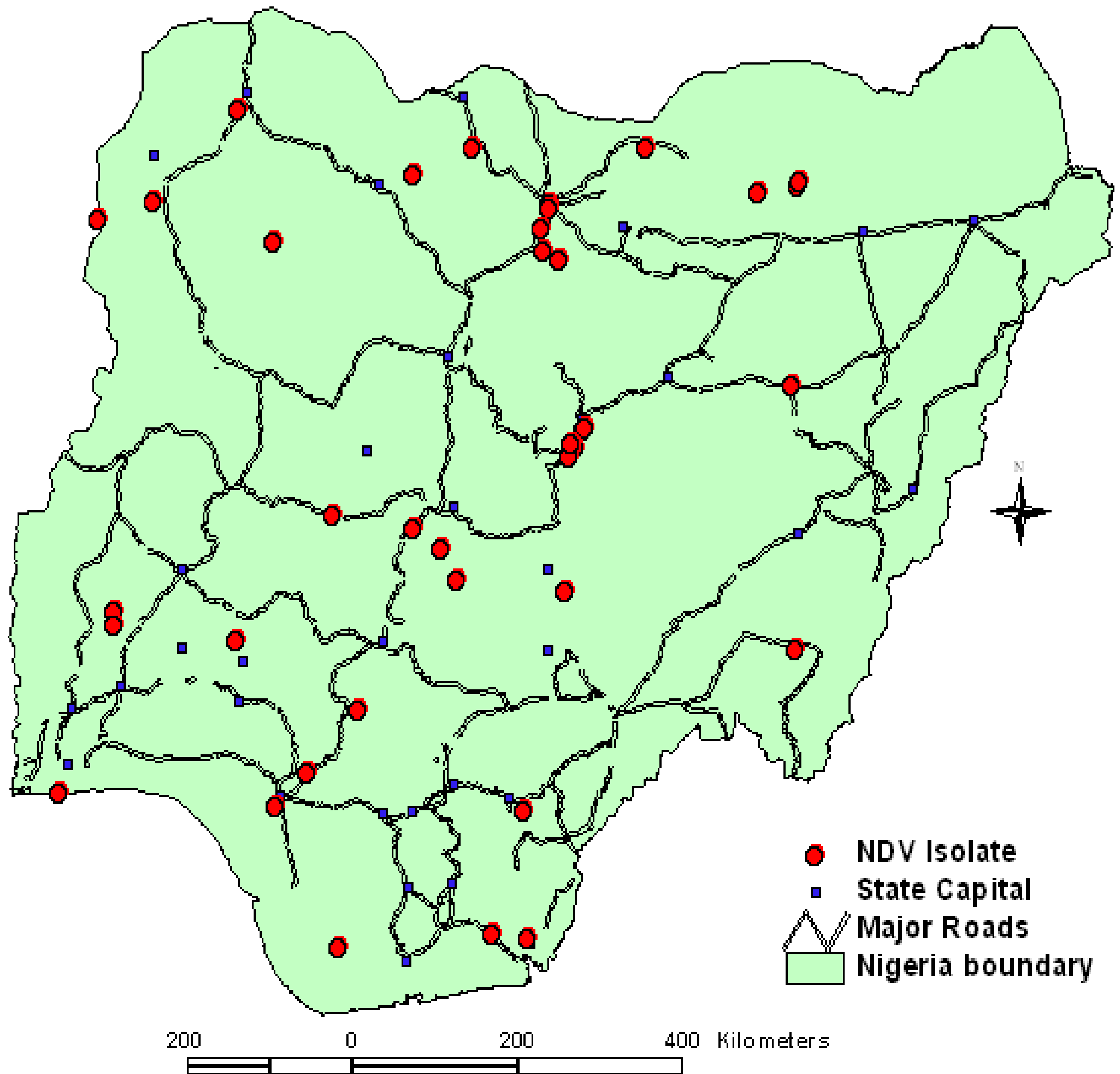

Figure 4.8: Map of Nigeria showing the distribution of isolates and the network of major roads

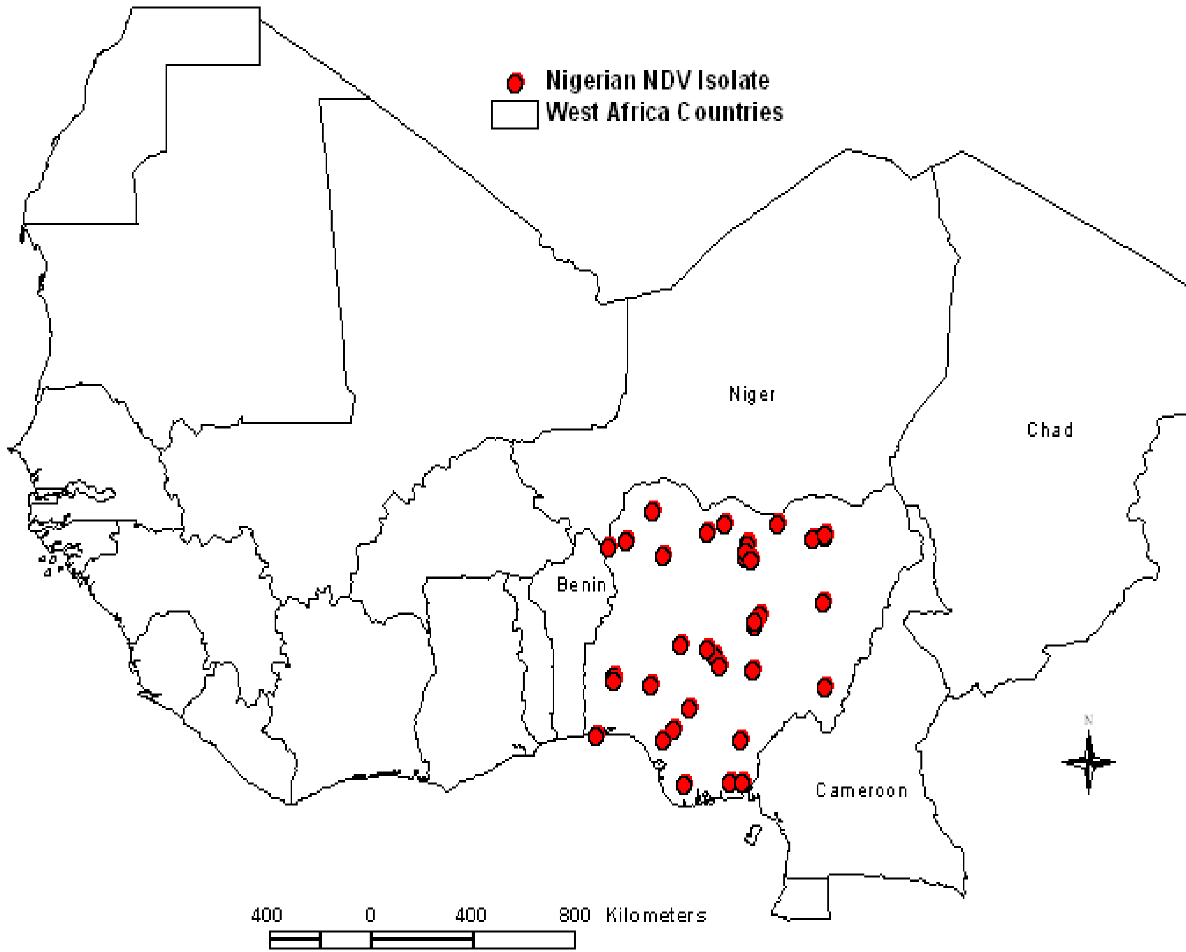

Figure 4.9: Map of West Africa showing international borders and distribution of Nigerian NDV isolate

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                                      70        80        90       100       110       120       130       140       150       160
                              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CK/Niger/38/2007              CTGTTGCTAAGCTGCATATGTCTGACAAGCTCCCTTGATGGTAGGCCTCTTGCAGCTGCAGGGATTGTAATAACAGGAGATAAGGCAATCAAGATATACA
NGCK1807                      ...A.............G....................C..C......T.................................................T.
NGCK2307                      ...A.AT........G.....A.AG.............C..C...........G........A........................G.......C....
NGCK2507                      ...A..................................C..C........................................................T.
NGDK2607                      ...A.A....A...T..G...A.A..............C..C...........G........A........................G.......C....
NGCK3207                      ...A.......T..TGC.C..A................C..C....T........G..........................................T.
NGCK4207                      ...A.A..G....AT......A......A.........C..C....T......G........A........................G.......C....
NGCK0908                      ...G.A........TG...A.A................C..C...........G........A........................G.......C....
NGCK0608                      ....................C....................C...................................................G......
NGCK1507                      ...A....................G..G..........C..C...........G............................................T.
NGCK2807                      ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
NGCK2907                      ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
NGCK3307                      ...A..................................C..C..........................................................
NGCK3407                      ...A........................A.........C..C........................................................T.
NGCK3807                      ...G.A........TG.C.A.A................C..C...........G........A..............G.........G.......C....
NGCK4407                      ...G.A........TG.C.A.A................C..C...........G........A..............G.........G.......C....
NGCK4807                      ...A..T...........C...................C..C..........................................................
NGDK2707                      ...G.A........TG.CCA.A................C..C...........G........A........................G.......C....
NGGF4907                      ...G.A.....T..TG.C.A.A................C..C...........G........A........................G.......C....
NGGF5307                      ...G.A.....T..TG.C.A.A................C..C...........G........A........................G.......C....
NGCK0108                      ........G..T......C......................C.........A................................................
NGCK0208                      ...A...C..............................C..C........................................................T.
NGCK0708                      ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
NGCK0808                      ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
NGCK1707                      ....................C....................C...................................................G......
NGCK4507                      ...A..................................C..C..........................................................
NGCK1108                      ...G.A........TG.C.A.A................C..C...........G........A........................G............
NGCK1308                      ........G..T......C......................C.........A................................................
NGCK1607                      ...G.A........TG.C.A.A................C..C...........G........A...A....................G.......C....
NGCK4307                      ...G.A....TG..T.C..A.A...........A....C..C...........G........A........................G.......C....
NGCK0508                      ...G.A........TG.C.A.A.............A..C..C...........G........A.................A......G.......C....
NGCK2007                      ...A..................................C..C..........................................................
