MOLECULARCHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATES FROM CHICKENSIN ZARIA AND ITS ENVIRONS, KADUNA STATE, NIGERIA

BY

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BY

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DECLARATION

I declare that, the work in this Dissertation entitled "**Molecular characterization of Newcastle disease virus isolates from chickens in Zaria and its environs, Kaduna State,Nigeria**" has been carried out by me in the Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Student SignatureDate

CERTIFICATION

This Dissertation entitled "MOLECULAR CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATES FROM CHICKENS IN ZARIA AND ENVIRONS, KADUNA STATE, NIGERIA by Tasiu Mallam HAMISU meets the regulations governing the award of the degree of Masters of Science in Veterinary Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literarypresentation.

Prof. H.M. Kazeem Chairman, Supervisory Committee	_____________ Signature	____________ Date
Dr. K.A. Majiyagbe Member Supervisory Committee	_____________ Signature	____________ Date
Prof. Lawal Sa'idu Member Supervisory Committee	_____________ Signature	____________ Date
Dr. (Mrs.) C.N. Kwanashie Head of Department	_____________ Signature	____________ Date
Prof. Kabir Bala Dean, School of Postgraduate Studies	_____________ Signature	____________ Date

DEDICATION

This study is dedicated to my late father, Mallam Hamisu Jibrin, my late mother, Hadiza M. Hamisu and my late brothers, Rabiu M. Hamisu andAdamu M. Hamisu for their love, support and encouragement before our separation. May Allah have mercy on all of them, amen.

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ABSTRACT

Newcastle disease virus (NDV) has a negative-sense,single-strand RNA genome.Although all NDVs are members of Avian Paramyxovirus-1 (APMV-1), antigenic and genetic diversity is recognized. Consequently, different genotypes of APMV-1 circulate in different parts of the world. Therefore, this study aimed to characterize circulating field NDV strains in Zaria and identify their genotype/sub-genotype. A total of 127cloacal swabs were collected from local chickens in live bird market and exotic chickens in commercial poultry farms in Zaria and environs, Nigeria between November, 2014 and January, 2015. Five commercial poultry farms and four live bird markets were purposively sampled.Molecular screening of NDV Matrix-gene (M-gene) was performed on all the samples using ReverseTranscriptase-Polymerase Chain Reaction (RT-PCR). Newcastle disease positive samples were further inoculated in to embryonated chicken eggs for isolation of NDV. Isolates were confirmed as NDV by haemaggulitination (HA) test and detection of the Fusion-gene using RT-PCR. The partial F-gene amplicons (containing nucleotide position 610 of NDV M-gene to position 581 of NDV F-gene) were sequenced in Inqaba Biotechnology, South Africa and the results were analyzed using BioEdit sequence analysis program and MEGA 6.6 software. Newcastle disease virus M-gene were detected in 16 out of 127 cloacal swabs; 13 from live bird markets and 3 from commercial poultry farms. However, only 10 NDVs were isolated in embryonated chicken eggs as confirmed by HA and RT-PCR. Eight of the 10 amplicons of the partial F-gene were successfully sequenced. Analysis of the 47-419 nucleotide region of the partial F-gene of these sequences revealed that all the NDVs isolated in this study were virulent and based on the bootstrap value of>60% and the tree topology, all the strains belonged to sub-genotype XIVb. Three strains from this study shared common amino acid mutations $P \rightarrow L^{10}$ and $A \rightarrow V^{11}$ as the

representatives of sub-genotype XIVb. In addition, one strain from this study shared$L \rightarrow P^{36}$ common amino acid mutation with two representative strains of sub-genotype XIVb from Nigeria and Benin Republic. However, when compared with two representatives of sub-genotype XIVb, all strains from this study had $R \rightarrow Q^{114}$ mutations. This study therefore, highlights the importance of continuous surveillance, characterisation and phylogenetic study of NDV in Nigeria so that effective control andpreventive measures against the disease can be implemented.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Newcastle Disease Virus (NDV), or avian paramyxovirus type 1 (APMV-1), is an enveloped, non-segmented, negative-sense, single-stranded RNA virus belonging to the Genus *Avulavirus* of the Family *Paramyxoviridae* (Mayor, 2002; Alexander and Senne, 2008).The NDV genome is composed of six genes and encodes their corresponding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN),RNA polymerase (L) (Chambers *et al.,* 1986). Among these genes, the Fusion protein mediates the fusion of virus with the cellular membranes and it originates from its precursor F_0. The cleavability of F_0 protein is the major determinant for virulence (Peeters *et al*., 1999). The variation in the virulence of NDV is mediated by amino acid variation at the cleavage site of F_0 and availability of protease enzymes to cleave F_0 in different tissues (Nagai *et al*., 1976; Chambers *et al*., 1986; Glickman *et al*., 1988). Fewer basic amino acids are present in the fusion protein cleavage site of lentogenic NDV isolates than in the proteins of either mesogenic or velogenic strains, which have similar cleavage site sequences (Glickman *et al*., 1988).

Newcastle disease (ND) results from infections with virulent Newcastle disease viruses (NDV), having intracerebral pathogenicity indices (ICPI) of 0.7 or greater than this, in day-old chickens (*Gallus gallus*) and/or having multiple basic amino acids (at least three arginine (R) or lysine (K) residues) at the C-terminus of F_2 starting at position 112 to 116, with a phenylalanine at position 117 of the N-terminus of F_1 (OIE, 2012).

Newcastle disease (ND)is one of the most important diseases in poultry production worldwide and remains a serious problem in spite of control measures, including vaccination which has been applied since 1950s (Czeglédi *et al*., 2006). Its global impact is enormous because ND servers as a major barrier to international trade in poultry and poultry products and a great drain on the world economy (Alexander, 2003).

Newcastle disease had been reported from all parts of Nigeria (Hill *et al*., 1953; Fatumbi and Adene, 1979; Ezeokoli *et al.,* 1984; Gomwalk *et al.,* 1985; Baba *et al.,* 1995). The disease was also reported to be more common during the dry harmattan season (November-March) (Sa'idu *et al*., 1994; Halle *et al*.,1999; Abdu *et al*.,2005). Cold stress has been known to worsen the outcome of Newcastle disease (Abdu *et al*., 1992). It is very clear that persistent virus infections or carrier states in virus infections are responsible for a wide range of human and animal diseases (Abe *et al*., 2007). In Nigeria, NDV has been noted to be widespread due to rapid expansion of the poultry industry, high stocking densities and inadequate biosecurity measures which created conditions conducive for the spread and maintenance of the endemicity of the disease (Okwor and Eze, 2010). The incubation period in ND infection after natural exposure varies from 2-15 days with the average of about 5 days (Kahn, 2005). Experimental data using Nigerian velogenic strain of NDV have shown an incubation period of 2-5 days depending on age and immune status of the birds (Okwor *et al*., 2007). The usual source of infection for healthy commercial and village chickens are infected chickens which are suffering from the disease and shedding the virus and those that are incubating the virus and shedding them at the same time (Nwanta *et al*., 2008). Virus excretion commences before clinical signs occur, therefore, those with longer incubation period possess greater danger.

Different NDV strains vary greatly in pathogenicity. NDV isolates can be broadly grouped into three pathotypes on the basis of clinical signs in infected chickens. These are; lentogenic, mesogenic, and velogenic strains (Alexander, 2000). Lentogenic strains are the least virulent, mesogenic strains are moderately virulent, and velogenic strains are the most virulent. Both mesogenic and velogenic strains are classified as virulent strains (Jindal *et al*., 2010). The virulent strains can cause high morbidity and mortality in commercial poultry, leading to considerable economic losses (Jindal *et al*., 2010).

The first documented outbreak of the disease in Nigeria occurred between December 1952 and February 1953 in and around Ibadan (Hill *et al.*, 1953). Since then the disease has been reported to be endemic in both Local and Commercial poultry with annual epidemics being recorded in highly susceptible poultry flocks (Adu *et al*., 1986; Sa'idu *et al*., 1994; Halle *et al*., 1999; Orajaka *et al*., 1999).

Newcastle disease is being controlled by routine vaccination in many countries (Okoye *et al*., 2001). Outbreaks of Newcastle disease in vaccinated flocks have been reported in many parts of Nigeria (Adu *et al*., 1999). It is often speculated that the frequent outbreaks of Newcastle disease in vaccinated flocks are due to many factors, such as presence of antigenic differences between strains of Newcastle disease virus, low maternal antibodies, improper vaccine administration and the use of a non-viable vaccines (Okoye *et al.*,2001; Sai'du *et al*.,2006). However, Adu *et al.*, (1989) reported that outbreaks of Newcastle disease in vaccinated flocks often reported in the field may not only be due to differences in the antigenicity of wild field strains and vaccine strains of the virus but could also be as a result of differences in the pathogenicity and virulence between different strains of Newcastle disease virus vaccines.

1.2 Statement of the Research Problem

Outbreaks of Newcastle disease were first reported for poultry from Java, Indonesia, and Newcastle-Upon-Tyne, England, in 1926.Since then, several genetic groups of Newcastle disease virus (lineages or genotypes) have been identified among the APMV-1(Aldous *et al*., 2003; Cattoli *et al*., 2010; Miller *et al*., 2010), and phylogenetic studies have shown that viruses of distinct genetic groups undergo simultaneous evolutionary changes in different geographic locations of the world (Miller *et al*., 2009).

An average of 200-250 outbreaks of Newcastle disease wass reported in Nigeria annually (Okeke and Lamorde, 1988). In Zaria, Newcastle disease was reported to be the most prevalent disease of local and exotic birds (Abdu *et al*.,1985; Sa'idu *et al*., 1994; Halle *et al*., 1999). The disease was reported to be the most important cause of death in chickens in Zaria (Abdu *et al*., 1992). Although there are various efforts such as vaccination aimed at controlling Newcastle disease in Nigeria, severe forms of ND continued to occur even in ND vaccinated flocks (Abdu *et al*., 2008).

1.3Justification of the Study

Poultry production remains a viable venture for sustainable livelihood to the average Nigerian family, both in terms of guaranteed supply of animal protein and economic returns and provision of employment to our teeming unemployed youth. 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 (Solomon, 2010).

Since the time of its first isolation in England in 1926, ND remained one of the deadly viral diseases of poultry in the world. It is also considered as one of the major economic threats to poultry population because of its high morbidity and mortality varying from 90-100% depending on the type of infecting Newcastle disease virus (Brandly, 1950). Most countries where poultry is raised commercially and where the disease is endemic rely on vaccination to keep the disease under control. However, researches have indicated that commercially available ND vaccines are not giving adequate protection against NDV (Czeglédi *et al*., 2006). While the causes of this apparent inadequate optimum performance in the field are not clear, the efficacy of commercially available vaccines is being questioned. Furthermore, the findings of previous studies suggested that available vaccines induced better protection against viruses that were isolated in past epizootics than against viruses that were circulating at the time of these studies (Kapczynski and King, 2005). Furthermore, it has also been shown that genotype-heterologous vaccines are less effective in preventing virus replication and shedding after challenge (Miller *et al*., 2007, 2009). Therefore, evolutionary dynamics impose significant difficulties for disease control and diagnosis (Kim *et al*., 2007b; Cattoli *et al*., 2010;Miller *et al*., 2010; Rue *et al.,* 2010). Additionally, as with other single-stranded RNA viruses, avian paramyxoviruses have a significant genetic variability. Two major genetic APMV-1 lineages have been described by Czegledi *et al*., (2006). In the previously years, Aldous *et al*., (2003) proposed that genotyping of NDV isolates should become part of diagnostic virus characterisation for reference laboratories. Such analysis should allow rapid epidemiological assessment of the origins and spread of the viruses responsible for ND outbreaks so that effective control measures against the disease can be implemented.

At present, limited data are available regarding molecular characterization of field isolates of NDV in Nigeria and Zaria in particular.

1.4Aim of the Study

To identify theNewcastle disease virus genotype circulating in live bird markets and commercial poultry farms in Zaria

1.5 Objectives of the Study

The objectives of this study were to:

1. Detect and isolate Newcastle disease virus in chickens from commercial poultry farms and live bird markets using Polymerase Chain Reaction and embryonated chicken eggs respectively
2. Determine partial nucleotides sequences of NDV F-gene in the isolates
3. Establish the relationship existing between circulating Newcastle disease virus strains in the study area and those reported from other parts of the world

1.6 Research Questions

1. Are there Newcastle disease viruses circulating in Zaria?
2. What are the strains of Newcastle disease viruses in Zaria?
3. To what genotype/sub-genotype do the circulating Newcastle disease viruses in Zaria belong?

CHAPTER TWO

LITERATURE REVIEW

2.1Newcastle Disease Virus

The three virus Families; *Rhabdoviridae*, *Filoviridae and Paramyxoviridae* form the Order *Mononegavirales*, i.e. viruses with negative sense, single-stranded, non-segmented, ribonucleic acid genomes (Kingsbury *et al.*, 1995). Newcastle disease is caused by avian paramyxovirus serotype 1 (APMV-1) viruses, which together with viruses of the other eight APMV serotypes (APMV-2 to APMV-9), have been placed in the Genus *Avulavirus*, Sub-family *Paramyxovirinae*, Family *Paramyxoviridae*, (Kingsbury *et al.*, 1995). Studies involving the sequencing of the whole ND virus (NDV) genome have suggested that avian paramyxoviruses are sufficiently different from other rubulaviruses to warrant the creation of a separate genus (DeLeeuw and Peeters, 1999).