NGCK2107                      ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
NGCK2207                      ...G.AA.......TG.G.A.A.............A..C..C...........G........A........................G.......C....
NGCK3507                      ...A..................................C..C........................................................T.
NGCK4007                      ...G.A........TG.C.A.A................C..C...........G........A...................G....G.......C....
NGPG5207                      ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
NGCK0408                      ...A...C..............................C..C........................................................T.
CK/Burkina Faso/SKF4.1/2006   ...A.ATC......T.......................C..C..AT.........................................G....A.......
CK/Burkina Faso/SKF4.2/2006   ...A.ATC......T.......................C..C..AT.........................................G....A.......
CKZA04AL500                   ...A..T.GG....T..CC...C.........T.....C..C....................A......G.................G....TG......
CKZA05UP1178                  ...A.AT.G........CC...C.........T.....C..C....................A......G.................G....TG......
CK/Sudan/04/2003              ...A.AT.GG....T..CC...C.........T.....C..C....................A......G.................G....TG......
CK/Sudan/05/04                ...A.AT.GG....T..CC...C.........T.....C..C....................A......G.................G....TG......
CK/Sudan/08/04                ...G.AT.GG....T.CCC...C.....A..AT.....C..C.............G......A......G.................G....TG......
Goose paramyxovirus HZ        ...A.AT.GG....T..CC...CT........T.....C..C....................A......G.................G....TG......
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JS/3/98/Go                      ...A.AT.GG....T..CC...C.........T.....C..C....................A......G.................G....TG......
NGCK0307                        ...A...C..............................C..C........................................................T.
FJ772449 avian913/33Nigeria2006 ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
FJ772475 CK/2602/605/Niger/2008 A..G.A........TG.C.A.A................C..C...........G........A........................G.......C....
FJ772478 CK3490/149Cameroon2008 ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
FJ772484 CK3490 147Cameroon2008 ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
FJ772481 CK/2602/625/Niger/2008 A..G.A........TG.T.A.A................C..C...........G........A........................G.......C....
FJ772486 avian/Nigeria2008       ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
FJ772472 CK/2602/468/Niger2008  ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
FJ772463 CK2415/580/B/ Faso2006 ...G.A...T....TG.C.A.A................C..C...........G........A........................G....A..C....
FM200805 CKB/ Faso/5.18/2006    ..AG.A...C....TG.C.A.A................C..C...........G........A........................G....A..C....
FM200804 CKB/ Faso/5.2/2006     ..AG.A...C....TG.C.A.A................C..C...........G........A........................G....A..C....
FJ772469 CK/2602/348/Niger2008  ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....
FJ772446 avian913/1Nigeria2006  ...G.A........TG.C.A.A................C..C...........G.................................G.......C....
FJ772458 CKB/ Faso/2008         ...G.A........TG.C.A.A................C..C...........G........A.................C......G....AG.C....
FM200796 fowlNigeriaNIE93/2007  ...G.A........TG.C.A.A................C..C...........G........A........................G.......C....

                                   170       180       190       200       210       220       230       240       250       260
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CK/Niger/38/2007                CCTCATCTCAGACTGGATCCATCATTGTCAAATTGCTCCCGAATATGCCCAAGGACAAGGAAGCATGTGCAAGAGCCCCATTAGAGGCGTACAACAGAAC
NGCK1807                        .............C...........A.....G...........C.................G..G...............C.......A.....T.....
NGCK2307                        .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A.......C...................
NGCK2507                        .............C...........A.....G...........C....................G...............C.......A.....T.....
NGDK2607                        ......T......A..G.TGGCA..A........A...........C........T..A..G..G.......A...............A........G..
NGCK3207                        .............C...........A.....GC..........C....................G...............C.......A..T..T.....
NGCK4207                        ..........AC.G..TG.ATCAT.AT......ACTC..............G.A.T..A..G..GCTG....A...............A........G..
NGCK0908                        .............A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
NGCK0608                        ...............................G..A......................................G..........................
NGCK1507                        .............C...........A.....G...........C....................G...............C.......A.....T.....
NGCK2807                        .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
NGCK2907                        .............A..G..G.....A.....G..A................G...T..A..G..G.......A...............A...........
NGCK3307                        .............C...........A.................C....................G...............C.......A.....T.....
NGCK3407                        .............C...........A.....G...........C....................G...............C.......A.....T.....
NGCK3807                        .............A..G..A.....A.....G..A....................T..A.....G.......A...............A...........
NGCK4407                        .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A.......C...................
NGCK4807                        .............C..T..............G...........C....................G...............C.......A.....T.....
NGDK2707                        .............A..G..G.....A.....G..A....................T..A..G..........A.......C.......A...........
NGGF4907                        .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A.......C...................
NGGF5307                        .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A.......C...................
NGCK0108                        ...............................G........................................................A..T........
NGCK0208                        .............C...........A.....G...........C....................G...............C.......A.....T.....
NGCK0708                        .............A..G..G.....A.....G..A....................T..A..G..G.......A..G............A...........
NGCK0808                        .......C.....A..G..G.....A.....G..A....................T..A..G..G..C....A...............A...........
NGCK1707                        ...............................G..A......................................G..........................
NGCK4507                        .............C...........A.....G...........C..A.................G...............C.......A.....T.....
NGCK1108                        .............A..G..A.....A.....G..A....................T..A..G..G.......A...............A...........
NGCK1308                        ...............................G........................................................A..T........
NGCK1607                        .............A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
NGCK4307                        ......A......A..G..G.....A.....G..A................G...T..A..G..G.......A...............A...........
NGCK0508                        .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A.......C...................
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NGCK2007                          .............C...........A.................C....................G...............C.......A.....T.....
NGCK2107                          .............A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
NGCK2207                          .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A...........................
NGCK3507                          .............C...........A.....G...........C....................G...............C.......A.....T.....
NGCK4007                          .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
NGPG5207                          .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A...........................
NGCK0408                          .............C...........A.....G...........C....................G...............C.......A.....T.....
CK/Burkina Faso/SKF4.1/2006       .............C.....T.....A.....G..........................A..G..G.......A......CC...................
CK/Burkina Faso/SKF4.2/2006       .............C.....T.....A.....G..........................A..G..G.......A......CC...................
CKZA04AL500                       ....G........A..G..A.....A.....G...................G...T........G.....G.A...............A..T........
CKZA05UP1178                      ....G........A..G..A.....C.....G........A..........G...T........G.......A...............A...........
CK/Sudan/04/2003                  ....G........A..G..A.....A.....G...................G...T.....G..G.......A...............A..T........
CK/Sudan/05/04                    ....G........A..G..A.....A.....G...................G...T.....G..G.......A...............A..T........
CK/Sudan/08/04                    ....G........A..G..A.....A.....G...................G...T.....G..G.......A...............A..T........
Goose paramyxovirus HZ            ....G........A..G..A.....A.....G...................G...T.....G..G.......A...............A..T........
JS/3/98/Go                        ....G........A..G..A.....A.....G...................G...T.G...G..G.......A...............A..T........
NGCK0307                          .............C...........A.....G...........C....................G...............C.......A.....T.....
FJ772449 avian913/33Nigeria2006   .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
FJ772475 CK/2602/605/Niger/2008   .............A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
FJ772478 CK3490/149Cameroon2008   .............A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
FJ772484 CK3490 147Cameroon2008   .............A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
FJ772481 CK/2602/625/Niger/2008   .............A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
FJ772486 avian/Nigeria2008        .......C.....A..G..G.....A.....G..A....................T..A..G..G...............C...................
FJ772472 CK/2602/468/Niger2008    .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A.......C...................
FJ772463 CK2415/580/B/ Faso2006   .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A...............A...........
FM200805 CKB/ Faso/5.18/2006      .......C.....G..G..G.....A.....G..A....................T..A..G..........A...............A...........
FM200804 CKB/ Faso/5.2/2006       .......C.....G..G..G.....A.....G..A....................T..A..G..........A...............A...........
FJ772469 CK/2602/348/Niger2008    .......C.....A..G..G.....A.....G..A....................T..A..G..G.......A.......C...................
FJ772446 avian913/1Nigeria2006    .......C.....A..G..G.....A.....G.......................T..A..G..G.......A...............A...........
FJ772458 CKB/ Faso/2008           .......C.....A..G..A.....A.....G..A....................T..A..G..G.......A...............A...........
FM200796 fowlNigeriaNIE93/2007    .......C.....A..G..G.....A.....G..A.....A..............T..A..G..G.......A.......C...................
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                                          270       280       290       300       310       320       330       340       350       360
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CK/Niger/38/2007                  ACTAACCACCTTACTCACTCCTCTTGGTGATTCCATCCGCAGGATACAAGGGTCTGTGTCCACATCTGGGGGAAGACGACAGAAACGCTTTATAGGTGCA
NGCK1807                          .......G.............C...............................................A..........G......T...G........
NGCK2307                          .T.........................C..C.......A................AC...T........A..G..GA......................C
NGCK2507                          .......G.............................................................A..........G......T...G........
NGDK2607                          .T.......T......G..........C..........A...............C.C...A........A..G..GA......................C
NGCK3207                          .......G.............................................................A..........G......T...G........
NGCK4207                          .T..............G..........C..............A.............C...T........A.....GA......................T
NGCK0908                          .T..............G..........C..........A.................C...A........A..G..GA......................C
NGCK0608                          ..................................................................A..A..............................
NGCK1507                          .......G.............................................................A..........G......T...G........
NGCK2807                          .T..G......................C..........A.................C...T........A..G..GA.G....................C
NGCK2907                          .T..............G..........C..........A.................C...T........A..G..GA......................C
NGCK3307                          .......G.............................................................A..........G......T...G.......G
NGCK3407                          .......G.........................................A...................A..........G......T...G........
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NGCK3807                     .T.........................C..........A.................C...T........A..G..GA......G...............C
NGCK4407                     .T.........................C..C.......A................AC...T........A..G..GA......................C
NGCK4807                     .............................................................G.......A..........G......T...G........
NGDK2707                     .T..............G..........C..........A...........A.....C...T........A..G..GA......................C
NGGF4907                     .T.........................C..C.......A................AC...T........A..G..GA......................C
NGGF5307                     .T.........................C..C.......A................AC...T........A..G..GA......................C
NGCK0108                     .....................................................................A.........................C....