Newcastle disease virus contains a non-segmented single-stranded RNA genome of negative polarity with a size of 15186 nucleotides (Krishnamurthy and Samal, 1998; Phillips *et al*., 1998; de Leeuw and Peeters, 1999; Römer-Oberdorfer *et al*.,1999) and contains six genes encoding the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin–neuraminidase protein (HN) and large polymerase protein (L) (Lamb and Kolakofsky, 1996).

Strains of NDV can also be distinguished on the basis of the clinical signs produced in infected chickens. Beard and Hanson (1984) defined the following five groups or pathotypes:

viscerotropic velogenic: viruses responsible for disease characterised by acute lethal infections, usually with haemorrhagic lesions in the intestines of dead birds;

neurotropic velogenic: viruses causing disease characterised by high mortality which follows respiratory and neurological disease, but in which gut lesions are usually absent;

mesogenic: viruses causing clinical signs consisting of respiratory and neurological signs, with low mortality;

lentogenic: viruses causing mild infections of the respiratory tract;

asymptomatic enteric: viruses causing avirulent infections in which replication appears to occur primarily in the gut.

Although these categories are useful for descriptive purposes, some overlapping does occur and some viruses are difficult to place (Beard and Hanson, 1984).

2.1.1 Biological properties of Newcastle disease virus

2.1.1.1 Haemagglutination activity

Newcastle disease virus agglutinates red blood cells through binding its haemagglutinin protein (HN) to the receptors on the surface of the red blood cells (OIE, 2012). The virus agglutinates red blood cells of all amphibians, reptiles and avian cells. All Newcastle disease virus strains agglutinate red blood cells of human, mouse and Guinea pig while Newcastle disease virus agglutination with red blood cells of cattle, sheep, swine and horse varies with the strain of Newcastle disease virus (Alexander and Senne, 2008).

2.1.2 Physico-chemical properties of Newcastle disease virus

Heat, irradiation, pH and several chemical agents destroy the infectivity of Newcastle disease virus. However, as Alexander and Senne (2008) reported, the virus strain, the duration of exposure, viral quantity and medium of suspension all affect the rate of the destruction.

2.2Classification of Newcastle Disease Virus into Genotypes/Lineages

Base on genomic size and the nucleotide sequences of the F and L genes, NDV strains can be categorized as class I or class II (Alexander, 2008). Class I NDVs, which have a genomic size of 15,198 nucleotides (Liu *et al*., 2009), are occasionally isolated from wild aquatic birds and domestic poultry and are mostly avirulent to chickens (Alexander, 2008). Class II NDVs comprise the majority of virulent NDV strains and some avirulent NDV strains (Alexander, 2008). The class II NDV is further divided into 11 genotypes (I–XI) (Zhang *et al*., 2011). The groups VI and VII can further be subdivided into sub–genotypes VIa to VI–h (Biagini *et al*., 1999; Herczeg *et al*., 1999) and VIIa to VII–f (Munir *et al*., 2012). Besides their genetic variations, these are characterized as individual antigenic subtypes (Alexander *et al*., 1997). Genotype VII has been reported to cause a number of outbreaks in East Asia and Western Europe (Munir *et al*., 2010; Munir *et al*., 2012a; Munir *et al*., 2012b). Early sublineages of Class II NDVs that occurred before the 1960s (genotypes I to IV) have a genomic size of 15,186 nucleotides, whereas late Class II NDV sublineages (genotypes VI to XI) have a genomic size of 15,192 nucleotides (Aldous *et al*., 2003). Aldous *et al.* of (2003) proposed the creation of lineages and sublineages in classifying NDVs to make it possible to rapidly type future virus isolates on the basis of their nucleotide sequence and make inferences about their origins. They proposed that NDVs could be divided into six broadly distinct groups (lineages 1 to 6), where lineages 3 and 4 were further subdivided into four sublineages (a to d) and lineage 5 was further subdivided into 5 sublineages (a to e). Genotypes I and II correspond to lineages 1 and 2, while genotype III corresponds to sublineage 3a; genotype IV to sublineage 3b; genotype V to sublineage 3c; genotype VIII to sublineage 3d; genotypes VIa and VIe to sublineage 4a; genotypes VIb, VIc and VId to sublineages 4b, 4c and 4d respectively; genotypes VIIa, VIIb, VIIc and VIId to sublineages 5a, 5b, 5c and 5d respectively; genotype VII NDVs from Taiwan and a quarantine isolate in UK that formed a separate cluster from other lineage 5 NDVs correspond to sublineage 5e; and lineage 6 represents a new NDV genogroup (Cattoli *et al.*, 2010). A novel lineage, provisionally named lineage 7 was reported in West and Central Africa (Cattoli *et al.*, 2010).

2.3Antigenic Relationships

Cross relationships in haemagglutination inhibition (HI) and other tests have been detected between some of the APMV serotypes (Alexander, 1993; Alexander, 1997).

However, use of a variety of serological and non-serological tests has tended to confirm the distinctiveness of the APMV serotypes (Box and Webb, 1988). Antigenic relationships between APMV-1 and other serotypes are important as these may affect diagnosis of ND (Box *et al.*, 1988). Generally, such cross-reactions in serological tests are low, but APMV-3 viruses may show sufficiently high levels of cross reactivity with conventional APMV-1 antisera to cause problems (Box *et al.*, 1988). Apparent antibodies to APMV-3 virus detected in chickens have been attributed to high antibody levels to NDV as a result of vaccination (Box and Webb, 1988).

Antigenic variation between viruses within APMV serotypes has been reported for most of the serotypes where more than a few isolates have been obtained. For NDV (APMV-1), differences detectable by conventional HI tests have been reported, although only rarely (Hannoun, 1977; Arias-Ibarrondo *et al*., 1978; Alexander *et al.*, 1984). One of the most noted variations of this kind has been the virus responsible for the panzootics in racing pigeons that occurred during the 1980s (Alexander and Parsons, 1986). The NDV responsible, referred to as pigeon APMV-1 (PPMV-1), was demonstrably different from standard strains in HI tests, although not sufficiently different that conventional ND vaccines were not protective against Newcastle disease (Alexander and Parsons, 1986).

Monoclonal antibodies (mAbs) raised against various strains of NDV have been used to establish the uniqueness of the variant NDV responsible for the panzootic of ND in pigeons and have proven particularly useful in identifying the spread of this virus around the world (Alexander *et al*., 1985; Alexander *et al*., 1987; Parson *et al*., 1987). Monoclonal antibodies to NDV have also been used to distinguish between specific viruses. For example, two groups have described mAbs that distinguish between the common vaccine strains, Hitchner Bl and La Sota (Erdei *et al*., 1987; Meulemans *et al.*, 1987), while other mAbs can separate vaccine viruses from epizootic virus in a given area (Srinivasappa *et al*., 1986).

Antigenic variation detected by mAb typing has also been used in epidemiological studies. In a large study of over 1,500 viruses, Alexander *et al*. (1997) used the ability of viruses to react with panels of mAbs, consisting initially of nine mAbs and later extended to twenty-eight mAbs, to place strains and isolates of NDV into groups on the basis of ability to react with the different mAbs (Alexander *et al.*, 1997). Viruses in the same mAb group shared biological and epizootiological properties. Use of such panels, particularly the extended panel, has indicated that viruses tend to remain fairly well conserved during outbreaks or epizootics and this often allows valuable assumptions to be made concerning the source and the spread of ND (Alexander *et al.*, 1997).

Variations within other serotypes have been reviewed by Alexander (1993). Marked variations have been recorded between viruses placed in APMV-2, APMV-3 and APMV-7 serotypes (Alexander, 1993). The variations recorded often showed two viruses with little relationship to each other, but each showing strong similarity with a third virus (Alexander, 1993). Ozdemir *et al*. (1990) , produced mAbs against APMV-2 virus and were able to classify isolates from a wide range of hosts and geographical locations into four groups. The antigenic variations in the APMV-3 group seem to have greater significance, as these appear to separate turkey isolates from psittacine isolates (Ozdemir *et al*., 1990). Anderson *et al.* (1987) produced mAbs to APMV-3 virus and the use of these suggested that antigenic variation within the APMV-3 serogroup may be more complicated than suggested using polyclonal antisera.

2.4Characterization of the Newcastle Disease Virus

2.4.1Biological characterization

The extreme variation in virulence of different NDV isolates and the widespread use of live vaccines means that the identification of an isolate of NDV from birds showing clinical signs does not confirm a diagnosis of ND (OIE, 2012). As indicated in the definition, an assessment of the virulence of the isolate by intracerebral pathogenicity index test or amino acid sequencing is also required. In the past, such tests as the mean death time in eggs, the intravenous pathogenicity test and variations of these tests have been used (Alexander and Senne, 2008).

2.4.1.1 Mean Death Time

This is an *in vivo*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).

2.4.1.2 Intracerebral Pathogenicity Index

The intracerebral pathogenicity index (ICPI) is carried out as follows;
Fresh infective allantoic fluid with an HA titre of greater than 24 (1/16) is diluted 1/10 in sterile isotonic saline, with no additives. This is followed by intracerebral injection of 0.05 ml of the diluted virus into each of ten one-day-old chicks hatched from a Specific Pathogen Free (SPF) flock. The birds are then examined every 24hours for eight days. At each observation, the birds are scored: 0 if normal, 1 if sick, and 2 if dead - the ICPI is the mean score per bird per observation over the eight-day period (OIE, 2012).The most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic strains will give values close to 0.0 (OIE, 2012).

2.4.1.3 Intravenous Pathogenicity Index

Fresh infective allantoic fluid with HA titre of >24is 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).

2.4.2 Molecular basis for determining Newcastle disease virus virulence using RT-PCR

NDV is an enveloped virus with two membrane proteins, one of which is involved in cell attachment and release (HN protein) and the other in mediating fusion of the viral envelope with cellular membranes (F protein) (Nagai *et al*., 1976). The F protein is synthesized as a precursor, F0, and is only fusogenic after cleavage into disulfide-linked F_1 and F_2 polypeptides, which is accomplished by host-cell proteases (Nagai *et al*., 1976; Morrison *et al*., 1985; Ogasawara *et al*., 1992). Thus, NDV becomes infective only when the precursor glycoprotein F_0 is cleaved into F_1 and F_2 (Morrison *et al.*, 1985). The ability to cleave F_0 varies among different strains of NDV and is the main determinant of mortality and morbidity in infected chickens (Nagai *et al.*,1976). Phylogenetic studies at the molecular level of the NDV F_0 cleavage site have determined the consensus amino acid sequence ^{112}R/K-R-Q-R/K-R-F^{117} for virulent and mesogenic strains and ^{112}G/E-K/R-Q-G/E-R-L^{117} for NDV strains of low virulence (Collins *et al*., 1993). However, two exceptions to the consensus amino acid sequence for virulent strains have been observed for the so-called pigeon paramyxovirus type 1 (PPMV-1) isolates (Collins *et al*., 1993) These virulent isolates show fusion protein cleavage sites of ^{112}**G**RQKRF^{117} (Collins *et al*., 1993) and ^{112}RR**K**KRF^{117} (Werner *et al*., 1999).

Since the initial studies comparing the deduced amino acid in the cleavage site of the F_0 precursor of a number of virulent and avirulent NDV strains, a large number of studies have confirmed the presence of multiple basic amino acids at that site in virulent viruses (Collins *et al.*, 1993). Therefore, the major influence of pathogenicity of NDV is amino acid motif at the F_0 cleavage site (Rott, 1979). The presence of basic amino acid at position 112-116 and Phenylalanine at position 117 in virulent strains means that cleavage can be affected by protease or proteases present in a wide range of host tissues and organs (Rott, 1979). However, the F_0 cleavage site of avirulent strains can only be cleaved by trypsin-like enzymes, which are found only on endodermal surfaces, such as in the intestinal and respiratory tracts. Consequently, NDV replication is limited to these surfaces (Alexander, 2000). As a distinguishing feature, avirulent viruses cannot produce plaques in tissue culture without trypsin being added to the overlay medium (Alexander, 2000). To date, using these criteria, only infections with velogenic or mesogenic NDVs have been classified as ND (Alexander, 2000).

2.4.3 Phylogenetic studies

Molecular phylogenetics converts the information in sequence data into an evolutionary tree based on the similarities or differences between sequences (Solomon, 2010). 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 (Solomon, 2010). 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 (Solomon, 2010). 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 in constructing phylogenetic trees (Saitou and Neil, 1985). 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, 1985). 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 (Solomon, 2010).

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 (Solomon, 2010). 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 (Felsentein, 1985).

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(Hill and Bull, 1993). 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% (Hill and Bull, 1993).

2.5Newcastle Disease

2.5.1 Historical background of Newcastle disease

The emergence of ND as a highly pathogenic disease of poultry in 1926, initially predominantly in South-East Asia, suggested that a sudden major change had occurred either in the virus or hosts, although earlier outbreaks may have been confused with HPAI (Alexander, 2000). Hanson (1972) considered that the various hypotheses proposed could be grouped in three categories, as follows:

1) The virulent virus has always existed in poultry in South East Asia, but the disease with its enormous economic impact was only recognized as a significant problem when the commercialization of the poultry industry commenced in that part of the world
2) The virus was enzootic in different species, possibly inhabiting tropical rainforests, and spread to domestic poultry due to the incursion of man into that habitat
3) A major mutation occurred in a precursor virus of low virulence

The first explanation remains a possibility (Alexander, 2000). Some consider that the disease is unlikely to have gone unreported if it was enzootic in village chickens, but even at present, village chickens throughout Africa, Asia and the Americas often show high levels of mortality, either regularly or as large die-offs every few years which largely go undiagnosed (Alexander, 2000). Similarly, occasional descriptions of disease outbreaks prior to1926 are very similar to ND (Alexander, 2000). Until recent years, the second explanation has been the one generally accepted to be most likely. This was fed by the finding, during the panzootic of 1970-1973, that movement of captive caged birds, particularly psittacines, which may be resistant excreters of NDV, was to some extent responsible for the introduction and spread of NDV in some countries, and most noticeably in California (Hanson, 1972; Walker *et al.*, 1973). However, viruses isolated from feral birds are usually of low virulence and caged birds are most probably infected after trapping (Alexander, 2000). The third explanation has usually been dismissed as representing too large a mutation to be within the bounds of probability of occurring, especially without any apparent evolutionary advantage that would result in selection (Alexander, 2000). However, viruses isolated from ND outbreaks in Ireland and Australia during the1990s had suggested that some virulent NDVs may emerge this way (Alexander, 2000).

The name “Newcastle disease”, (after the geographical location of the first outbreak in Great Britain), was coined by Doyle as a temporary measure because he wished to avoid descriptive name that might be confused with other diseases (Doyle, 1935). The name has, however, continued to be used although when referring to the ND virus, the synonym ‘avian paramyxovirus type 1’ (APMV-1) is now often employed (Alexander, 1998).

ND was first reported in West Africa in The Gambia (Fatumbi and Adene, 1979). The first documented outbreak of the disease in Nigeria occurred in Ibadan in 1952 (Hill *et al*., 1953).

2.5.2 Synonyms of Newcastle disease

Alexander and Senne (2008) reported different synonyms of Newcastle disease to include 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.

2.5.3 Definition of Newcastle disease

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 F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term ‘multiple basic amino acids’ refers to at least three arginine or lysine residues between residues 112 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test.

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F_0 gene; 112-116 corresponds to residues -4 to -1 from the cleavage site (OIE, 2012).

2.5.4 Epidemiology of Newcastle disease

Newcastle disease in poultry continues to occur in almost all continents of the world, except in Europe, where sporadic epizootics of the disease usually occur (Munir *et al.*, 2012; Naveen *et al.*, 2013).

Different parts of the world recorded major panzootics of Newcastle disease at different periods of time. The first panzootic was reported in 1926 in Southeast Asia from Java, Indonesia and in Europe from Newcastle-Upon-Tyne, England (Seal *et al.*, 1995; Arifin *et al.*, 2011), and it remained till late 1950s (Qiu *et al.*, 2011). This wass followed by another panzootic that started in Middle East in late 1960s and spread to other countries of the world till 1973 (Ashraf and Shah, 2014). The most devastating was the third panzootic that occurred in the Middle East in late 1970s which was caused by pigeon paramyxovirus type 1. In 1981, Newcastle disease reached Europe and then spread rapidly throughout the globe (Mase *et al.*, 2002). The fourth pandemic occurred in late 1980s in which many continents were affected- Far East, South Africa, and Europe (Qiu *et al.*, 2011). The first documented outbreak of Newcastle disease in Nigeria occurred between December 1952 and February 1953 in and around Ibadan (Hill *et al.*, 1953). Since then, Newcastle disease was reported from all parts of the country (Hill *et al.*, 1953; Fatumbi and Adene, 1979; Ezeokoli *et al.*, 1984; Gomwalk *et al.*, 1985; Baba *et al.*, 1995). The disease had also been reported to be endemic in both Local and Commercial poultry with annual epidemics being recorded in highly susceptible poultry flocks (Adu *et al*., 1986; Sa‟idu *et al*., 1994; Halle *et al*., 1999; Orajaka *et al*., 1999).

2.5.5 Susceptibility and reservoir hosts of Newcastle disease

All domestic and wild bird species are susceptible to infection with NDV. Kaleta and Baldauf (1988) reported that NDV infections had been established in at least 241 species of birds representing 27 of the 50 orders of the class *Aves*. These include both local and exotic chickens (Fatumbi and Adene, 1979; Abdu *et al.,* 1985*;* Echeonwu *et al.,* 1993; Baba *et al.,* 1995; Oyeduntan and Durojaiye, 1999), and Guinea fowls and turkeys (Oame, 1983; Ezeifeka *et al*., 1992; Echeonwu *et al.,* 1993; Haruna *et al*., 1993; Sa'idu *et al*., 1994). Wild water birds seem to be the reservoir of avirulent strains, whereas poultry are the most likely reservoir of virulent viruses, but both hosts exchange viruses (Chantal *et al*., 2013).

2.5.6 Transmission of Newcastle disease

Transmission is primarily through direct contact between healthy birds and bodily discharges of infected birds (Solomon, 2010). Inhalation of excreted droplet particles and/or the ingestion of infective material such as faeces were reported to be the major routes of transmission of Newcastle disease (Alexander, 2000). However, egg-associated vertical transmission with virulent strains is also possible although uncommon, because the embryo may die except if the viral titre is low (Chen and Wang, 2002). There is also little experimental evidence that administration of live vaccines by aerosol demonstrates infection of Newcastle disease via the respiratory route. This is because the success of this route of transmission will depend on many environmental factors, such as temperature, humidity and stocking density (Alexander, 2000).

Other potential ways of spread of viral infections include movement of contaminated fomites, feed or water and introduction of carrier birds to susceptible flocks. Migratory wild birds and mixed poultry populations can equally help in spread of viral infections (Adene, 2007; CIDRAP, 2008).

2.5.7 Clinical signs of Newcastle disease virus

The clinical signs are variable depending on the pathotype, species of bird, concurrent disease and preexisting immunity (Beard and Hanson, 1984). The incubation period ranges from 2-15 days and mortality ranges from 0-100%, depending on the strain (Nanthakumar *et al*., 2000). Generally, the clinical signs of ND seen in chickens were: gasping, stretching of the neck, sneezing, coughing, tracheal rales, dyspnoea and opisthotonus (Fatumbi and Adene, 1979; Okoye *et al*., 2000). There are also ruffled feathers, oedema of the head, cloudy eyes, conjunctivitis, severe depression, yellow and whitish diarroea, dehydration, emaciation and sudden death (Abdu *et al*., 2004). Oladele *et al*., (2005) reported a rise in rectal temperature following infection by NDV.

2.5.8 Pathological lesions

2.5.8.1 Gross lesions

Generally, there are no pathognomonic lesions associated with any form of Newcastle disease virus (Alexander, 1982; Alexander, 1988). However, viscerotropic velogenic Newcastle disease viruses are usually distinguished from neurotropic velogenic Newcastle disease viruses by the presence of haemorrhagic intestinal lesions of affected chickens (Alexander, 1988).

Pronounced gross lesions such as swelling of the head or periorbital region and oedema of the interstitial tissue of the neck especially near the thoracic inlet are usually found in birds infected with velogenic strains (Alexander, 1998; Brown *et al*., 2008). Avirulent strains are also associated with localized congestion and mucoid exudates in the respiratory tract, opacity and thickening of the air sacs (Brown *et al.,* 2008). The most important suggestive lesions of Newcastle disease in chickens are haemorrhages, ulcers, oedema and /or necrosis which are often found in various organs and tissues especially the caecal tonsils and lymphoid tissues of the intestinal wall (Alexander, 1998).

2.5.8.2 Histopathological lesions

Histopathological lesions are as varied as clinical signs and gross lesions and can be affected by virus pathotype, species of bird, concurrent disease and preexisting immunity (Kumbish, 2013).

Hyperemia, oedema and haemorrhages are found in the blood vessels of many organs (Alexander, 1982). There is also hydropic degeneration of the media and hylination of capillaries and arterioles (Alexander, 1982). Marked degeneration of the madullary region is seen in the bursa (Alexander, 1988). In the respiratory system, oedema, cellular infiltration and increased thickening of the air sacs may occur in chickens (Alexander, 1988).

2.5.9 Diagnosis of Newcastle disease

2.5.9.1 Viral isolation of Newcastle disease virus

Two good sources of Newcastle disease virus for isolation in living birds were reported to be tracheal and cloacal swabs (OIE, 2000). Swabbing may harm small delicate birds, but the collection of fresh faeces may serve as an adequate alternative (OIE, 2012). Newcastle disease virus can also be isolated from homogenized organs of dead birds (Alexander, 2008). Samples from dead birds consist of oro-nasal swabs, as well as samples collected from lung, air sac, intestine (including contents), spleen, brain, liver and heart tissues (OIE, 2012).

The samples are placed in a phosphate buffered saline containing antibiotics. Though the antibiotics should vary according to local conditions, however, it can generally contain penicillin (2,000 units/ml), streptomycin (2 mg/ml), gentamycin (0.05 mg/ml) and mycostatin (100 units/ml) for tissues and tracheal swabs, but at concentrations 5-fold higher for faeces and cloacal swabs (OIE, 2012). The suspensions in an antibiotic solution are inoculated into the allantoic cavity of 9–11-day-old embryonated chicken eggs. The eggs are incubated at 37°C for 4–7 days. The allantoic fluid of any egg containing dead or dying embryos, as they arise, and all eggs at the end of the incubation period are tested for haemagglutinating activity and/or by use of validated specific molecular methods (OIE, 2012).

2.5.9.2 Haemagglutination activity

The HA test is carried out as follows:

0.025 ml of PBS was dispensed into each well of a plastic microtitre plate (V-bottomed). This is followed by placing 0.025 ml of virus suspension (i.e. infective allantoic fluid) in the first well. For accurate determination of the HA content, this was carried out from a close range of an initial series of dilutions, i.e. 1/3, 1/4,1/5,1/6, etc. Two-fold dilutions of 0.025 ml amounts of the virus suspension are made across the plate. A further 0.025 ml of PBS was dispensed to each well. 0.025 ml of 1% v/v of chicken red blood cells was dispensed to each well; mixed by tapping the plate gently and the RBC was allowed to settle for approximately 40 minutes (either at room temperature or 4°C if ambient temperatures are high). Haemagglutination is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the red blood cells. The titration is read to the highest dilution giving complete haemagglutination (no streaming); this represents 1 HA unit (HAU) and is calculated accurately from the initial range of dilutions (OIE, 2012).

2.5.9.3 Haemagglutination inhibition

The HI test is based on the principle that the haemagglutinin on the viral envelope can bring about the agglutination of chicken red blood cells and that this can be inhibited by specific antibodies (FAO, 2004). V-bottomed microtitration plates are used. The serum samples are diluted in serial twofold dilutions in phosphate buffered saline and then a fixed quantity of viral antigen is added to each well. Usually, 4 haemagglutination units are used, according to the method of Allan and Gough (1974). Following incubation, a suspension of red blood cells is added to each well and the plate is incubated again. In the absence of any antibody against the virus, haemagglutination occurs, appearing as a diffuse red colour at the bottom of the well (FAO, 2004). In the wells where the antibody against the virus is of a sufficient level, haemagglutination is inhibited and the red blood cells sediment and appear as small pellets at the bottom of the well. The presence or absence of agglutination is accurately assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing RBCs and PBS only) should be considered to show inhibition (FAO, 2004). The HI titre is the reciprocal of the highest dilution of serum which completely inhibits haemagglutination and is usually and most conveniently expressed as the logarithm to the base 2

(FAO, 2004). Although the test is difficult to standardise between laboratories, the HI titre gives an indication of the immune status of the bird. A titre of $\log_2 3$ is indicative of protection and a titre of $\log_2 6$ or more suggests a recent infection by the virus. If no vaccination has taken place, diagnosis of the infection can be made on this basis, although it cannot be determined exactly when it took place. Sequential samples taken at different times can indicate whether the titre is rising - indicative of a recent infection - or declining (FAO, 2004). When the HI titre is used as a measure of immunity (for example, when testing flock immunity following vaccination), it is recommended that an avirulent strain such as V4 or Ulster 2C be used as the viral antigen. The La Sota antigen has been found to be unsuitable for this purpose when vaccination is done by the same strain as it results in an overestimation of protective serum antibody titres (Maas *et al*. 1998).

2.5.9.4 Enzyme-Linked Immunosorbent Assay

The ELISA works on the principle of recognition of anti-NDV antibodies, attached to a plate coated with viral antigen, by antibodies produced in another species against chicken antibodies (FAO, 2004). The NDV ELISA kit will measure the amount of antibody to NDV in the serum of chickens. Microtitre plates have been pre-coated with inactivated NDV antigen. Chicken serum samples are diluted and added to the microtitre wells where any anti-NDV antibodies present will bind and form an antigen-antibody complex. Non specific antibodies and other serum proteins are then washed away. Anti-chicken IgG labelled with the enzyme alkaline phosphatase is then added to the wells and binds to any chicken anti-NDV antibodies bound to the antigen. After another wash to remove unreacted conjugate, substrate is added in the form of pNPP chromogen. A yellow colour is developed if anti-NDV antibody is present and the intensity is related to the amount of anti-NDV antibody present in the sample (www.biochek.com).