NGCK0208                     .......G.............................................................A..........G......T...G........
NGCK0708                     .T..............G..........C..........A.................C...T........A..G..GA.G...........C........C
NGCK0808                     .T.........................C..........A.................C...T........A..G..GA......................C
NGCK1707                     ..................................................................A..A..............................
NGCK4507                     .......G.............................................................A..........G......T...G........
NGCK1108                     .T..............G..........C..........A.................C...A........A..G..GA...................A..C
NGCK1308                     .....................................................................A..............................
NGCK1607                     .T..............G..........C..........A.................C...A........A..G..GA......................C
NGCK4307                     .T.............TG..........C..........A.................C...A........A..G..GA...................C..C
NGCK0508                     .T.........................C..C.......A................AC...T........A..G..GA......................C
NGCK2007                     .......G.............................................................A..........G......T...G.......G
NGCK2107                     .T..............G..........C............................C...T........A..G..GA....................TAC
NGCK2207                     .T.........................C..........A................AC...T........A..G..GA......................C
NGCK3507                     .......G.............................................................A..........G......T...G........
NGCK4007                     .T................C........C..........A.................C...T........A..G..GA......................C
NGPG5207                     .T.........................C..........A................AC...T........A..G..GA......................C
NGCK0408                     .......G.............................................................A..........G......T...G........
CK/Burkina Faso/SKF4.1/2006  ............G........C.....C.............A........................C..A......A...GA.........G........
CK/Burkina Faso/SKF4.2/2006  ............G........C.....C.............A........................C..A......A...GA.........G........
CKZA04AL500                  ...G..T..T..G..............C..C..........A...C.................G...........GA....A.................T
CKZA05UP1178                 ...G..T..G..G..............C..C..........A...C.................G.....A.....GA....A..................
CK/Sudan/04/2003             ...G..T..T..G..............C..C..........A...C.................G.....A.....GA....A.................T
CK/Sudan/05/04               ...G..T..T..G..............C..C..........A...C.................G.....A.....GA....A.................T
CK/Sudan/08/04               ...G..T..T..G..............C..C..........A...C.................G.....A.....GA....A.................T
Goose paramyxovirus HZ       ...G..T..T..G..............C..C..........A...C.................G.....A.....GA....A.................T
JS/3/98/Go                   ...G..TG.T..G..............C..C..........A...C.................G.....A.....GA....A.................T
NGCK0307                     .......G.............................................................A..........G......T...G........
FJ772449 avian913/33Nigeria2006 .T.........................C..........A.................C...T........A..G..GA......................C
FJ772475 CK/2602/605/Niger/2008  .T..............G..........C..........A.................C...T........A..G..GA.G...........C........C
FJ772478 CK3490/149Cameroon2008  .T..............G..........C..........A..R.........R....C...TG.......A..G..GA......................C
FJ772484 CK3490 147Cameroon2008  .T..............G..........C..........A..A.........A....C...TG.......A..G..GA......................C
FJ772481 CK/2602/625/Niger/2008  .T..............G..........C..........A.................C...T........A..G..GA.G...........C........C
FJ772486 avian/Nigeria2008       .T.........................C..........A................AC...T........A..G..GA......................C
FJ772472 CK/2602/468/Niger2008   .T.........................C..C.......A................AC...T........A..G..GA......................C
FJ772463 CK2415/580/B/ Faso2006  .T.........................C..........AT................C...T........A..G..GA......................C
FM200805 CKB/ Faso/5.18/2006     .T.........................C..........A.................C...T........A..G..GA......................C
FM200804 CKB/ Faso/5.2/2006      .T.........................C..........A.................C...T........A..G..GA......................C
FJ772469 CK/2602/348/Niger2008   .T.........................C..C.......A................AC...T........A..G..GA....A.................C
FJ772446 avian913/1Nigeria2006   .T.........................C..........A.....................T........A.....GA......................C
FJ772458 CKB/ Faso/2008          .T.........................C..........A..A..............CA..T.T......A..G..GA......................C
FM200796 fowlNigeriaNIE93/2007   .T.........................C..C.......A................AC...T........A..G..GA......................C
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                                         370
                                ....|....|....
CK/Niger/38/2007                GTCATTGGCAGTGT
NGCK1807                        ..T.....T.....
NGCK2307                        ..............
NGCK2507                        ..T.....T.....
NGDK2607                        ..T...........
NGCK3207                        ..T.....T.....
NGCK4207                        ..T...........
NGCK0908                        ..............
NGCK0608                        ..............
NGCK1507                        ..T.....T.....
NGCK2807                        ..T...........
NGCK2907                        ..T...........
NGCK3307                        ..T..C..T.....
NGCK3407                        ..T.....T.....
NGCK3807                        ..T...........
NGCK4407                        ..............
NGCK4807                        ..T.....T.....
NGDK2707                        ..T...........
NGGF4907                        ..............
NGGF5307                        ..............
NGCK0108                        ..............
NGCK0208                        ..T.....T.....
NGCK0708                        ..T...........
NGCK0808                        ..T...........
NGCK1707                        ..............
NGCK4507                        ..T.....T.....
NGCK1108                        ..T...........
NGCK1308                        ..............
NGCK1607                        ..T...........
NGCK4307                        ..T...........
NGCK0508                        ..............
NGCK2007                        ..T..C..T.....
NGCK2107                        ..T...........
NGCK2207                        ..............
NGCK3507                        ..T.....T.....
NGCK4007                        ..T...........
NGPG5207                        ..............
NGCK0408                        ..T.....T.....
CK/Burkina Faso/SKF4.1/2006     ..T..C........
CK/Burkina Faso/SKF4.2/2006     ..T..C........
CKZA04AL500                     A.T...........
CKZA05UP1178                    ..T...........
CK/Sudan/04/2003                ..T...........
CK/Sudan/05/04                  ..T...........
CK/Sudan/08/04                  ..T...........
Goose paramyxovirus HZ          ..T...........
JS/3/98/Go                      ..T...........
NGCK0307                        ..T.....T.....
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FJ772449 avian913/33Nigeria2006 ..T...........
FJ772475 CK/2602/605/Niger/2008 ..T...........
FJ772478 CK3490/149Cameroon2008 ..T...........
FJ772484 CK3490 147Cameroon2008 ..T...........
FJ772481 CK/2602/625/Niger/2008 ..T...........
FJ772486 avian/Nigeria2008      ..............
FJ772472 CK/2602/468/Niger2008  ..............
FJ772463 CK2415/580/B/ Faso2006 ..T...........
FM200805 CKB/ Faso/5.18/2006    ..T...........
FM200804 CKB/ Faso/5.2/2006     ..T...........
FJ772469 CK/2602/348/Niger2008  ..............
FJ772446 avian913/1Nigeria2006  ..T...........
FJ772458 CKB/ Faso/2008         ..T...........
FM200796 fowlNigeriaNIE93/2007  ..............
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Figure 4.10: Nucleotide sequence alignment (nucleotide 61- 374) for lineage 5 with sequences from this study indicated in boldface

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                                         70        80        90       100       110       120       130       140       150
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DQ202263 grouseRussiaKhabarovs   CTGATCCTGAGCTGCACCTGCCCGACGAACTCTCTTGACGGCAGGCCTCTTGCAGCTGCGGGGATTGTGGTAACAGGAGATAAAGCGATC
AY471782 PAEPI97415             T....A..........T...........G...................................................C.....A...
AY471781 PAEPI96210             T....A..........T...........G...................................................C.....A...
AY175751 PAEPI96210             T....A..........T...........G...................................................C.....A...
AY471785 PAEKE99364             .....A..........T....T......G......................................................G..A...
AY471784 PAEKE98398             .....A..........T....T......G......................................................G..A...
AY471783 PAEKE98373             .....A..........T....T......G......................................................G..A...
AY471779 PAEPI98367             T....A....A.....T...........G...................................................C.....A...
AY471780 PAEPI99361             T....A..........T......A....G...................................................C.....A...
DQ289796 I-1                    .....A........T.T...........G.............................................................
AY734536 PigeonArgentina3/97    .....A..........T...TT......G......................................................G..A...
AY471773 PTRBU95211             .....A........T.T...........G.............................................................
FM200798 parrotNigeriaNIE139/07 .....A..........T.........A.G..............................A..........................A...
AY150115 IT-125/87              .....A..........T........T..G.............................................................
AY175776 TRCK95111              .....A........T.T...........G.............................................................
FJ410147 PPMV-1/Maryland/1984   .....AT.........T...........G.........................................................AG..
FJ410145 PPMV-1New York/1984    .....AT.........T...........G.........................................................AG..
EF520716 pigeon/NY/US/1984      .....AT.........T...........G.........................................................AG..
AY288996 pigeon/Italy/1166/00   .....A..........T.....T.....G..................A......................................A...
AY471857 strain PIQPI78442      .....AT.........T.........T.G.........................................................AG..
NGCK1208                        ................T.....................................................................A...
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                                           160       170       180       190       200       210       220       230       240
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DQ202263 grouseRussiaKhabarovs    AATATATACACCTCATCTCAGACAGGGTCAATCATAGTCAAGTTGCTCCCGAATATGCCCAAGGACAAGGAGGCATGTGCAAAAGCCCCG
AY471782 PAEPI97415               ..........................................................................................
AY471781 PAEPI96210               ....................................A.....................................................
AY175751 PAEPI96210               ....................................A.....................................................
AY471785 PAEKE99364               .............................................................................C............
AY471784 PAEKE98398               ..C.......................................................................................
AY471783 PAEKE98373               ..C.......................................................................................
AY471779 PAEPI98367               ..........................................................................................
AY471780 PAEPI99361               .........................................................................................A
DQ289796 I-1                      ....................................................................A.....................
AY734536 PigeonArgentina3/97      ..........................A....................................ACT..............G.........
AY471773 PTRBU95211               ....................................................................A.....................
FM200798 parrotNigeriaNIE139/07   ........T..T..................................................A.....A.....................
AY150115 IT-125/87                ....................................................................A.....................
AY175776 TRCK95111                ....................................................................A.....................
FJ410147 PPMV-1/Maryland/1984     .................C..................................................A.....................
FJ410145 PPMV-1New York/1984      .................C..................................................A.....................
EF520716 pigeon/NY/US/1984        .................C..................................................A.....................
AY288996 pigeon/Italy/1166/00     ....................................................................A...........G.........
AY471857 strain PIQPI78442        ....................................................................A.....................
NGCK1208                          ..............................................................A...........................
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                                           250       260       270       280       290       300       310       320       330
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DQ202263 grouseRussiaKhabarovs    TTAGAAGCATACAACAGAACACTGACCACTTTACTCACCCCCCTTGGTGACTCCATCCGCAGGATACAAGGGTCTGTGTCCACGTCAGGA
AY471782 PAEPI97415               .....................................................T.............................A......
AY471781 PAEPI96210               ...................................................................................A......
AY175751 PAEPI96210               ...................................................................................A......
AY471785 PAEKE99364               ...................................................................................A......
AY471784 PAEKE98398               ...................................................................................A......
AY471783 PAEKE98373               ...................................................................................A......
AY471779 PAEPI98367               .............................C.....................................................A......
AY471780 PAEPI99361               ...................................................................................A......
DQ289796 I-1                      .....G............................................T................................A......
AY734536 PigeonArgentina3/97      ...................................................................................A......
AY471773 PTRBU95211               .....G............................................T................................A......
FM200798 parrotNigeriaNIE139/07   ..................................................T................................A......
AY150115 IT-125/87                C....G............................................T................................A......
AY175776 TRCK95111                .....G............................................T.............................T..A......
FJ410147 PPMV-1/Maryland/1984     ..................................................T................................A......
FJ410145 PPMV-1New York/1984      ..................................................T................................A......
EF520716 pigeon/NY/US/1984        ..................................................T................................A......
AY288996 pigeon/Italy/1166/00     .....G............................................T....................A...........A......
AY471857 strain PIQPI78442        ..................................................T................................A......
NGCK1208                          .........................................T.........................................A......
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                                           340       350       360       370
                                ....|....|....|....|....|....|....|....|....
DQ202263 grouseRussiaKhabarovs  GGAAGGAGGCAGAAGCGTTTTATAGGTGCCATTATAGGCAGTGT
AY471782 PAEPI97415             ............................................
AY471781 PAEPI96210             ............................................
AY175751 PAEPI96210             ............................................
AY471785 PAEKE99364             ............................................
AY471784 PAEKE98398             ............................................
AY471783 PAEKE98373             ............................................
AY471779 PAEPI98367             ............................................
AY471780 PAEPI99361             ...C........................................
DQ289796 I-1                    .................C..........................
AY734536 PigeonArgentina3/97    ............................................
AY471773 PTRBU95211             .................C..........................
FM200798 parrotNigeriaNIE139/07 ........AA..................................
AY150115 IT-125/87              .................C..........................
AY175776 TRCK95111              .................C..........................
FJ410147 PPMV-1/Maryland/1984   ..............A..C.................C........
FJ410145 PPMV-1New York/1984    ..............A..C.................C........
EF520716 pigeon/NY/US/1984      ..............A..C.................C........
AY288996 pigeon/Italy/1166/00   .................C..........................
AY471857 strain PIQPI78442      ..............A..C.................C........
NGCK1208                        ..........................................C.
```

Figure 4.11: Nucleotide sequence alignment (nucleotide 61- 374) for lineage 4 with sequence from this study indicated in boldface

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                                  70        80        90       100       110       120       130       140       150
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AY845400 LaSota          CTGGTACTGAGTTGCATCTGTCCGGCAAACTCCATTGATGGCAGGCCTCTTGCAGCTGCAGGAATTGTGGTTACAGGAGACAAAGCCGTC
GQ901891MB061/06         ..........................................................................................
FJ938174 sparrow/Guangxi ..........................................................................................
FJ810494 NDV08-071       ..........................................................................................
FJ810485 NDV08-060       ..........................................................................................
FJ810483 NDV08-058       ..........................................................................................
FJ810482 NDV08-057       ..........................................................................................
FJ810435 NDV08-009       ..........................................................................................
EU418738                 ..........................................................................................
EU296496 TW/84-361       ..........................................................................................
FM200802 chicken/Nigeria ..........................................................................................
FM200801                 ..........................................................................................
NGCK1008                 ..........................................................................................

                                 160       170       180       190       200       210       220       230       240
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AY845400 LaSota          AACATATACACCTCATCCCAGACAGGATCAATCATAGTTAAGCTCCTCCCGAATCTGCCCAAGGATAAGGAGGCATGTGCGAAAGCCCCC
GQ901891MB061/06         ..........................................................................................
FJ938174 sparrow/Guangxi ..........................................................................................
FJ810494 NDV08-071       ..........................................................................................
FJ810485 NDV08-060       ..........................................................................................
FJ810483 NDV08-058       ..........................................................................................
FJ810482 NDV08-057       ..........................................................................................
FJ810435 NDV08-009       ..........................................................................................
EU418738                 ..........................................................................................
EU296496 TW/84-361       ..........................................................................................
FM200802 chicken/Nigeria ..........................................................................................
FM200801                 ..........................................................................................
NGCK1008                 ..........................................................................................

                                 250       260       270       280       290       300       310       320       330
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AY845400 LaSota          TTGGATGCATACAACAGGACATTGACCACTTTGCTCACCCCCCTTGGTGACTCTATCCGTAGGATACAAGAGTCTGTGACTACATCTGGA
GQ901891MB061/06         ..........................................................................................
FJ938174 sparrow/Guangxi ..........................................................................................
FJ810494 NDV08-071       ..........................................................................................
FJ810485 NDV08-060       ..........................................................................................
FJ810483 NDV08-058       ..........................................................................................
FJ810482 NDV08-057       ..........................................................................................
FJ810435 NDV08-009       ..........................................................................................
EU418738                 ..........................................................................................
EU296496 TW/84-361       ..........................................................................................
FM200802 chicken/Nigeria ..........................................................................................
FM200801                 ..........................................................................................
NGCK1008                 ..........................................................................................
```

```
                                  340       350       360       370
                              ....|....|....|....|....|....|....|....|....
AY845400 LaSota               GGGGGGAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGT
GQ901891MB061/06              ............................................
FJ938174 sparrow/Guangxi      ............................................
FJ810494 NDV08-071            ............................................
FJ810485 NDV08-060            ............................................
FJ810483 NDV08-058            ............................................
FJ810482 NDV08-057            ............................................
FJ810435 NDV08-009            ............................................
EU418738                      ............................................
EU296496 TW/84-361            ............................................
FM200802 chicken/Nigeria      ............................................
FM200801                      ............................................
NGCK1008                      ............................................
```

Figure 4.12: Nucleotide sequence alignment (nucleotide 61- 374) for lineage 2 with sequence from this study indicated in boldface

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                                     70        80        90       100       110       120       130       140       150
                             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FJ810487 NDV08-063           TTGGCACTGAGTTGCGTCTGTCCGACCAGCGCCCTTGATGGCAGGCCTCTTGCAGCTGCAGGGATTGTGGTAACAGGAGACAAAGCAGTC
EU847310 NIAH16566-06        ..........................................................................................
EU547760 APMV/duck/10/07     ..........................................................................................
EU419321 NDV07-64            ..........................................................................................
AF532743 ZA37401/X/01        ..........................................................................................
AF532741 ZA341/P/99          ..........................................................................................
AF532147 ZA348/B/00          ..........................................................................................
FM200800 chicken/Nigeria     ..........................................................................................
EU547759 APMV/duck/09/07     ................................T.........................................................
AY175657 AU--90100           ................A..A..........T...........................................................
AY175660 AUCK98028           ..........................................................................................
AY935499 I-2                 C.......................G............................G................................G...
AF503639 shelduck/France     C.............................T...........................................................
NGCK1907                     C.........................................................................................

                                    160       170       180       190       200       210       220       230       240
                             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FJ810487 NDV08-063           AACATATACACCTCATCTCAGACAGGGTCAATCATAATCAAGTTACTCCCAAATATGCCCAAGGATAAAGAGGCGTGTGCAAAAGCCCCG
EU847310 NIAH16566-06        ..........................................................................................
EU547760 APMV/duck/10/07     ..........................................................................................
EU419321 NDV07-64            ..........................................................................................
AF532743 ZA37401/X/01        ..........................................................................................
AF532741 ZA341/P/99          ..........................................................................................
AF532147 ZA348/B/00          ..........................................................................................
FM200800 chicken/Nigeria     ..........................................................................................
EU547759 APMV/duck/09/07     ..........................................................................................
AY175657 AU--90100           ..........................................................................................
AY175660 AUCK98028           ..........................................................................................
AY935499 I-2                 ...........A......................................G...................................A...
AF503639 shelduck/France     ....................................G................C........A..........................A
NGCK1907                     ..........................................................................................

                                    250       260       270       280       290       300       310       320       330
                             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FJ810487 NDV08-063           TTGGAGGCATACAACAGGACATTGACTACTTTGCTCACCCCCCTTGGTGATTCTATCCGTAGGATACAAGAGTCTGTGACCACGTCCGGA
EU847310 NIAH16566-06        ..........................................................................................
EU547760 APMV/duck/10/07     ..........................................................................................
EU419321 NDV07-64            ..........................................................................................
AF532743 ZA37401/X/01        ..........................................................................................
AF532741 ZA341/P/99          ..........................................................................................
AF532147 ZA348/B/00          ..........................................................................................
FM200800 chicken/Nigeria     ......................................................................G...................
EU547759 APMV/duck/09/07     ..........................................................................................
AY175657 AU--90100           ...........T.......................................................................A......
AY175660 AUCK98028           ..............................C.A........T................................................
AY935499 I-2                 .........................................T................................................
AF503639 shelduck/France     ........G..........................................................................A..T.A.
NGCK1907                     ..........................................................................................
```

```
                                       340       350       360       370
                               ....|....|....|....|....|....|....|....|....
FJ810487 NDV08-063             GGAGGGAAACAGGGACGTCTTATAGGCGCCATTATCGGTGGTGT
EU847310 NIAH16566-06          ............................................
EU547760 APMV/duck/10/07       ............................................
EU419321 NDV07-64              ............................................
AF532743 ZA37401/X/01          ............................................
AF532741 ZA341/P/99            ............................................