2.5.10 Control and prevention of Newcastle disease

2.5.10.1 Vaccines and vaccination

Today, there are commercial live and inactivated oil adjuvant vaccines (IOAV) which are very effective as immunization antigens (Bennejean *et al.*, 1978). The live ones are produced from lentogenic and mesogenic virus strains (Lancaster, 1981). Live lentogenic strains namely F, Hitchner B1 and La Sota and mesogenic virus strains namely Mukteswar and Komarov are commercially available (Chansiripornchai and Sasipreeyajan, 2006). The ND Ulster virus strain has been reported to induce a very mild or inapparent disease in susceptible chickens after vaccination (Waterson *et al.*, 1967; McFerran *et al.*, 1968; McFerran and Nelson, 1971). Its efficacy of a drinking water-administered, and its potential as an aerosol vaccine have been reported by Beard (1971) and Gough and Allan (1974). The Ulster strain is known to trigger immunity in the vaccinated chickens with less efficacy than strain Hitchner B1 when given as aerosol vaccine (Chansiripornchai and Sasipreeyajan, 2006). However, Ulster strain vaccine causes less side effects than B1 (Gough and Allan, 1976; vanEck and Goren, 1991; vanEck *et al.*, 1991; Villegas, 1997).

However, live vaccines have traditionally been heat-sensitive and require storage at or below 4°C. Complete and effective cold chains are expensive and difficult to maintain and extensively raised birds are difficult to catch, hence making vaccination labour intensive (Spradbrow, 1992). Two heat resistant vaccine strains have been developed, known as V-4 and I-2, and their potential advantages for use in village production systems have been underscored (Oakeley, 1998).

Thermostable ND vaccines exhibit a relative resistance to inactivation on exposure to elevated temperatures. They are prepared from a strain of NDV that retains its ability to infect cells after storage outside a cold chain for a short period of time and involves isolation of naturally occurring thermostable variants of the virus and increasing the thermostability of this variant by artificial selection in the laboratory (Spradbrow, 1992). The heat resistant V4 (NDV4-HR) vaccine against ND has yielded encouraging results in many countries in Africa and Southeast Asia (Alders and Spradbrow, 2001). NDV4-HR vaccine is a living vaccine which is thermostable, retaining its activity for 12 weeks at a temperature of 28°C in freeze-dried form (Spradbrow, 1992). Its ease of administration makes it suitable for use by village farmers and the vaccine strain can be transmitted by contact from vaccinated to non-vaccinated birds. Although this vaccine has been commercialized, it is only available in large doses that the smallholder farmer cannot afford. The I-2 strain, which has been tested in a number of countries has also proved to be protective against local virulent strains of the ND virus (Tu *et al.*, 1998). The thermostable V4 vaccine was reported to stimulate very good antibody response in local chickens (Nwanta, 2003).

2.5.10.2 Biosecurity measures against Newcastle disease

Good biosecurity measures are essential to prevent Newcastle disease in poultry flocks. Commercial flocks should not have any contact with domesticated poultry or wild birds or any pet birds. Workers should avoid contact with birds outside the farm. Biosecurity measures include bird-proof houses, feed and water supplies, minimizing travel on and off the facility, disinfecting vehicles and equipments that enter the farm. Pests such as insects and mice should also be controlled. If possible, employees should shower and change into dedicated clothing prior to entry into the poultry farm (Ashraf and Shah, 2014).

2.5.11 Public health significance of Newcastle disease

Humans are among the many species that can be infected by NDV in addition to avian species (Alexander, 2000). NDV may cause conjunctivitis in humans, when a person has been exposed to large quantities of the virus (Alexander, 2000). Mostly, laboratory workers and vaccinators are affected. The use of personnel protective equipment and biological safety cabinet has reduced the exposure of laboratory workers. Infection is rarely seen in the workers of a farm; moreover, persons handling or consuming poultry products do not appear to be at risk (Nolen, 2003). The conjunctivitis usually resolves rapidly, but the virus is usually shed in ocular discharges from 4 to 7 days. In some cases, mild, self-limiting, influenza-like disease with fever and headache has also been reported in humans (Alexander, 2000; OIE, 2012). There is no evidence found to support human to human transmission but the potential for human to bird transmission exists (Alexander, 2000; David and Daniel, 2003).

2.6Newcastle Disease in Nigeria

Newcastle disease (ND) was the leading cause of death and is even the Nigeria's single most dreaded disease of the poultry industry (Adu *et al*., 1985). Since the first report of the disease in Nigeria by Hill *et al.*, (1953), there have been several reports of the disease throughout the country. The disease has been reported to be endemic in both local and commercial poultry with annual epidemics being recorded in highly susceptible poultry flocks (Adu *et al*., 1986; Sa‟idu *et al.,* 1994; Halle *et al*., 1999; Orajaka *et al.,* 1999). Newcastle disease was also described as one of the most economically important disease of poultry in Nigeria (Okeke and Lamorde, 1988). The disease was also reported to be more common during the dry harmattan period (Saidu *et al*., 1994; Halle *et al*., 1999) and the stress of the harmattan period is known to worsen the outcome of the disease (Abdu *et al*., 1992).

The first laboratory confirmation of ND 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 (West, 1972; Nawathe *et al.,* 1975). 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 by Okoh (1979). However, there was no virus isolation (Solomon *et al*., 2010).

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

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

Zaria, the second largest city inKaduna State is the study area. It is located between longitude $7^o36'$ to $7^o42'$ E and latitude $11^o00'$ to $11^o10'$ N of the equator (Ubogu *et al*., 2011). Zaria is found on the high plains of northern Nigeria, in sub-Saharan Africa (Ubogu *et al*., 2011). The relatively deep tropical ferruginous soils and climate conditions of Zaria are suitable for and sustain a good cover of savanna woodland (Northern Guinea Savanna) with a variety of grasses, woody shrubs and short trees (www.abu.edu.ng).The nodal function of the city has helped to encourage the rapid growth of the city. Its growth is due to the fact that historically Zaria was an important trading centre between 16th and 20^{th}centuries with a well-established powerful oligarchy (Ubogu *et al*., 2011). The space economy of Zaria comprises of commercial, residential, educational, transportation, agricultural and industrial landuse withthe industrial activities presently dominated by small-scale informal sector (Ubogu *et al*., 2011). However, Zaria's economy is primarily based on agriculture (Britannica Encyclopedia). Zaria is also a market town for the surrounding areas; in addition to being a home of numerous artisans, from traditional crafts like leather work, dyeing and cap making, to tinkers, print shops and furniture makers (Gihring, 1984).

In terms of its ethnic composition, the city by virtue of its vibrant socio-cultural system coupled with its historical past has become a choice place for migrants of several ethnic groups aside from the Hausa-Fulani that constitute the majority of the inhabitants (Ubogu *et al*., 2011).

The sampled locations for this study were distributed within Zaria and environs (Figure I)

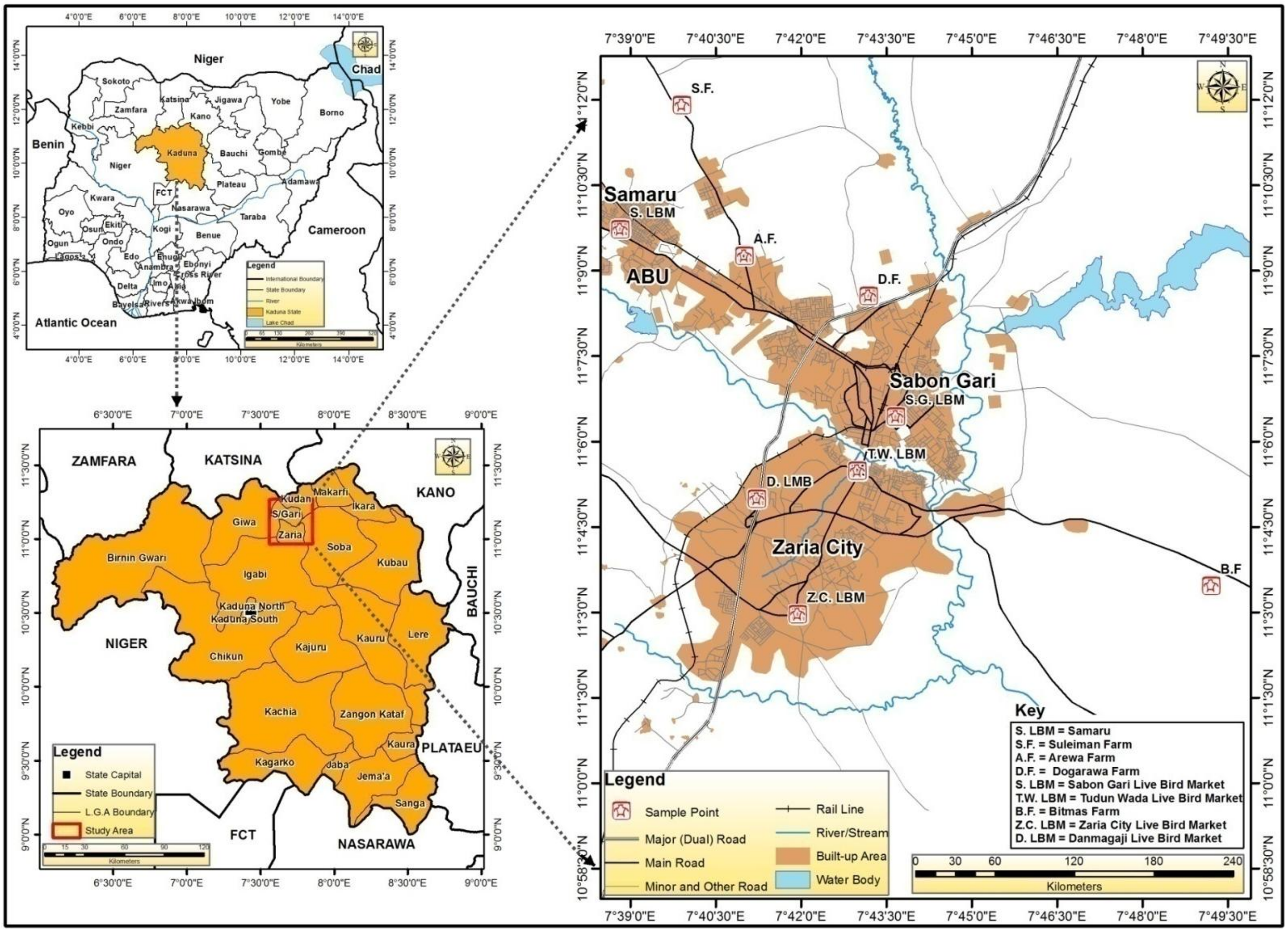

Figure 1: Map of Zaria and environs showing sampled locations

Source: Fieldwork/Geography Department, ABU, Zaria

3.2Category of Farms/ Markets Selected

3.2.1 Commercial poultry farms

This isa facility for raising various types of domestic birds commercially for the purpose of meat, eggs and feather production. The most common and widely raised poultry birds are chicken. The chickens which are raised for eggs are called layer chicken, and the chickens which are raised for their meat production are called broiler chickens (www.roysfarm.com/poultry-farming).

3.2.2 Live bird markets

Live-bird markets are a places where birds are assembled from various other locations for further sale (www.upc-online.org) or Live bird markets are places where customers can buy live bird to be slaughtered for food to take home (www.cdc.gov/az).

3.3 Category of Birds Sampled

Local chickens were sampled from live bird markets and only exotic chickens were sampled from commercial poultry farms.

3.4 Inclusion Criteria for Sampling

Only chickens showing clinical signs of disease were sampled.

3.5Sampling of Birds

A total of 127 symptomatic birds were purposively sampled in both commercial poultry farms andlive bird markets in Zaria and environs.Samples were collected in four commercial poultry farms and five live bird markets; 69 samples from commercial farms and 58 samples from live bird markets (Table 3.1 and 3.2).

The samples were properly labeled with a designated sample I.D, Farm name and date of sample collection amongst other information.

3.6 Sample Collection

A cloacal swab was collected from each bird between November, 2014 and January 2015 and transported in virus transport medium (Hanks' transport medium) containing 2000 U/ml of penicillin, 2 mg/ml of streptomycin, 0.05 mg/ml of gentamycin and 100 U/ml of mycostatin in cold flask. The samples were stored in a refrigeratorat -20 ^{0}Cin the Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, prior to transporting them to Virology Laboratory, NVRI Vom, Plateau state, where they were stored at -20 °C until Newcastle disease virus detection.

Table 3.1: Number of chickens sampled per commercial farm in Zaria and environs Nigeria

S/No.	Name of commercial farms	Number of chickens sampled
1	Suleiman farm	22

2	Arewa farm	20
3	Dogarawa farm	17
4	Bitmas farm	10
Total		69

Table 3.2: Number of Chickens sampled per live bird market in Zaria and environs, Nigeria

S/No.	Name of live bird markets	Number of chickens sampled
1	Samaru live bird market	12

2	Sabon Gari live bird market	10
3	Tudun Wada live bird market	13
4	Zaria City live bird market	11
5	Danmagaji live bird market	12
Total		58

3.7Molecular Detection of Newcastle Disease Viruses

3.7.1Viral RNA extraction

Viral RNA was extracted directly from the cloacal swab samples collected from sick birds. Newcastle disease virus extraction was performed in the Virology laboratory, National

Veterinary Research Institute Vom, Plateau State Nigeria, using QIAgen RNeasy Mini Kit (QIAgen, Hilden, Germany) according to the protocol provided with the QIAgen RNeasy® kit.