AF532147 ZA348/B/00            ............................................
FM200800 chicken/Nigeria       ............................................
EU547759 APMV/duck/09/07       ............................................
AY175657 AU--90100             ............................................
AY175660 AUCK98028             ...A...G......G.............................
AY935499 I-2                   ...A.A...........C........A.................
AF503639 shelduck/France       .................C..C......................C
NGCK1907                       ................................C...........
```

Figure 4.13: Nucleotide sequence alignment (nucleotide 61- 374) for lineage 1 with sequence from this study indicated in boldface

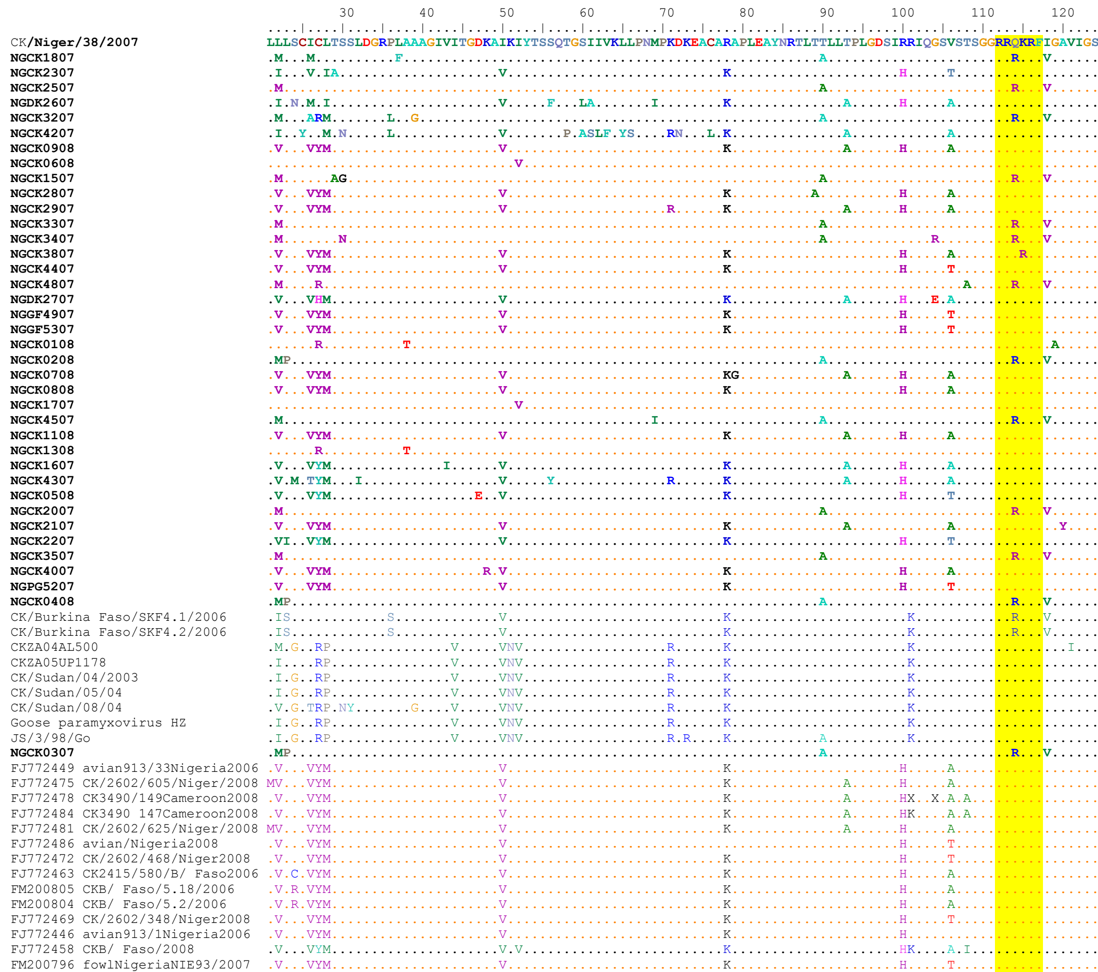

Figure 4.14: Amino acid alignment (residue21-124) of the partial fusion protein gene sequences of lineage 5, with the sequences from this study are in boldface and the fusion cleavage motif site is shaded.

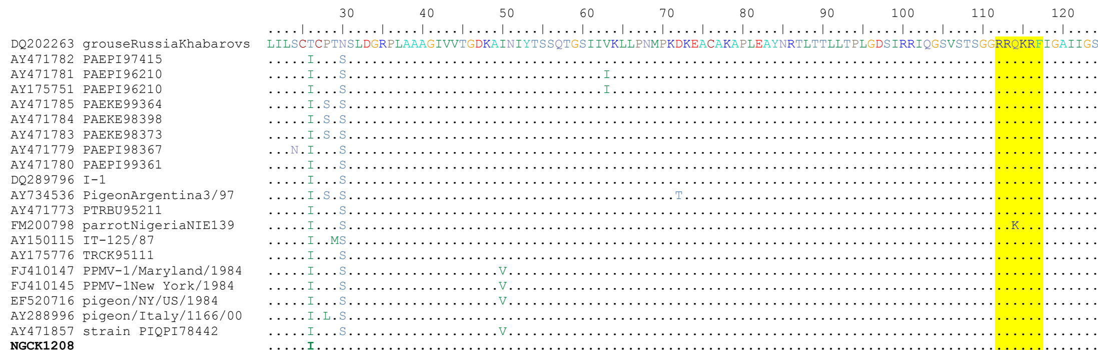

Figure 4.15: Amino acid alignment (residue21-124) of the partial fusion protein gene sequences of lineage 4, with the sequence from this study in boldface, and the fusion cleavage motif site is shaded.

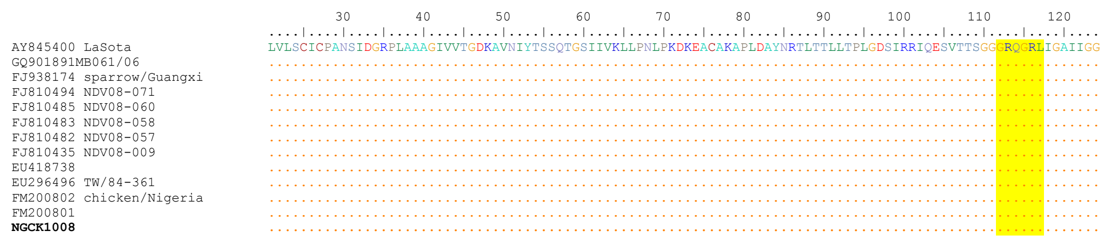

Figure4.16: Amino acid alignment (residue21-124) of the partial fusion protein gene sequences of lineage 2, with the sequence from this study in boldface, and the fusion cleavage motif site is shaded.

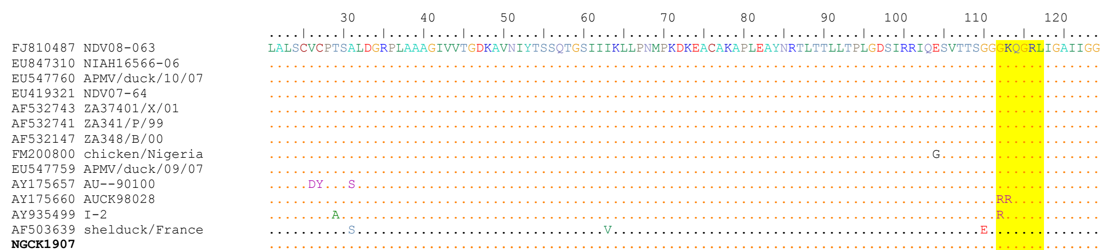

Figure 4.17: Amino acid alignment (residue21-124) of the partial fusion protein gene sequences of lineage 1, with the sequence from this study in boldface, and the fusion cleavage motif site is shaded.

CHAPTER FIVE: DISCUSSION AND CONCLUSIONS

In this study phylogenetic characterization of 41 Nigerian NDVs isolated in 2007 and 2008 were performed. These viruses were isolated from a variety of hosts and locations, including 36 recovered from live bird markets (LBMs) and 5 from outbreaks in backyard and commercial farms.

The purpose of the investigation was to identify the genetic lineage(s) of NDVs circulating in Nigeria and the variety of poultry species that harbours them, and establish if there is a linkage between the NDV lineage(s) from the LBMs and the lineages responsible for outbreaks in backyard and commercial farms. A commonly used region that spans nucleotide 61 to nucleotide 374 of the Fusion protein, including the F_0 cleavage site, was analysed.

Based on sequence analysis, 39 of the isolates were classified as virulent, whereas 2 of the isolates were classified as avirulent.

High sequence similarity (100%) of NGC1008 from a backyard farm with the LaSota vaccine strain (lineage 2) is suggestive that it is a derivative of a vaccine strain and may be a reflection of the use of this vaccine in the poultry operation of this category in Nigeria. Moreover, the farm where this strain was recovered is located within the same Local Area Council with the National Veterinary Institute, Vom; that produces poultry and other vaccines for national use; hence access to the vaccine may not be a problem. Also, the practice of buying vaccinated spent hens from commercial farms to increase backyard stock, could be a reason for the prevalence of this strain around the city.

The similarities (96%) shared by the lineage 1 strain identified in this study with the I-2 vaccine suggests that it could be a vaccine derived strain, even though there are no official commercial sales I-2 or the closely related V4 vaccines in Nigeria. It is more likely this strain spread to Nigeria through trans-border trade in chickens with neighbouring Cameroon (where I-2 vaccine is used) or perhaps the vaccine was obtained illegally from neighbouring countries. The use of the lineage 1, I-2 vaccine in Nigeria is still at the trial level, with ongoing research focusing on identifying the most appropriate carrier for delivery. The 38 virus strains in lineage 5 were further divided into

2 sub-lineages viz. 5f and 5g, also reported by Snoeck *et al*., (2009). This finding supports the report that distinct genetic lineages circulate in West Africa and Nigeria in particular. A major finding of this study was the identification of clusters within these sub-lineages which were not reported by previous investigators that analysed recent Nigerian NDV isolates. Of particular interest are the 13 strains from the 5f group which formed a distinct clade not reported in similar studies. The identification of ongoing genetic drift raises concerns about the efficacy of current control measures in the country.