The procedure was carried out as follows;

560µL of prepared Buffer AVL containing carrier RNA were pipetted into a 1.5ml microcentrifuge tube. It was then followed by adding 140µL of cloacal swab contained in the viral transport media to the Buffer AVL-carrier RNA in the microcentrifuge tube. In order to yield a homogeneous solution, this mixture was properly mixed and pulse-vortexed for 15 seconds. The mixture was then incubated at room temperature for 10 minutes. In order to remove drops from inside of the lid, the tubes were briefly centrifuged and 560µL of ethanol (96-100%) were added to the samples, pulse-vortexed for 15 seconds and briefly centrifuged. 630µL of this solution were carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube). This was followed by centrifugation at 8000rpm for 1 minute. The QIAamp spin column was placed in to a clean 2ml collection tube and the tube containing the filtrate was discarded. The QIAamp spin columns were opened and the remaining 630µL of the solution were applied, the cap was closed and centrifuged at 8000rpm. The QIAamp mini spin columns were again placed into a clean 2ml collection tube and the tube containing the filterate was discarded. The QIAamp Mini spin columns were opened and 500µL of Buffer AW1 were added. This was centrifuged at 8000rpm for 1 minute after closing the cap. The QIAamp Mini spin columns were placed in clean 2ml collection tubes and the tubes containing the filtrate were discarded. The QIAamp Mini spin columns were opened and 500µL of Buffer AW2 were added. The tubes were centrifuged at 14,000rpm for 3 minutes. The QIAamp Mini columns were placed in new 2ml collection tubes and the old collection tubes containing the filtrate were discarded. These were centrifuged at 14,000rpm for 1 minute.

Finally, the QIAamp Mini spin columns were placed in clean 1.5ml microcentrifuge tubes. The old collection tubes containing the filtrate were discarded. The QIAamp Mini spin columns were carefully opened and 60µL of Buffer AVE equilibrated to room temperature were added. The caps of the tubes were closed and the tubes incubated at room temperature for 1 minute, then centrifuged at 8000rpm for 1 minute.

3.7.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

One-step RT-PCR was performed for the amplification of 280 base pair fragment for the Matrix gene using the following primers;

APMV-1 M4079F 5′-AAGGAGCCTTGATCTATCTGTCGG-3′ Forward

APMV-1 M4337R 5′-TGTGCCCCTTCTCCAGCTTAGTA-3′ Reverse.

Using 25 µL reaction volume, reaction mixtures containing 11.5 µL Nuclease free water, 5.0 µL 5x PCR buffer, 0.5 µL dNTPs, 0.5 µL Avian Myeloblastosis Reverse transcriptase enzyme, 0.5 µL Rnase Inhibitor, 5.0 µL of RNA template and 1.0 µL each of the primers were first cycled in a 96-well thermo cycler (Applied Biosystems) at 50 ^{0}C for 30 minutes for reverse transcription. This was followed by initial denaturation at a temperature of 94 ^{0}C for 2 minutes. A 35 cycles of denaturation, annealing and elongation of the templates were achieved at the cycling conditions of 94 ^{0}C for 30 seconds, 55 ^{0}C for 30 seconds and 72 ^{0}C for 1 minute. Final extension of the templates was achieved at 72 ^{0}C for 7 minutes.

The products were electrophoresed at 120V for 30 minutes in 2% agarose gel stained with ethidium bromide and bands were visualized using a gel documentation system.

3.8Virus Isolation

Hank's viral transport media containing NDV positive samples by RT-PCR were vortexed to ensure thorough mixing. 0.2ml of the fluid from cloacal samples was inoculated using 1 ml syringe into each of three 9-day-old embryonated chicken eggs obtained from Poultry farm of National Veterinary Research Institute Vom, Plateau State, Nigeria, via the allantoic route. The inoculated eggs were incubated at 37^{o}C and candled with a 25 voltage bulb twice daily for 4 days. Eggs showing embryonic death at 24hours and up to day 4 were chilled at 4^{0}C. Eggs that were viable after 4 days of incubation were also chilled. Allantoic fluid from each of the inoculated eggs was harvested and tested for haemagglutination (HA) using 1% chicken red blood cells. The HA-negative samples were passaged once more in to each of two embryonated chicken eggs. The isolates were kept at -20^{0}C before further amplification of the partial F-gene by reverse transcription-polymerase chain reaction (RT-PCR).

3.9Partial Amplification of the Newcastle Disease Virus F-gene by RT-PCR

Total RNA was extracted from the isolates. Viral extraction was performed in Virology laboratory, National Veterinary Research Institute Vom, Plateau State Nigeria, using QIAgen RNeasy Mini Kit (QIAgen, Hilden, Germany) according to the protocol provided with the QIAgen RNeasy® kit.

One-step RT-PCR was performed for the partial amplification of 1181 base pair fragment for the Newcastle disease virus at position 610 of M-gene to position 581 of F-gene, including the F_0 cleavage site using the following primers as synthesized and used by Solomon, (2010);

M610 5′-CTGTACAATCTTGCGCTCAATGTC-3′ forward primer

F581 5′-CTGCCACTGCTAAGTTGTGATAATCC-3′ Reverse primer

Using 25 µL reaction volume, reaction mixtures containing 11.5 µL Nuclease free water, 5.0 µL 5x PCR buffer, 0.5 µL dNTPs, 0.5 µL Avian Myeloblastosis Reverse transcriptase enzyme, 0.5 µL Rnase Inhibitor, 5.0 µL of RNA template and 1.0 µL each of the primers were first cycled in a 96-well thermo cycler (Applied Biosystems) at 50 ^{0}C for 30 minutes for reverse transcription. This was followed by initial denaturation at a temperature of 95 ^{0}C for 15 minutes. A 35 cycles of denaturation, annealing and elongation of the templates were achieved at the cycling conditions of 94 ^{0}C for 30 seconds, 53 ^{0}C for 30 seconds and 72 ^{0}C for 1 minute. Final extension of the templates was achieved at 72 ^{0}C for 7 minutes.

The products were electrophoresed at 120 V for 30 minutes in 1.5 % agarose gel stained with ethidium bromide and bands were visualized using a gel documentation system.

However the cycling conditions were modified from that of Solomon, (2010) because Solomon, (2010) used Molony Murine Leukemia Virus-Reverse transcriptase enzyme as enzyme mix, while in this study, Avian Myeloblastosis Reverse transcriptase was used. Avian Myeloblastosis Reverse transcriptase is shown to have stronger *Rnase H* activity than Molony Murine Leukemia Virus-Reverse transcriptase, hence the reason for the increase in temperature for reverse transcription from 42 ^{0}C for 20 minutes from that of Solomon, (2010) to 50 ^{0}C for 30 minutes in this study.

3.10DNA Sequencing

Each sequencing tube was added with 2 µl of sequencing buffer, 4 µl of sequencing mix, deionized water to a total volume of 20 µl, 1 µl of reverse primer (F581) and 40ng of NDV amplicons and was calculated based on a recommended protocol for Big Dye Terminator sequencing (Applied Biosystems). The standard Big Dye thermal cycling conditions used were 96 ^{0}C for 1 minute for initial denaturation and 25 cycles of96 ^{0}C for 10 seconds, denaturation, 50 ^{0}C for 5 seconds, annealingand 60 ^{0}C for 4 minutes,extension.

3.10.1 Sequencing reaction precipitation

A total volume of each sequencing reaction was transferred into 0.5ml tubes containing a combined mixture of 50µl of 100% ethanol and 2µl of 3ml sodium acetate (NaOAc). Reactions were incubated at room temperature in the dark for 10 minutes. The tubes were centrifuged at 13,000rpm for 15 minutes. Using micropipette, the fluid was removed. 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.

3.10.2 DNA sequencing reaction

10 µl of highly deionized formamide (Applied Biosystems) were added to each PCR 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 96-well thermal cycler (Applied Biosystems) and denatured at 96 ^{0}C for 2 minutes, then cooled at 4 ^{0}C. Reactions were analyzed on 3130 Genetic Analyser (Applied Biosystems).

3.11Data Analyses

3.11.1 Editing of the sequences

Nucleotide sequences were edited using BioEdit(v7.1.11) software. Using this, sequences were checked for basecalling, and length edited before subsequent exporting in Fast Adaptive Shrinkage Thresholding Algorithm (FASTA) format to notepad.

3.11.1.1 Sequence alignment

The sequences, in FASTA format from the notepad, were blasted for homology searches (http://www.ncbi.nlm.nih.gov/blast) so as to identify closely related sequences to be included in multiple sequence alignments. Representative of genotypes and subgenotypes were also retrievedfrom the GeneBank for comparison. Multiple sequence alignments were carried out using ClustalW in BioEdit software.

3.11.2 Nucleic acid translation

The sequences were translated into amino acid so as to determine the strain of the virus circulating in the study area.

3.11.3 Phylogenetic analyses

The Neighbour joining tree method (Satou and Nei, 1985) was selected as the most suitable amongst Unweighted-pair group method with Arithmetic Mean Algorithm (UPGMA), Minimum Evolution (ME) and Maximum Parsimony, in constructing a phylogenetic tree. The evolutionary distances were computed using the Kimura 2-Parameter method (Felsenstein, 1985), and are in the unit of base substitution per site (Kimura, 1980). Evolutionary analyses were conducted in Molecular Evolutionary Genetic Analysis 6 (MEGA 6) (Tamura*et al.*, 2013).

3.11.4 Evolutionary distance analyses

Homology between strains was deduced from matrix relationship table and presented in tables.

Other data were also presented in tables

CHAPTER FOUR

RESULTS

4.1 Result ofMolecular Detection of NDV

A 280 base pair (bp) DNA fragment was amplified for each of the 127 samples using Reverse Transcriptase Polymerase Chain Reaction. The results showed that sixteen samples were positive for Newcastle Disease Virus; threefrom commercial poultry farms and thirteenfrom live bird markets(Plate I and II).

The M-gene RT-PCR test was carried out on all the cloacal swab samples. However, only M-gene positive samples were inoculated in to embryonated eggs for virus isolation. The RT-PCR protocol detected the presence of APMV-1 RNA in 16 out of 127 clinical samples included in thisresearch. Ten of the samples positive by RT-PCR were also positive by virus isolation. Sixsamples;3 from commercial farms and 3 from live bird markets were positive by RT-PCR but gave a negative result by virus isolation.

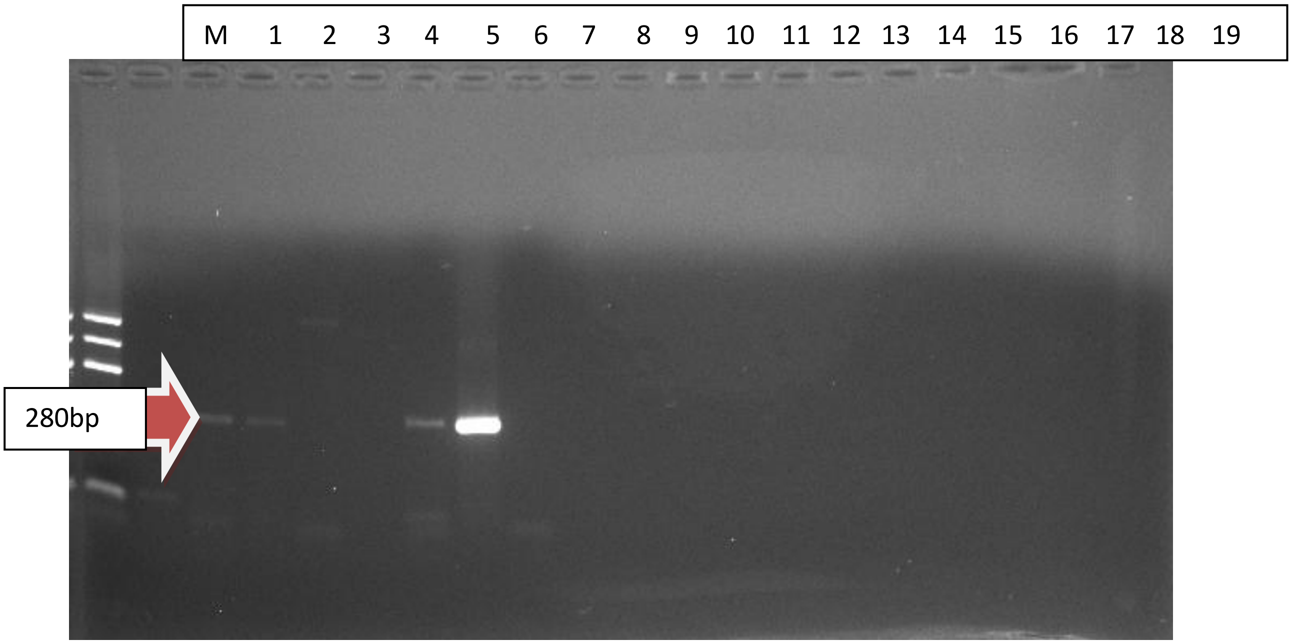

Plate I:RT-PCR product bands (Lanes 2, 3 and 6) of partially amplified Newcastle disease virus M-gene from Cloacal swabs of chickens in commercial poultry farms. The expected amplicon size is 280 bp. Lane 7 and 8 are positive and negative controls, respectively. M represents 100 bp ladder.

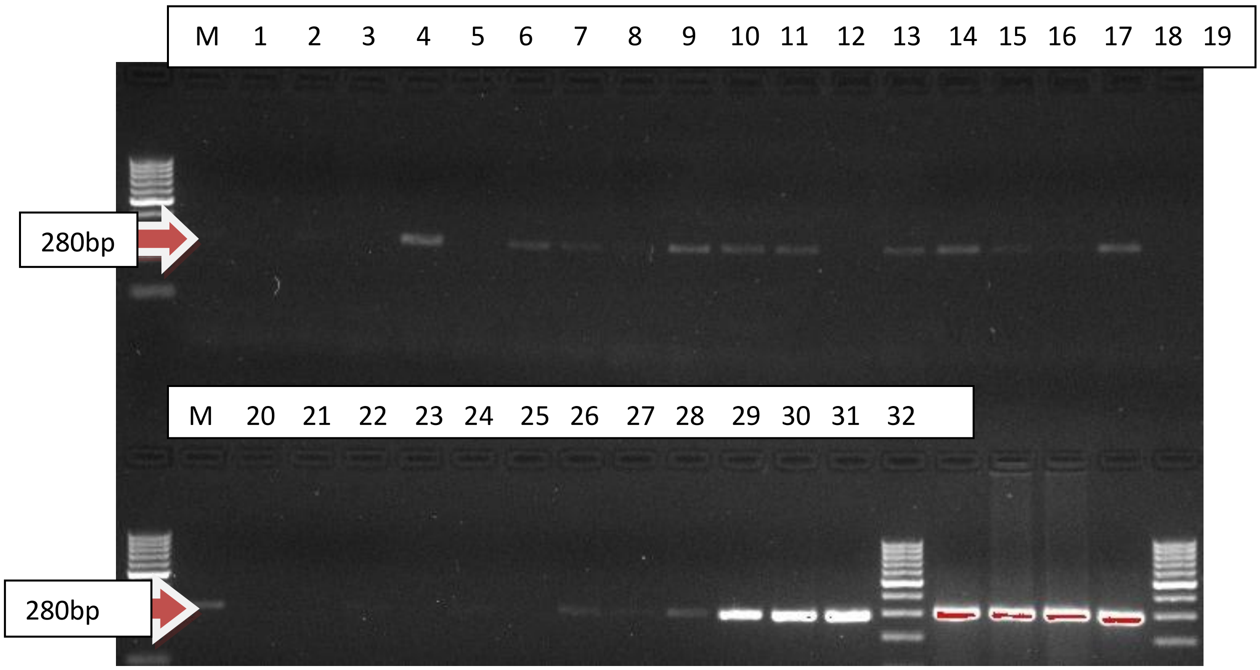

Plate II: RT-PCR product bands (Lanes 5, 7, 8,10, 11, 12, 14, 15, 16, 18, 20, 27, and 29) of partially amplified Newcastle disease virus M-gene from Cloacal swabs of chickens in live bird markets. The expected amplicon size is 280 bp. Lane 30, 31 and 32 are positive controls. M represents 100 bp Plus ladder.

4.2 Result of NDV Isolationin Embryonated ChickenEggs

A total of 10 isolates were arrived at, at the end of the virus isolation in embryonated eggs (Table 4.1). In all the isolates,embryos died within 48 to 72 hours post inoculation.

Embryonic mortality was observed with some isolates within 24-hour period. This, according to OIE, (2012) should be considered dying from non specific cause until subjected to further batch/batches of passage/passages.

Consequently, thefour HA-negative samples from first inoculation were subjected to first passageand the result showed that all the four samples were positive for Newcastle disease virus as confirmed by HA (Appendix 12). These samples were previously confirmed by RT-PCR as Newcastle disease virus positives.

Table 4.I: Results of inoculating Newcastle disease positive samples in to embryonated chicken eggs

Samples I.D	Embryonic mortality	H.A result	Comment
01NGZCK015	+	+	NDV isolated
02NGZCK015	+	+	NDV isolated
03NGZCK015	+	+	NDV isolated
04NGZCK015	+	+	NDV isolated
05NGZCK015	+	+	NDV isolated
06NGZCK015	+	+	NDV isolated
07NGZCK015	+	+	NDV isolated
08NGZCK015	+	+	NDV isolated
09NGZCK015	+	+	NDV isolated
10NGZCK015	+	+	NDV isolated
11NGZCK015	+	-	Non specific
12NGZCK015	+	-	Non specific
13NGZCK015	+	-	Non specific
14NGZCK015	+	-	Non specific
15NGZCK015	+	-	Non specific
16NGZCK015	+	-	Non specific

4.3 Amplificationof Newcastle Disease Virus Partial F-Gene

A 1181 base pair (bp) DNA fragment spanning position 610 of the matrix gene to position 581 of the fusion gene, including the F_0 cleavage site, was amplified for each of the10 isolates. The result showed that all the10 isolates were amplified (Plate III).

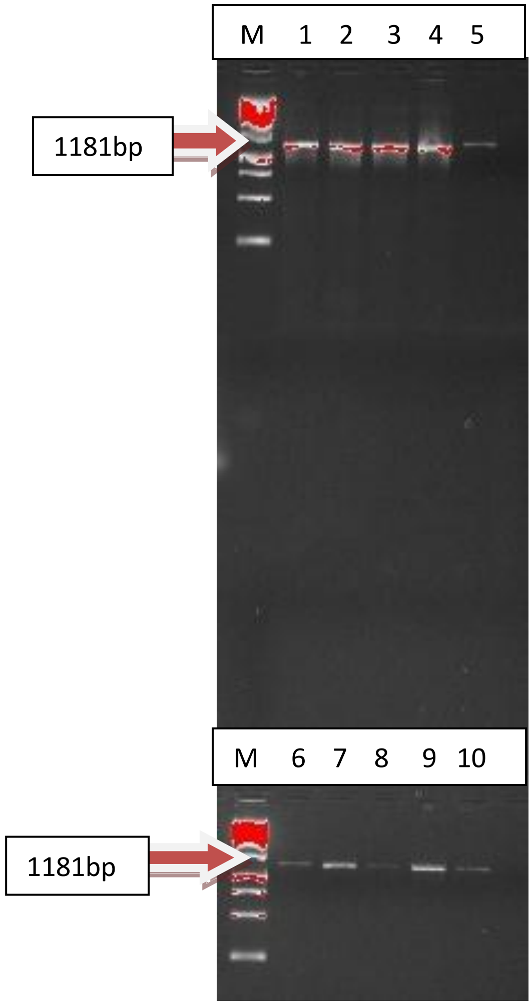

Plate III: RT-PCR product bands (Lanes 1-10) of partially amplified Newcastle disease virus position 610 of M-gene region to position 581of F-gene region from isolates obtained from chickens in commercial poultry farms and live bird markets.Theexpected amplicon size is 1181bp.M representswide rangeladder.

4.4Analysis of the Amino Acids at the F_0 Cleavage Site

The DNA sequences of the isolates together with some strains fromGenBank were translated in to amino acid residues. The deduced amino acid motif at the F gene cleavage site was analysed in order to determine the pathotypes of the strains.

The results revealed that all the Newcastle disease viruses from this study contained multiple basic amino acids at the cleavage site which is a characteristic of virulent strains. All the strains exhibited the sequence motif ^{112}RRQKR^{116}-F(Table 4.2). None of these strains had monobasic amino acid sequences, which is a characteristic of lentogenic strains. However, strains from this study and the three vaccine strains (La Sota, V4 and Komarov) shared the amino acid Arginine at position 16.

Two Newcastle disease virus isolates were not characterized because of low templates quality.

In addition, analysis of the variable region of the partial F-gene (47-419 nt) of these sequences identified in Zaria and environs revealed many amino acids substitutions in various positions (Table 4.2).

These substitutions were identified by comparing sequences from this study with reference vaccine strains (La Sota, V4 and Komarov) and other representative strains of various genotype/subgenotype from genbank.

Table 4.2: Amino acid substitutions in the variable region (47-419 nt) and some neutralizing epitopes ofNewcastle disease virus partial F-gene sequences fromZaria and environs.

Position	T/wada1	D/magaj1	T/wada2	S/gari	S/man1	Arewa	D/magaj2	S/man2	Benin	Nigeria	L Sota	V 4	Komarov
4	K	-	-	-	-	-	-	-	-	-	R	R	R
5	P	-	-	-	-	*	-	-	-	-	-	S	-
10	P	L	-	L	-	*	L	*	L	L	-	-	-
11	A	V	-	V	-	*	V	*	V	V	V	V	T
16	I	-	-	-	-	-	-	-	-	-	T	T	T
18	L	-	-	-	-	-	-	-	-	-	R	R	R
19	I	-	-	T	-	-	-	-	-	-	V	V	V
26	T	I	-	I	-	*	-	*	I	I	I	V	I
27	R	C	-	C	-	*	C	C	C	C	C	C	C
28	P	L	-	-	-	*	L	*	L	L	-	-	-
36	L	-	-	-	-	-	-	P	P	P	P	P	P
50	I	-	-	-	-	-	-	-	-	-	V	V	V
51	K	-	-	-	-	-	-	-	-	-	N	N	N
63	V	-	-	-	-	-	-	-	-	-	-	I	-
69	M	-	-	-	-	-	-	-	-	-	L	-	L
78	R	-	-	-	-	-	-	-	-	-	K	K	K
82	E	-	-	-	-	-	-	*	-	-	D	-	D
90	A	-	-	-	-	-	-	-	-	-	T	T	T
104	G	-	-	-	-	-	-	-	-	-	E	E	E
107	S	-	-	-	-	-	-	-	-	-	T	T	T
112	R	-	-	-	-	-	-	-	-	-	G	G	-
113	R	-	-	-	-	-	-	-	-	-	-	K	-
114	Q	-	-	-	-	-	-	-	R	R	-	-	-
115	K	-	-	-	-	-	-	-	-	-	G	G	-
116	R	-	-	-	-	-	-	-	-	-	-	-	-
117	F	-	-	-	-	-	-	-	-	-	L	L	-
118	V	-	-	-	-	-	-	-	-	-	I	I	I

KEY
* = any amino acid except proline or aspartic acid

4.5 Substitutions of Some Amino Acid Residues

Based on analysis of deduced amino acid sequences of partial F-gene for the 8 strains of this study, including representatives of different genotypes, many amino acid substitutions were identified and highlighted below;

When compared with the two representativesof sub-genotype XIVb from Nigeria and Benin republic(JQ039390, JX546245), strains from this study and other strains retrieved from Genbank shared $R \rightarrow Q^{114}$ substitution.

All strains from this study had $R \rightarrow K^{4}$ substitution when compared with the three vaccinal strains (La Sota, V4 and Komarov).

Strains from this study shared $S \rightarrow P^{5}$ substitution with V4 vaccine strain (JX524203) when compared with the other vaccine strains La Sota and Komarov (AY845400, KT445901).

Strains from this study shared $T \rightarrow I^{16}$ substitution with representative strains of subgenotype XIVb from Nigeria and Benin Republic in comparison with the three vaccine strains La Sota, V4 and Komarov

Strains from this study had $I \rightarrow V^{63}$ substitution when compared with the vaccine strain V4 (JX524203).

In comparison with LaSota and V4 vaccine strains, strains from this study had unique $D \rightarrow E^{82}$ substitution.

All strains from this study shared $T\rightarrow S^{107}$ substitution with the representative strains of subgenotype XIVb from Nigeria and Benin republic when compared with the vaccine strains La Sota, V4 and Komarov.

Three strains of this study: Danmagaji1, Sabon Gari and Danmagaji2 (02NGZCK015, 04NGZCK015 and 09NGZCK015) shared unique $P\rightarrow L^{10}$ and $A\rightarrow V^{11}$ substitutions with two representative strains of sub-genotype XIb from Nigeria and Benin republic when compared with other strains from this study, while the five other strains from this study had unique $L\rightarrow P^{10}$, $V\rightarrow A^{11}$, $I\rightarrow T^{26}$, $C\rightarrow R^{27}$, and $L\rightarrow P^{28}$ substitutions when compared with the two representative strains of sub-genotype XIVb.

Only strains from this study and the representative strains of sub-genotype XIVb shared common $R/Q\rightarrow L^{18}$ substitution when compared with all other strains.

When compared with the 3 vaccine strains (La Sota, V4 and Komarov), strains from this study shared $V\rightarrow I^{19}$ substitution except strain from Sabon Gari (04NGZCK015) which has $V\rightarrow T^{19}$ substitution.

Except one strain from this study (Suleiman2=10NGZCK015), all other seven strains from this study had unique $P\rightarrow L^{36}$ substitution when compared with all other strains.

Strains from this study and four other representative strains from genotype XIV (JX546245, JQ039390 and FJ772452) shared $V\rightarrow I^{50}$ substitution in comparison with other strains.

Strains from this study had $N\rightarrow K^{51}$ substitution when compared with the reference La Sota, V4 and Komarov vaccines.

Strains from this shared common L→M^{69} substitution with the vaccine strain V4 when compared with the two vaccine strains La Sota and Komarov.