Previous studies reported that the movement of live poultry, especially rural and spent or culled poultry in Nigeria is usually from the North to the South (Adene and Oguntade, 2006). The retail and supply system of the live poultry business in Nigeria is a complex operation. Usually the live bird vendors travel by road on motorbike, buying live poultry from villages which are pooled and loaded into open trucks. The birds are then transported to the city centres within Northern Nigeria, and eventually to Southern Nigeria via major roads with occasional stopovers at state capitals and villages along the road. In the course of these movements some of the birds are sold at some points to road side food vendors and other live bird marketers.

Geographic information system mapping based on the sampling approach revealed that the majority of the isolates lay along major transportation routes and around state boundaries, which is a reflection of the locations of live bird markets in Nigeria, situated mostly within the city centres and in villages along major roads leading to the cities.

The observed similarities between strains from the North East and North Central regions with strains from Kano state in the North West presents an interesting scenario: Kano state is the commercial nerve centre of Northern Nigeria, with a daily influx of people from all parts of the North for business that includes the live bird trade. Both villagers and city dwellers come to sell and buy from the market to either re-stock or increase their existing backyard flocks. Strains recovered in 2007 from Sokoto and Kano in the far North showed high similarity with the strains recovered in 2008 from Jos in North Central Nigeria.

The 100% sequence homology of strains NGCK0208, NGCK0308 and NGCK0408 isolated within the same year but from different geopolitical zones, indicates a spatial spread and the uncontrolled extensive movement of live poultry within the country.

The sequence similarity (97.4%) of both NGCK0108 from Kebbi state and NGCK1707 from Sokoto state and the Niger strain suggests a multiple source of entry of the Niger strain into Nigeria especially since NGCK0108 and NGCK1707 were recovered from border towns were a high volume of live bird trade occurs.

The sub-lineage-specific residue substitutions of the strains analysed in this study (e.g. $N \rightarrow K^{51}$ and $I \rightarrow V^{44}$) are consistent with NDVs circulating in West Africa and Nigeria in particular, described in previous investigations (Hassan *et al.*, 2010; Catolli *et al.*, 2009 and Snoeck *et al.*, 2009). Observed sequence similarity between strains recovered from outbreaks in backyard and commercial farms in Jos, Plateau state and those from live bird markets in Sokoto and Kano states may be due to horizontal spread by live poultry vendors from rural settlements and LBMs to commercial and backyard poultry houses. This is because the same group of persons who move from villages to buy live poultry are often the ones who go to farm to buy spent or culled chickens to sell in bigger markets in the cities.

The high genetic variability observed within the lineage 5 may suggest that the co-mingling of species of birds such as; chickens, guinea fowls, ducks and pigeons from the rural poultry and backyard farms; which are either not vaccinated or with a history of sub-optimal vaccination, could have lead to the evolution of variant strains. However, the largely unvaccinated poultry population is the likely reason for the continuous shedding and spread of velogenic strains of NDVs in Nigeria.

Strain NGCK1208, a lineage 4 (PPMV-1) virus was isolated from a dead chicken. The virus shared more sequence similarities with a grouse strain from Russia, which might suggest spread by migratory birds, aided by bridge bird species which occasionally mixed with the domestic poultry on the one hand and the importation of fancy and ornamental birds on the other hand.

Bayelsa state, where the isolate was recovered, is located in the Niger delta area of Nigeria, and has a wetland of international importance viz. the Apoi Creek Forests, which is composed mainly of marshes, mangrove forests and fresh water swamps and constitutes an important breeding area for a significant number of wild bird species and other wildlife (Ramsar, 2000).The state is also known for oil exploration activities, and has attracted many foreign oil companies with a large population of expatriates who keep exotic fancy birds as a hobby. Also, some wealthy Nigerians are known to import different species of fancy and ornamental birds including exotic domestic water fowls. All of these could be likely sources of spread. The possibility of spread by migratory doves also exists. Pigeon racing is not practiced in Nigeria, hence, not a likely source of spread.

The identification of sub-lineages 5f and 5h in all the Nigerian geopolitical zones country, in a variety of species, illustrates how the mixing of different species of poultry in LBMs and at stop-overs during transportation provides opportunity for virus spread and perpetuate virus lineages both within and between zones. The NDV strains thus seem to have been disseminated by the activities of live bird vendors who transport large number of live poultry (mostly indigenous) to the South through Jos. Also, close similarity of strains recovered from Abuja (FCT) in 2007 and those from Kano in the same year may be attributed to road transport of live poultry through Abuja, the Federal capital to the Southern part of the country. This is because; Jos and Abuja in central Nigeria are the major gateways to the South through land transportation

Similarities in the sequences of strains recovered from LBMs with those associated with outbreaks in backyard and commercial farms suggests a spill over from the LBMs which may be due to poor bio-security practices in the backyard and some of the commercial operations. Moreover, farmers occasionally buy birds from LBMs and introduce them into their premises without consideration for their disease status. Both apparently healthy and sick birds from rural households are taken to the LBMs to be sold. This practice was responsible for the last official outbreak of highly pathogenic avian influenza (HPAI) outbreak in Katsina state, where mortalities due to the highly pathogenic avian influenza virus (H5N1) were reported within a few days of the

introduction of sick guinea fowl purchased from a LBM into a backyard poultry farm (personal communication with affected farmer during post-outbreak investigation).

Trans-border transmission of animal diseases between Nigeria and its neighbours has been reported, and the trans-border activity, especially in live bird trade, contributes to the spread of avian influenza and other avian diseases (Fasina, 2007). The identification of phylogenetically-distinct lineages in Niger republic in 2006, which shared a high similarity with NGCK0108 recovered from a village chicken in 2008 from a live bird market in Kamba, (Kebbi State) illustrates this point. Kamba is a border town between Nigeria and Niger Republic with extensive trans-border trade activities that include live bird trading. The 97% homology between the Niger strains and two Nigerian strains, viz. NGCK0108 and NGCK1707 that were recovered from chickens in Kebbi and Sokoto states in consecutive years, suggests multiple introductions. Isolation dates suggest that the virus spread from Niger Republic to Nigeria, however, the possibility of continuous introduction and re-introduction of these viruses between these countries exists because of the porous borders.

Similarly, the observed relatedness between Nigerian viruses from Damaturu in Yobe state (50km from Borno state which borders Cameroon to the east of Nigeria) and two strains from Cameroon indicate a potential trans-border transmission of viruses through the trade in live birds along the Nigerian and Cameroonian borders. The spread between across the Niger and Cameroon borders is encouraged because most of the Nigerian communities that border these countries share common cultural and language identities.

In spite of the wide spread and economic importance of this disease, vaccination is limited to commercial farms with the vast majority of rural poultry remaining unvaccinated. Although vaccination of chickens with existing Newcastle disease vaccines induces protection against morbidity and mortality from virulent NDV challenge, these vaccines do not fully protect birds so vaccinated birds continue to shed NDV during infection. (Kapcznski and King, 2005). However, studies have shown that vaccination of chickens with homologous ND vaccines can significantly reduce the level of virus shedding when compared with chickens vaccinated with genotypically

heterologous vaccines (Miller *et al.*, 2009). Therefore, the identification of the different genetic lineages of NDV circulating in Nigeria, and their genetic variability together with the variety of hosts that harbours them is necessary to better understand the epidemiology of ND in the region and, by extension the control of ND in Nigeria. This is hinged on the assumption that with the wide spread sub-optimal vaccination practices and the widespread circulation of virulent ND strains in Nigeria, this may facilitate the evolution of virulent NDV (Miller et al., 2007; Miller et al., 2010).. The identification of these variants may help in the production of homologous vaccines that can induce greater protection and reduce virus shedding and spread.

In this study, sequence data (314bp) for 38 additional Nigerian NDV isolates identified as sub-lineages 5g and 5f is presented. This represents the single largest Nigerian NDV sequence data set to date, significantly increasing the molecular information in public databases for that country. Unlike previous studies (Snoeck *et al.*, 2009; Cattoli *et al.*, 2009) where the isolates analysed were obtained from only two geopolitical zones (North West and South West), in the present study, the isolates analysed were obtained from all six geopolitical zones of Nigeria; providing the most comprehensive epidemiological investigation of NDV in Nigeria to date.

Whereas Snoeck *et al.*, 2009 only reported the identification of the new virulent strains in non-commercial farms in Nigeria, this study identified the new strains in live bird markets, backyard and commercial farms and reports for the first time on the epidemiological links between the velogenic NDV (sub-lineages 5f and 5g) from LBMs and the strains recovered from outbreaks in backyard and commercial poultry farms in Nigeria.

The marked heterogeneity observed in the lineage 5 in this study, particularly the identification of a specific clade in sub-lineage 5f (Figure 4.7) which was not reported by Snoeck *et al.,* (2009) and Cattoli *et al* (2009) suggests continuing genetic drift of Nigerian NDV strains.

Previous studies reported that NDVs have a long history of evolution in Southern Asia and that the region was one of the original locations for the emergence of new lineages

and the transmission of ND and ND panzootics (Li *et al.,* 2001; Otim *et al.,* 2004; Tsai *et al.,* 2004). Whereas in China the genetic divergence of NDVs isolated has been reported to be a reflection of the fact that village poultry production has flourished extensively along with a large intensive commercial operation where a strict vaccination programme against ND has been practiced for over 20 years (Liu *et al.,* 2002). The high evolutionary rate of NDV is therefore, attributed to the selective immune pressure on the host in response to intensive vaccination programmes (Li *et al.,* 2001).

In Nigeria however, the poultry production system is largely the semi-intensive backyard to the extensive village operation. The majority of the operators in these categories get no financial assistance from government, thus in an attempt to cut production costs, they rely on questionable suppliers and untrained personnel both vaccines and vaccine administration respectively, with poor results.

Another dimension to the problem is the buying of exotic breeds, especially cockerels, with history of limited ND vaccination from commercial hatcheries. Poultry vendors raise them for a few weeks before trading them to rural dwellers that mix them with their own multi-specie indigenous stock. These practices, aided by unauthorised importation of a wide range of vaccines, including ND vaccines, from Europe and other parts of the world has lead to wide-scale abuse and indiscriminate use of ND vaccines.