Strains from this study had K→R^{78} substitution when compared with the vaccine strains.

Strains from this study and two representative strains of sub-genotype XIVb shared common T→A^{90} substitution when compared with other representative strains.

Strains from this study had E→G^{104} substitution when compared to the three vaccine strains (La Sota, V4 and Komarov).

All strains from this study shared common G→R^{112} substitution with Komarov vaccine strain when compared with the two vaccine stains La Sota and V4.

Strains from this study shared common K→RA^{113} substitution with two vaccine strains La Sota and Komarov when compared with the V4 vaccine strain.

Strains from this study shared common G→K^{115} substitution with Komarov vaccine strain when compared with La Sota and V4 strains.

All strains from this study and two representative strains of subgenotype XIVb shared the amino acid, Arginine with the vaccine strains (La Sota, V4 and Komarov) at position 16.

Strains from this study shared L→F^{117} substitution with Komarov vaccine strain when compared with La Sota and V4 strains.

Finally, all strains from this study and two representative strains of sub-genotype XIVb shared unique I→V^{118} substitution when compared with all other representative strains.

4.6 Phylogenetic Analysis

Mega v6.6 software was used in constructing Neigbour Joining tree to compare the relationship between the partial nucleotide sequences of F-gene of NDV isolated from this study with representatives of some genotype/sub-genotype retrieved from GenBank. All the sequences recovered in this study clustered with representatives of sub-genotype XIVb (figure 2).

The topology of the tree (figure 2) indicates that 3 distinct sub clusters can be deduced from subgenotype XIVb:

As observed, the representatives of subgenotype XIVb, which are from Katsina State, Nigeria and Benin Republic, retrieved from genbank formed a subcluster; another subcluster is a strain from commercial poultry farm identified in this study; and the major subcluster is the one that comprises all Newcastle disease virus strains from live bird markets in Zaria and environs and two strains from commercial poultry farms.

The phylogenetic analysis also revealed that most strains from Zaria and environs had more than 20% divergence with vaccine strains La Sota, V4 and Komarov (Appendix 2). The bootstrap values between strains from this study and vaccine strains further confirmed their divergence (figure 2).

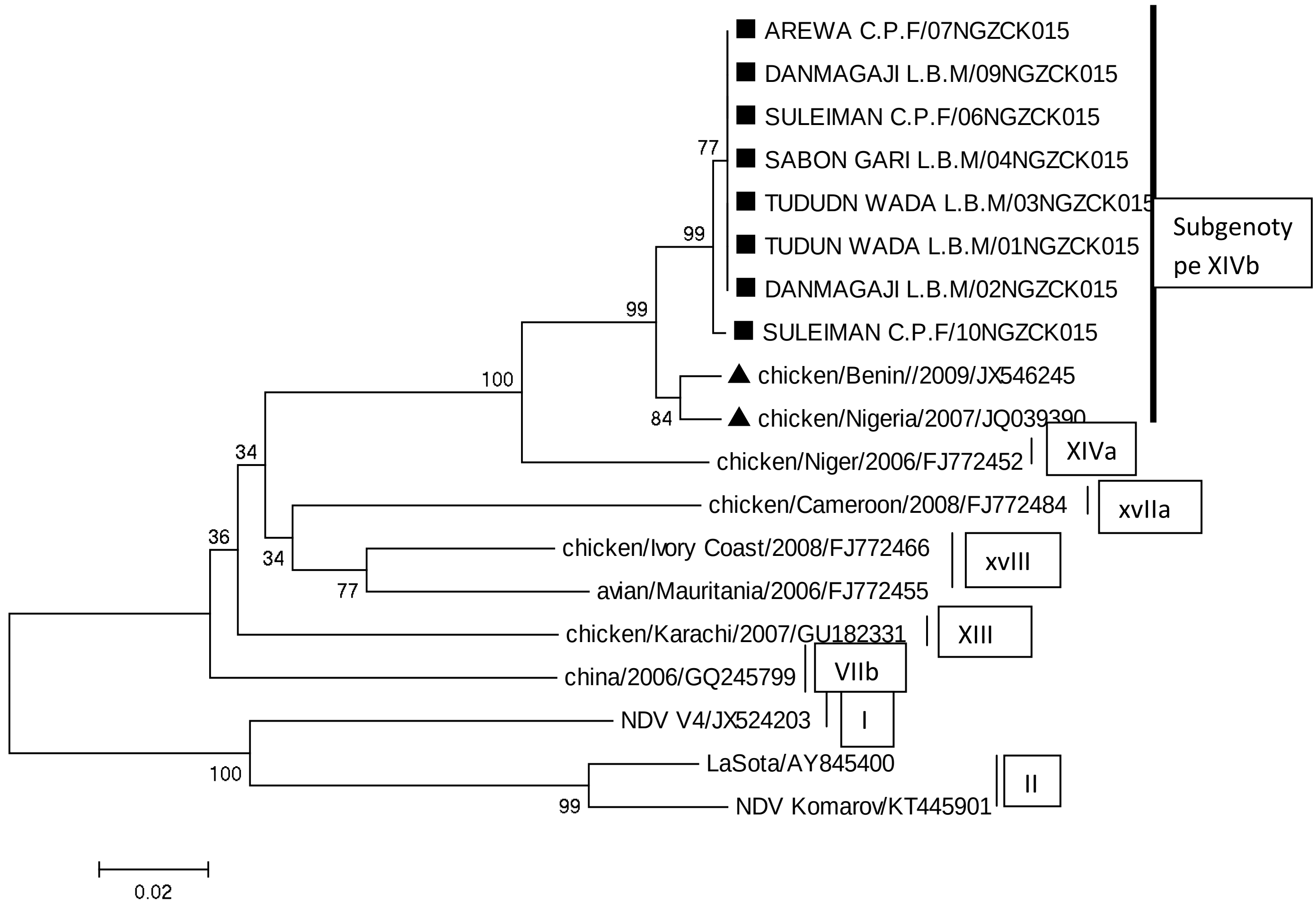

Figure 2: Neighbour joining tree based on a variable region (nt 47 to 419) of the partial F-gene of Newcastle disease virus characterized in this study with representative strains of different genotypes/sub-genotypes. Strains from this study were designated 01NGZCK015, 02NGZCK015, 03NGZCK015, 04NGZCK015, 06NGZCK015, 07NGZCK015, 09NGZCK015, and10NGZCK015.

4.7 Genetic Distance Similarities and Divergence

Based on thedistance comparison of the nucleotide sequences using Sequence Identity Matrix, the genetic distance similarities and divergence between strains of this study and reference strains was computed as follows;

Among the strains of this study, sequence homology ranges from 97.6% to 100% with maximum divergence of 2.4% mostly seen with strain 10NGZCK015 from commercial farm. The mean divergence between three strains of this study (02NGZCK015, 04NGZCK015, 09NGZCK015) with two reference strains from genotype XIVb (JQ039390, JX546245)was 2.5%; with strains 02NGZCK015 and 09NGZCK015 having2.4% divergence and strain 04NGZCK015, with 2.7% divergence. Apart from the above three strains, the other five strains had a mean divergence of 4.2% when compared with the two reference strains of sub-genotype XIVb representing 3.9% with strain 10NGZCK015, 4.1% with strains 01NGZCK015, 03NGZCK015, 06NGZCK015 and maximum divergence of 4.9% with strain 07NGZCK015.

When compared with the vaccine strains, none of the strains in this study clustered with any of the vaccine strains. For instance, the mean distance similarities of strains from this study with the three reference vaccine strains were 77.9%, 79.5% and 77.7, with mean difference of 22.1%, 20.5% and 22.3% for strains La Sota, V4 and Komarov respectively.

The mean distance similarity of three strains identified from commercial farms (06NGZCK015, 07NGZCK015, 10NGZCK015) with strains recovered from live bird markets were 98.5% for sequence 01NGZCK01, 98.1% for sequence 02NGZCK015, 98.5% for sequence 03NGZCK015, 97.9% for sequence 04NGZCK015 and 98.1% for sequence 09NGZCK015, with mean difference of 1.5%, 1.9%, 1.5%, 2.1% and 1.9% respectively.

CHAPTER FIVE

DISCUSSION

The total of sixteensamples detected by RT-PCR; three from commercial farms and thirteen from live bird markets can be seen as low when compared to the number of samples collected and prevalent nature of ND reported by many researchers in the study area. However, this low detection rate may be due to the presence of hemagglutinating viruses/ other avian paramyxovirus serotypes (e.g serotype 2, 3, 6, 7) that can cause infection in chickens other than NDV in the samples. However, this finding disagreed with the finding of Hasan *et al*.(2010) who molecularly identified 20 samples (58.8%) out of 34 clinically diagnosed samples from layers and broilers. Variation between these results probably occurred because Hasan *et al*. (2010) collected his samples from chickens clinically diagnosed with Newcastle disease on the basis of postmortem lesions, while samples from this study were collected on the basis of apparent clinical signs of the disease. The faint bands seen may be due to low virus concentration in the samples because of site of sample collection and late stage of infection in which samples were collected during this study. This is in agreement with Haque *et al*. (2010) who reported that NDV concentration is higher in trachea than cloacal site and this is because the major rout of the spread of NDV is aerosol, and therefore there is high probable chance of the virus concentration in the trachea. Keneth *et al*. (2014) reported that low viral concentrations might also be due to the period of sampling during early and advanced stages of the disease. Also, this low number of isolates may be due to non-viability of the virus in the samples. As PCR can detect even samples containing non-viable viruses provided the genome or target gene is intact, only samples with viable virus can be amplify in embryonated chicken eggs. In addition, virus concentration was found to be low in cloacal swab when compared with other clinical samples. This finding agreed with the finding of Haque *et al*. (2010) who reported that virus isolation rate was higher from tracheal swab and spleen than compared cloacal swab.

The protein sequence motif ^{112}RRQKR^{116}-F at the F-gene cleavage site discovered in this study is a characteristic of virulent strain. This is in line with what Aldous and Alexander (2001) reported. Solomon *et al*. (2010) also reported similar finding in his research on exotic chickens in Nigeria.

In this study, the identification of same sub-genotype XIVb in all the strains recovered in Zaria and environs and high sequence similarities from commercial farms and that of live bird markets (98.5%, 98.1%, 98.5%, 97.9%, 98.1%) may be due to horizontal spread through fomites by people who often interact with chickens in Live Bird Markets and move to commercial poultry farms for other businesses- to buy spent or culled chickens, to buy eggs, treatment, to work, etcetera. Consequently, spill over from live bird market to commercial farms may occur, which invariably entails the poor nature of bio-security practices in live bird market and some commercial farms.This finding agreed with the finding of Solomon *et al*.(2010) who reported that similarities in the sequences of strains recovered from LBMs and those associated with outbreak in commercial farms suggests spill over from the LBMs which may be due to poor bio-security practices.In addition, environmental factors such as the harmattan wind which can transmit the virus from one place to another might have played an important role in the spread of NDV between live bird markets and commercial farms.

As observed in this study, sub-genotype XIVb strains were also found in commercial farms in Zaria that had reportedly vaccinated their chickens with la Sota strain vaccine from different countries- U.S, Australia. However, vaccine-related strains were not detected from commercial farms and live bird markets. Phylogenetic analyses revealed that most strains had more than 20% percent divergence with vaccine strainsl. This is of clinical significance because it may affect NDV shedding in faeces. As reported by Cattoli *et al.* (2010), similarities between field and vaccine strains less than or equal to 90% percent were significantly related to higher viral shedding. Patty *et al*. (2007), also reported that vaccinating birds with more antigenically similar vaccines with the challenged strain showed reduced viral shedding. Miller *et al*. (2007) also reported that NDV shedding in chickens correlates with the similarities between vaccine strains and challenged NDV HN and F genes.

It is generally known that most of African NDV strains shared the residues D72, E74, A75, K78, A79 on the F gene (Snoeck *et al.,* 2013). Strains from this study shared some of these neutralizing epitopes with the vaccine strains, however, variations in others such as seen in position 78, were lysine in vaccine strain is replaced with arginine in field virus; proline is replaced by leucine at position 36; asparagine is replaced by leucine at position 51, e.t.c., can influence the activity of the virus. This is similar to the report of Estevez *et al*. (2007) who stated that a single amino acid substitution can affects viral activity without necessarily affecting viral pathotype.

Fasina (2007) reported that trans-border activity such as live bird trade, contributes in transmission of avian influenza and other avian diseases between Nigeria and its neighbouring countries. This point was illustratedin this study since all the strains of this study are similar in topology with representative strains from Nigeria and neighbouring Benin republic isolated in 2009. Nigeria shares border with Benin republic through Kogi and Kebbi states in the north;the latter is located in the same political zone with the study area.Certain towns serve as trans-border commercial nerve centers were extensive trans-border activities occur. For example, the extensive live bird trade in Kamba, which is a border town between Nigeria and Niger republic in Kebbi state, may likely encompasses poultry from neighbouring Benin republic and thus, transmission of avian disease like ND is likely. These NDV strains can then easily be disseminated by the activities of live bird vendors who transport large number of poultry to different towns and cities in Nigeria, like Zaria which has large concentration of students and civil servant.