Therefore, it seems likely that vaccination pressure due indiscriminate intensive vaccination by some commercial farms may have lead to selective immune pressure which have contributed to the accelerated genetic drift observed in Nigerian poultry systems. Alternatively, improperly applied vaccines may have lead to emergence of immune escaped mutants. Also, it is likely that the progenitors of the new sub-lineages could have entered Nigerian flocks some time ago without detection, and the accumulation of point mutations might have been going on in the largely unvaccinated rural poultry population over an extended period. The interplay between the supply and retail system of the live bird markets might have helped in the spread with spill over to the commercial flock.

In this study, sub-lineage 5g, previously reported in Burkina-Faso (Snoeck *et al.*, 2009) was not identified in Nigeria. Although it is recognised that the strain may be present in Nigeria yet undetected in the limited present isolates, its absence could be attributed to several factors too: trans-border live bird trade between the two countries may be low. Moreover, the two countries do not share direct international borders. Similarly, the same reason may be advanced for the lack of detection of the sub-lineage 5d reported for South Africa, Sudan and some parts of the world. However, routine surveillance for NDVs in all sectors of the poultry operations may reveal otherwise, because there are cases of illegal importation of poultry and poultry products from Egypt and the Middle East by certain farmers.

So far, molecular studies on Nigerian NDV isolates reported the detection of PPMV-1 in pigeon and a dove (Snoeck *et al.*, 2009) .This study reports for the first time the isolation and molecular characterization of PPMV-1 in a chicken in Nigeria. If allowed to circulate, PPMV-1 can increase in virulence for chickens (Alexander and Parsons, 1986; Kissi and Lomniczi, 1988; Kommers, *et al.*, 2001).

This study provide insights on the strains of NDVs that circulate in a variety of poultry species found in Nigerian LBMs, which were found to be largely virulent strains. The result is in agreement with previous reports that strains prevalent in Nigeria are the highly virulent and are kept in circulation by a population of scavenging rural fowl including the domestic water fowl (Nawathe *et al.*, 1975; Adu *et al.*, 1985).

Based on genetic distance measurement, phylogenetic tree analysis, residue substitution analysis and GIS mapping and analysis, it can be concluded that, velogenic NDV circulates in Nigerian live bird markets and by extension the Nigerian poultry industry, and currently consists principally of lineage 5f and 5g strains.

Even though this study was able to establish that NDVs in Nigeria circulate in LBMs in the North and spread to the South through major transport roads by live bird vendors, the widespread identification of strains with high similarities across the six zones raises questions as to whether there are other ways which aid in the spread.

The activities of nomadic herdsmen suggest they could play a role in the spread of NDVs. The Fulani tribe; who are the largest nomadic group in the world, are found mainly in West Africa. They are primarily nomadic herders and traders, who through their nomadic way of living; have established numerous trade routes in West Africa (http://wwwafriquide.com/culture/tribes.html:IRIN).

In Nigeria, animal husbandry is mainly of the pastoral type: a nomadic system under which the herdsmen move around with their herds (cattle, camel, sheep and goats) and a limited number poultry like chickens ducks and guinea fowl which they often sell in live bird markets along their routes.

There are about 15 million pastoralists in Nigeria whose livelihood depends on nomadic herding; they move Southwards as far as the deciduous forest during the dry season (October to April) and Northwards as far as the Sahel during the wet–rainy (May to September) season. This movement system has an international dimension as herdsmen from neighbouring West African countries move into Nigeria during the dry seasons for grazing. It would be interesting to establish whether or not the activities of the nomadic Fulani in Nigeria, and by extension in other West African countries, contribute to the spread of ND within the country and between countries across international borders. A collaborative international surveillance between the Economic Community of West African Countries (ECOWAS) for NDV in poultry kept by the nomadic Fulani would facilitate this.

Recommendations /Further studies

The elimination of the LBMs in Nigeria, like most parts of the world, remains an impossible task largely due to traditions and consumer preference. However, with proper information and regular meetings between LBM operators and the local veterinary authorities to discuss and adopt bio-security measures, the risk of LBMs acting as reservoirs or source of velogenic ND and other poultry diseases can be mitigated.

The detection of velogenic NDVs in the majority of the samples analysed is of great concern, thus the need for continuous surveillance and characterization of NDV from Nigeria to understand the emergence of new lineages and sub-lineages in Nigerian poultry and by extension the West African sub-region. Also, a targeted ND surveillance in poultry kept by nomads in the Nigeria should be carried out, alongside a collaborative surveillance initiative by member countries of the West African sub-region.

In spite of the devastating effect of ND on the Nigerian poultry industry, it is only recently that the causative genetic lineages have been determined. Prompt detection and differentiation of evolving NDV strains is essential for the control of ND. However, the challenge in Nigeria is that diagnostic capacity to differentiate and characterise the various circulating and emerging NDV strains is lacking.

The global threat of the highly pathogenic avian influenza (HPAI) which was officially reported in Nigeria in 2006 (Joannis *et al.,* 2006) was a wake-up call. Since the report of the first outbreak, the Nigerian government in collaboration with other organizations funded avian influenza (AI) surveillance. Some of the ND viruses used in this study were recovered during the course of this AI surveillance. It is therefore hoped that this will continue on a regular basis so as to expand the data base of ND in Nigeria.

There is also the need for collaborative work with better equipped laboratories in Africa and other part of the world, both in terms of capacity building and sample analysis.

With increasing reports of outbreaks of ND in vaccinated flocks, it suggests that these new NDV variants are becoming more virulent and the poultry may not be completely

protected by the present conventional vaccines. Hence, cross-protection testing need to be done to determine whether the emergence of new lineages and sub-lineages could be responsible for Newcastle disease outbreaks in vaccinated flocks along with studies on the molecular determinants responsible for the antigenic variation. It is also anticipated that challenge studies using the emerging strains need to be carried out with a view to produce homologous vaccines that would further reduce shedding and promote better control.

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APPENDIX: A GPS LOCATION AND DATE OF COLLECTION OF ISOLATES

DATE	LAB.CODE	SPECIE	STATE	LATITUDE	LONGITUTE
17/07/2008	KB/KMB/C22-26	Chicken	Kebbi	11.866389	3.656944
16/07/2008	KT/CH/C18-21	Chicken	Katsina	12.541944	7.706667
26/11/2007	07/676WK	Chicken	Taraba	7.7638	11.22418
06/02/2008	08/302A	Chicken	Akwa Ibom	5.0317	7.92906
10/03/2008	08/514A	Chicken	Cross River	4.993611	8.316667
05/03/2008	08/225	Chicken	Plateau	9.862778	8.916944
06/03/2008	08/166	Chicken	Plateau	9.679722	8.833889
18/04/2008	08/201	Chicken	Plateau	9.866667	8.933333
25/03/2008	08/149	Chicken	Plateau	9.6	8.766667
16/04/2008	08/196	Chicken	Plateau	9.733333	8.783333
16/07/2008	08/316A	Chicken	Gombe	10.290001	11.17793
30/06/2008	08/378B	Chicken	Bayelsa	4.91922	6.26805
17/07/2008	KB/KMB/22-26B	Chicken	Kebbi	12.033333	4.25
19/11/2007	07/652	Chicken	Kano	11.562222	8.4825
20/10/2007	07/640A	Chicken	FCT	7.37467	8.72523
13/11/2007	07/624C	Chicken	Sokoto	12.90462	5.16768
21/11/2007	07/644	Chicken	Edo	6.5825	5.911111
11/11/2007	07/609	Chicken	Ekiti	7.836111	5.152222
19/12/2007	07/590	Chicken	Zamfara	7.0831112	12.300125
12/03/2007	07/687K-D	Chicken	Nassarawa	8.309167	8.725833
19/11/2007	07/625K4	Chicken	Kano	8.65918	11.48878
12/07/2007	07/648A	Chicken	Oyo	3.81065	8.11623
14/12/2007	07/595	Chicken	Edo	7.1875	6.463333
11/04/2007	07/597D3	Duck	Yobe	12.18592	11.25127
11/04/2007	07/597D1	Duck	Yobe	12.18592	11.25127
02/08/2007	07/97D	Chicken	Kano	8.55662	12.02224
26/11/2007	07/675FGC	Chicken	Kano	8.55	11.166666
23/11/2007	07/649Y1	Chicken	Yobe	12.12161	10.826
26/11/2007	07/675/DBC	Chicken	Kano	8.466666	11.783333
12/07/2007	07/648A D4	Chicken	Oyo	3.81666	7.98333
24/12/2007	07/734A	Chicken	Jigawa	12.09984	9.1124
06/04/2007	07/691A	Chicken	Edo	6.251111	5.58
12/03/2007	07/660A	Guinea-fowl	Zamfara	5.55357	11.55315
24/12/2007	07/736C	Chicken	FCT	7.0751	8.90583
12/03/2007	07/687/K-PG	Pigeon	Nassarawa	8.42765	7.53332
23/11/2007	07/649Y2	Chicken	Yobe	12.22716	11.26035
12/03/2007	07/687K-C	Chicken	Nassarawa	8.42765	7.53322
17/12/2007	07/722C	Chicken	Kano	8.65918	11.48878
24/12/2007	07/732A	Chicken	Lagos	6.37727	3.22746
26/11/2007	07/673-GR-GF	Guinea-fowl	Niger	9.033611	6.190278

25/08/2007	07/697	Chicken	Ebonyi	6.220833	8.263611

APPENDIX: B

agriculture
Department:
Agriculture
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF AGRICULTURE
REPUBLIC OF SOUTH AFRICA

Directorate of Veterinary Services
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa

Tel: (27)-012-3197514
Fax: (27)-012-3298292

IMPORTER:
ARC-ONDERSTEPOORT VETERINARY INSTITUTE
OLD SOUTPAN ROAD
PRETORIA
0110

PERMIT NO: 13/1/1/30/2/9/6-137
Valid from: 2008-12-05
Expiry date: 2009-06-05

VETERINARY IMPORT PERMIT FOR SPECIMENS
(Issued in terms of the Animal Diseases Act, 1984)

Authority is hereby granted for you to import 55 VIALS CHEMICALLY-INACTIVATED NEWCASTLE DISEASE VIRUS STRAIN (IN LYSIS BUFFER-ROCHE) into Republic of South Africa:

from: DR. SOLOMON PONMAN, VETERINARY RESEARCH INSTITUTE, P.O. BOX 01, VOM, PLATEAU STATE, NIGERIA
subject to the following conditions:

1. the consignment must be accompanied by this original permit;
2. the NEWCASTLE DISEASE VIRUS STRAINS to be securely packed and transported in leakproof containers, sealed by an authorised official of the Veterinary Administration of the exporting country;
3. the consignment must be airfreighted through port of entry O.R.TAMBO INTERNATIONAL AIRPORT. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
4. the **NEWCASTLE DISEASE VIRUS STRAINS** must be kept and used for purposes of testing/research at the laboratories of ARC-ONDERSTEPOORT VETERINARY INSTITUTE, ONDERSTEPOORT under the personal supervision of DR. C. ABOLNIK
5. on completion of tests/research the NEWCASTLE DISEASE VIRUS STRAINS must be destroyed by incineration;
6. The State Veterinarian: KEMPTON PARK Tel: 011-973 2827 must be advised timeously of the arrival of the consignment.
7. **This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import.**
8. This permit is subject to amendment or cancellation by the Director Veterinary Services at any time and without prior notice being given.
9. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.

M Lewis
For **DIRECTOR: VETERINARY SERVICES**

NOTE:
From 1st January 2005 any consignment imported into South Africa packed with either wood packing material or dunnage, will require treatment to remove any pests present (by heat or methyl bromide fumigation). Treatment must be indicated on packing material. [Enquiries: Directorate Plant Health & Quality Fax: 012 319 6350 or www.nda.agric.za]

APPENDIX: C: ESTIMATES OF EVOLUTIONARY DIVERGENCE BETWEEN SEQUENCES

Model: Nucleotide: Maximum Composite Likelihood method in MEGA4
There were a total of 314 positions in the final dataset
Lineage 1 sequence in this study in boldface

[1] FJ810487_NDV08-063
[2] EU847310_NIAH16566-06
[3] EU547760_APMV/duck/10/07
[4] EU419321_NDV07-64
[5] AF532743_ZA37401/X/01
[6] AF532741_ZA341/P/99
[7] AF532147_ZA348/B/00
[8] FM200800_chicken/Nigeria/SH11/2005
[9] EU547759_APMV/duck/09/07
[10] AY175657_AU--90100
[11] AY175660_AUCK98028
[12] AY935499_I-2
[13] AF503639_shelduck/France/MB20/76
[14] NGCK1907

[	1	2	3	4	5	6	7	8	9	10	11	12	13	14]
[1]														
[2]	0.000													
[3]	0.000	0.000												
[4]	0.000	0.000	0.000											
[5]	0.000	0.000	0.000	0.000										
[6]	0.000	0.000	0.000	0.000	0.000									
[7]	0.000	0.000	0.000	0.000	0.000	0.000								
[8]	0.003	0.003	0.003	0.003	0.003	0.003	0.003							
[9]	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.006						
[10]	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.019	0.019					
[11]	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.023	0.023	0.036				
[12]	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.043	0.043	0.057	0.046			
[13]	0.043	0.043	0.043	0.043	0.043	0.043	0.043	0.046	0.046	0.046	0.064	0.071		
[14]	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.010**	**0.010**	**0.023**	**0.026**	**0.040**	**0.043**	

Model: Nucleotide: Maximum Composite Likelihood method in MEGA4
There were a total of 314 positions in the final dataset
Lineage 2 sequence in this study in boldface

[1] AY845400_LaSota
[2] GQ901891MB061/06
[3] FJ938174_sparrow/Guangxi/NN10/07
[4] FJ810494_NDV08-071
[5] FJ810485_NDV08-060
[6] FJ810483_NDV08-058
[7] FJ810482_NDV08-057
[8] FJ810435_NDV08-009
[9] EU418738
[10] EU296496_TW/84-361
[11] FM200802_chicken/Nigeria/N18/2006
[12] FM200801
[13] NGCK1008

[	1	2	3	4	5	6	7	8	9	10	11	12	13]
[1]													
[2]	0.000												
[3]	0.000	0.000											
[4]	0.000	0.000	0.000										
[5]	0.000	0.000	0.000	0.000									
[6]	0.000	0.000	0.000	0.000	0.000								
[7]	0.000	0.000	0.000	0.000	0.000	0.000							
[8]	0.000	0.000	0.000	0.000	0.000	0.000	0.000						
[9]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
[10]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
[11]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
[12]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
[13]	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	

Model: Nucleotide: Maximum Composite Likelihood method in MEGA4
There were a total of 314 positions in the final dataset
Lineage 4 sequence in this study in boldface

[1] DQ202263_grouseRussiaKhabarovsk2004
[2] AY471782_PAEPI97415
[3] AY471781_PAEPI96210
[4] AY175751_PAEPI96210
[5] AY471785_PAEKE99364
[6] AY471784_PAEKE98398
[7] AY471783_PAEKE98373
[8] AY471779_PAEPI98367
[9] AY471780_PAEPI99361
[10] DQ289796_I-1
[11] AY734536_PigeonArgentina3/97
[12] AY471773_PTRBU95211
[13] FM200798_parrotNigeriaNIE139/2007
[14] AY150115_IT-125/87
[15] AY175776_TRCK95111
[16] FJ410147_PPMV-1/Maryland/1984
[17] FJ410145_PPMV-1New_York/1984
[18] EF520716_pigeon/NY/US/1984
[19] AY288996_pigeon/Italy/1166/00
[20] AY471857_strain_PIQPI78442
[21] NGCK1208

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
[1]																					
[2]	0.026																				
[3]	0.026	0.006																			
[4]	0.026	0.006	0.000																		
[5]	0.026	0.019	0.019	0.019																	
[6]	0.026	0.019	0.019	0.019	0.006																
[7]	0.026	0.019	0.019	0.019	0.006	0.000															
[8]	0.029	0.010	0.010	0.010	0.023	0.023	0.023														
[9]	0.033	0.013	0.013	0.013	0.026	0.026	0.026	0.016													
[10]	0.029	0.029	0.029	0.029	0.029	0.029	0.029	0.033	0.036												
[11]	0.043	0.036	0.036	0.036	0.023	0.023	0.023	0.040	0.043	0.046											
[12]	0.029	0.029	0.029	0.029	0.029	0.029	0.029	0.033	0.036	0.000	0.046										
[13]	0.047	0.040	0.040	0.040	0.040	0.040	0.040	0.043	0.047	0.036	0.057	0.036									
[14]	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.036	0.040	0.010	0.050	0.010	0.040								
[15]	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.036	0.040	0.003	0.050	0.003	0.040	0.013							
[16]	0.043	0.036	0.036	0.036	0.036	0.036	0.036	0.040	0.043	0.026	0.054	0.026	0.043	0.029	0.029						
[17]	0.043	0.036	0.036	0.036	0.036	0.036	0.036	0.040	0.043	0.026	0.054	0.026	0.043	0.029	0.029	0.000					
[18]	0.043	0.036	0.036	0.036	0.036	0.036	0.036	0.040	0.043	0.026	0.054	0.026	0.043	0.029	0.029	0.000	0.000				
[19]	0.043	0.036	0.036	0.036	0.036	0.036	0.036	0.040	0.043	0.019	0.047	0.019	0.043	0.023	0.023	0.033	0.033	0.033			
[20]	0.043	0.036	0.036	0.036	0.036	0.036	0.036	0.040	0.043	0.026	0.054	0.026	0.040	0.030	0.030	0.006	0.006	0.006	0.033		
[21]	**0.019**	**0.026**	**0.026**	**0.026**	**0.026**	**0.026**	**0.026**	**0.030**	**0.033**	**0.036**	**0.043**	**0.036**	**0.040**	**0.040**	**0.040**	**0.043**	**0.043**	**0.043**	**0.043**	**0.043**	

Model: Nucleotide: Maximum Composite Likelihood method in MEGA4
There were a total of 314 positions in the final dataset
Lineage 5 sequence in this study in boldface

[1] CK/Niger/38/2007
[2] NGCK1807
[3] NGCK2307
[4] NGCK2507
[5] NGDK2607
[6] NGCK3207
[7] NGCK4207
[8] NGCK0908
[9] NGCK0608
[10] NGCK1507
[11] NGCK2807
[12] NGCK2907
[13] NGCK3307
[14] NGCK3407
[15] NGCK3807
[16] NGCK4407
[17] NGCK4807
[18] NGDK2707
[19] NGGF4907
[20] NGGF5307
[21] NGCK0108
[22] NGCK0208
[23] NGCK0708
[24] NGCK0808
[25] NGCK1707
[26] NGCK4507
[27] NGCK1108
[28] NGCK1308
[29] NGCK1607
[30] NGCK4307
[31] NGCK0508
[32] NGCK2007
[33] NGCK2107
[34] NGCK2207
[35] NGCK3507
[36] NGCK4007
[37] NGPG5207
[38] NGCK0408
[39] CK/Burkina Faso/SKF4.1/2006
[40] CK/Burkina Faso/SKF4.2/2006
[41] CKZA04AL500
[42] CKZA05UP1178
[43] CK/Sudan/04/2003
[44] CK/Sudan/05/04
[45] CK/Sudan/08/04
[46] Goose_paramyxovirus_HZ
[47] JS/3/98/Go
[48] NGCK0307
[49] FJ772449_avian913/33Nigeria2006
[50] FJ772475_CK/2602/605/Niger/2008
[51] FJ772478_CK3490/149Cameroon2008
[52] FJ772484_CK3490_147Cameroon2008
[53] FJ772481_CK/2602/625/Niger/2008
[54] FJ772486_avian/Nigeria2008
[55] FJ772472_CK/2602/468/Niger2008
[56] FJ772463_CK2415/580/B/_Faso2008
[57] FM200805_CKB/_Faso/5.18/2006
[58] FM200804_CKB/_Faso/5.2/2006
[59] FJ772469_CK/2602/348/Niger2008
[60] FJ772446_avian913/1Nigeria2006
[61] FJ772458_CKB/_Faso/2008
[62] FM200796_fowlNigeriaNIE93/2007