Strains from this study also clustered with one representative strain isolated in 2007 in Katsina state, Nigeria. Though previously classified by the authors as belonging to Lineage 7d, but recent NDV unified nomenclature proposed by Diel *et al*, (2012) and adopted and used by Snoeck *et al*, (2013) grouped the strain in to sub-genotype XIVb. Katsina state shared boarder with Kaduna state and the role of local poultry vendors in spreading ND between this neighbouring states cannot be underestimated.

Based on the results from this study, higher numberof NDVisolateswere isolated from live-bird markets than birds from commercial farms. This may be due to various supply origins manned by semi-professional intermediate traders with poultry from rural house holds more likely contribute to the enzootic circulation of NDV.Three distinct clusters were observed in this sub-genotype XIVb of this study. The representative strains clustered together; one strain from commercial farm formed another cluster; and the major cluster is the one that comprises all NDV strains from live bird market and two strains from commercial farms. This finding agreed with Van Borm *et al*., (2012), who observed two distinct clusters in sub-lineage 7a (currently sub-genotype X1Va), and one additional cluster in sub-lineage 7d (currently sub-lineage XIVb). It also agreed with the finding of Solomon *et al*. (2012), who reported genetic diversification of NDV strains in live bird market.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study shows that:

Newcastle Disease Virus circulating in Zaria has the fusion protein cleavage site motif of ^{112}RRQKRF^{117}, which is a characteristic of virulent strain and clustered with the representatives of sub-genotype XIVb.

There are high genetic similarities between strains identified from commercial farms and those identified in live bird markets.

The NDV from Zaria are not related to the vaccine strains (LaSota, V4 and Komarov).

Based on the phylogenetic analysis, strains from this study are similar with a Nigerian strain from Katsina (JQO39390) and Benin republic strain (JX546245).

6.2 Recommendations

The following recommendations are made:

There is need for continuous surveillance and characterization of NDV in Zaria so that an existing circulating NDV strain and or genotype at a particular period will be known.

There is need for further research to develop a vaccine that will match the antigenic diversity of current NDV strain circulating in Zaria.

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APPENDICES

Appendix I:NDV sequences characterized from this study

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Appendix II: Sequence identity matrix

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Sequence Identity Matrix
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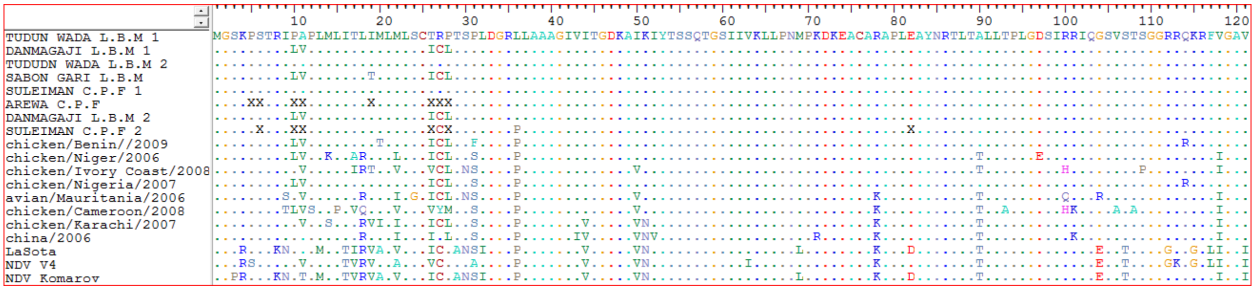
Copy.TXT

Seq->   TWADA   DMGJI   TWADA   SGARI   SLMAN   AREWA   DMGJI   SLMAN   Benin
        Niger   ICost   Nigra   Mtani   Croon   Kraci   china   LSota   NDVV4
        Kmrov
TWADA   ID      0.983   1.000   0.980   1.000   0.980   0.983   0.976   0.959
        0.916   0.864   0.959   0.861   0.842   0.857   0.854   0.780   0.795
        0.778
DMGJI   0.983   ID      0.983   0.997   0.983   0.980   1.000   0.980   0.976
        0.933   0.876   0.976   0.873   0.859   0.869   0.857   0.783   0.802
        0.780
TWADA   1.000   0.983   ID      0.980   1.000   0.980   0.983   0.976   0.959
        0.916   0.864   0.959   0.861   0.842   0.857   0.854   0.780   0.795
        0.778
SGARI   0.980   0.997   0.980   ID      0.980   0.980   0.997   0.978   0.973
        0.930   0.878   0.973   0.871   0.857   0.866   0.854   0.780   0.800
        0.778
SLMAN   1.000   0.983   1.000   0.980   ID      0.980   0.983   0.976   0.959
        0.916   0.864   0.959   0.861   0.842   0.857   0.854   0.780   0.795
        0.778
AREWA   0.980   0.980   0.980   0.980   0.980   ID      0.980   0.985   0.957
        0.914   0.861   0.957   0.857   0.840   0.852   0.845   0.771   0.788
        0.769
DMGJI   0.983   1.000   0.983   0.997   0.983   0.980   ID      0.980   0.976
        0.933   0.876   0.976   0.873   0.859   0.869   0.857   0.783   0.802
        0.780
SLMAN   0.976   0.980   0.976   0.978   0.976   0.985   0.980   ID      0.961
        0.919   0.869   0.961   0.866   0.845   0.857   0.847   0.778   0.792
        0.776
Benin   0.959   0.976   0.959   0.973   0.959   0.957   0.976   0.961   ID
        0.938   0.871   0.985   0.869   0.859   0.869   0.857   0.780   0.795
        0.778
Niger   0.916   0.933   0.916   0.930   0.916   0.914   0.933   0.919   0.938
        ID      0.869   0.933   0.864   0.866   0.866   0.864   0.795   0.807
        0.795
ICost   0.864   0.876   0.864   0.878   0.864   0.861   0.876   0.869   0.871
        0.869   ID      0.873   0.928   0.890   0.888   0.880   0.819   0.830
        0.807
Nigra   0.959   0.976   0.959   0.973   0.959   0.957   0.976   0.961   0.985
        0.933   0.873   ID      0.869   0.861   0.876   0.864   0.778   0.797
        0.776
Mtani   0.861   0.873   0.861   0.871   0.861   0.857   0.873   0.866   0.869
        0.864   0.928   0.869   ID      0.880   0.890   0.878   0.811   0.816
        0.809
Croon   0.842   0.859   0.842   0.857   0.842   0.840   0.859   0.845   0.859
        0.866   0.890   0.861   0.880   ID      0.869   0.861   0.800   0.807
        0.804
Kraci   0.857   0.869   0.857   0.866   0.857   0.852   0.869   0.857   0.869
        0.866   0.888   0.876   0.890   0.869   ID      0.883   0.809   0.826
        0.802
```

china	0.854	0.857	0.854	0.854	0.854	0.845	0.857	0.847	0.857	0.864	0.880	0.864	0.878	0.861	0.883	ID	0.809	0.823	0.802
LSota	0.780	0.783	0.780	0.780	0.780	0.771	0.783	0.778	0.780	0.795	0.819	0.778	0.811	0.800	0.809	0.809	ID	0.869	0.957
NDVV4	0.795	0.802	0.795	0.800	0.795	0.788	0.802	0.792	0.795	0.807	0.830	0.797	0.816	0.807	0.826	0.823	0.869	ID	0.859
Kmrov	0.778	0.780	0.778	0.778	0.778	0.769	0.780	0.776	0.778	0.795	0.807	0.776	0.809	0.804	0.802	0.802	0.957	0.859	ID

Appendix III:Amino acid sequence alignment



Appendix IV:Thermal cycler (PCR machine) used for this study

Appendix V:The researcher doing RNA extraction in Virology laboratory, National Veterinary Research Institute, Vom, Plateau State, Nigeria

Appendix VI:Molecular ladder

Appendix VII:Gel documentation system

Appendix VIII; Eppendorf centrifuge machine

Appendix IX:Innoculated embryonated ND-free chickens eggs in the course of this research

Appendix X:Quotation for sequencing from Inqaba biotech.

inqaba biotec

Quotation

Quote Prepared For
H.A Kazeem
Ahmadu bello University zaria, Nigeria
Department of Vet. Microbiology
Facult of Vet. Medicine, A.B.U
Zaria, Kaduna State NGA
+2348029314296

Quote Date 19-Mar-2015
Quote Number 201500124

TIN/VAT No: 17949735-0001
Co.Reg.No: RC1232028

Sequencing Primers and Reactions

Item #	Description	Qty	Price	Extended Price
IB SE0005	FULL SERVICE sequencing reactions, no repeat	2	$8.20	$16.40
IB OL0006	Sequencing primer (internal use)	2	$7.15	$14.30

Naira equivalent: N6,931.6

Exchange rate: N215/USD

Sequencing Primers:
NDVM610 F-CTG TAC AAT CTT GCG CTC AAT GTC
NDVF581 R-CTG CCA CTG CTA AGT TGT GAT AAT CC

Total Items	$30.70
Labor / Shipping	$0.00
VAT @ 5.00%	$1.54
Total Quote	**$32.24**

All Quotes are valid for 14 days unless otherwise stated.

Origin of products: IB = inqaba biotec / FE, F, AB, DH, OBS and ND = Thermo Scientific / NEB = New England BioLabs / ZR = Zymo Research / SG= Seegene / BC = Biocom / CAPP = CAPP / CCR = Clare Chemical Research / GV = Genevac / LU = Lucigen / CLC = CLC bio / MIR =Mirus Bio / SC = ScienCell / Bioo = Bioo Scientific / BL =BioLytix /DV = Devyser /AM = Amresco / BPS = BPS Bioscience / ED = Edvotek / EL = Elchrom / RA = Rapigen / VB = Vilber

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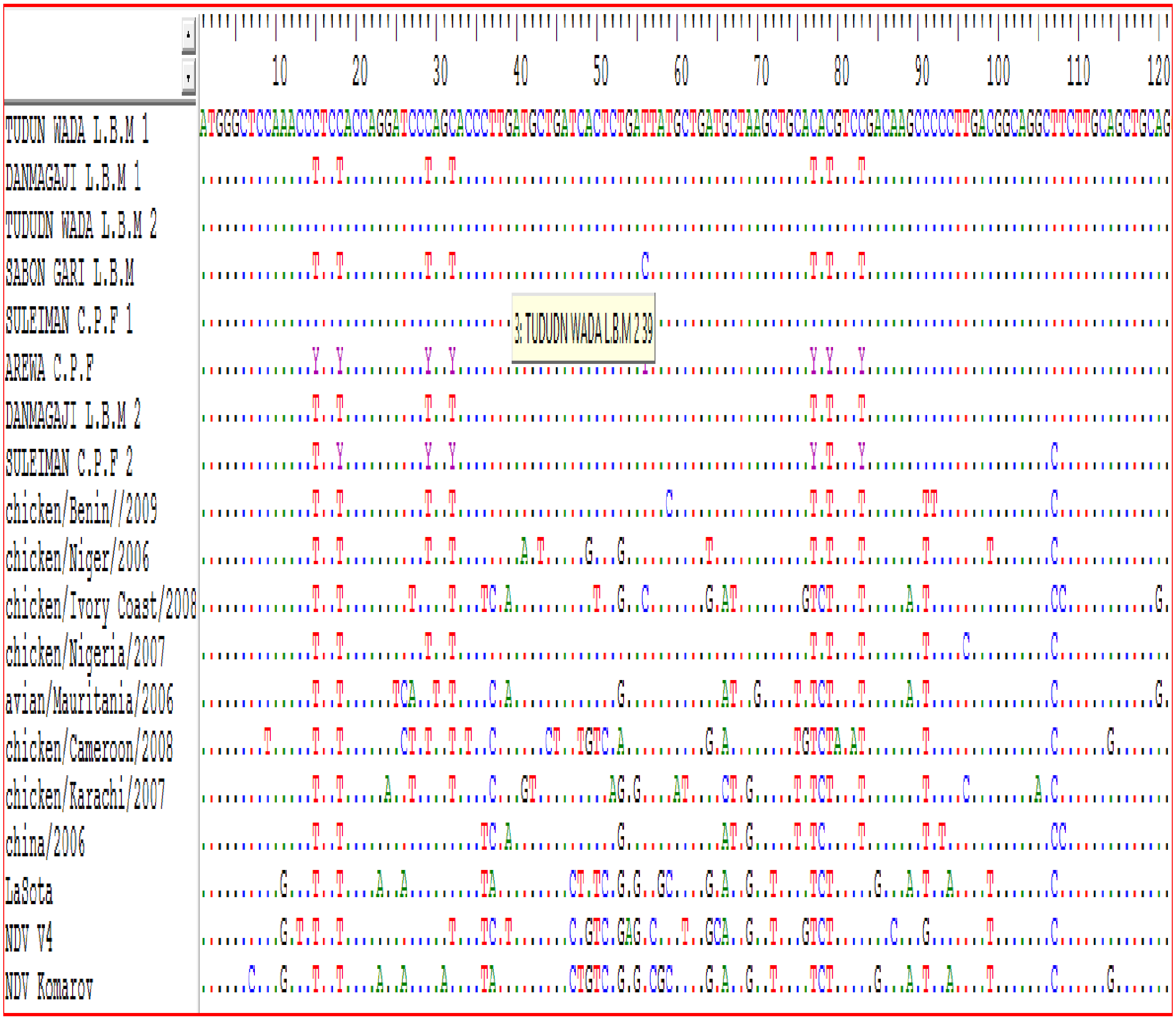
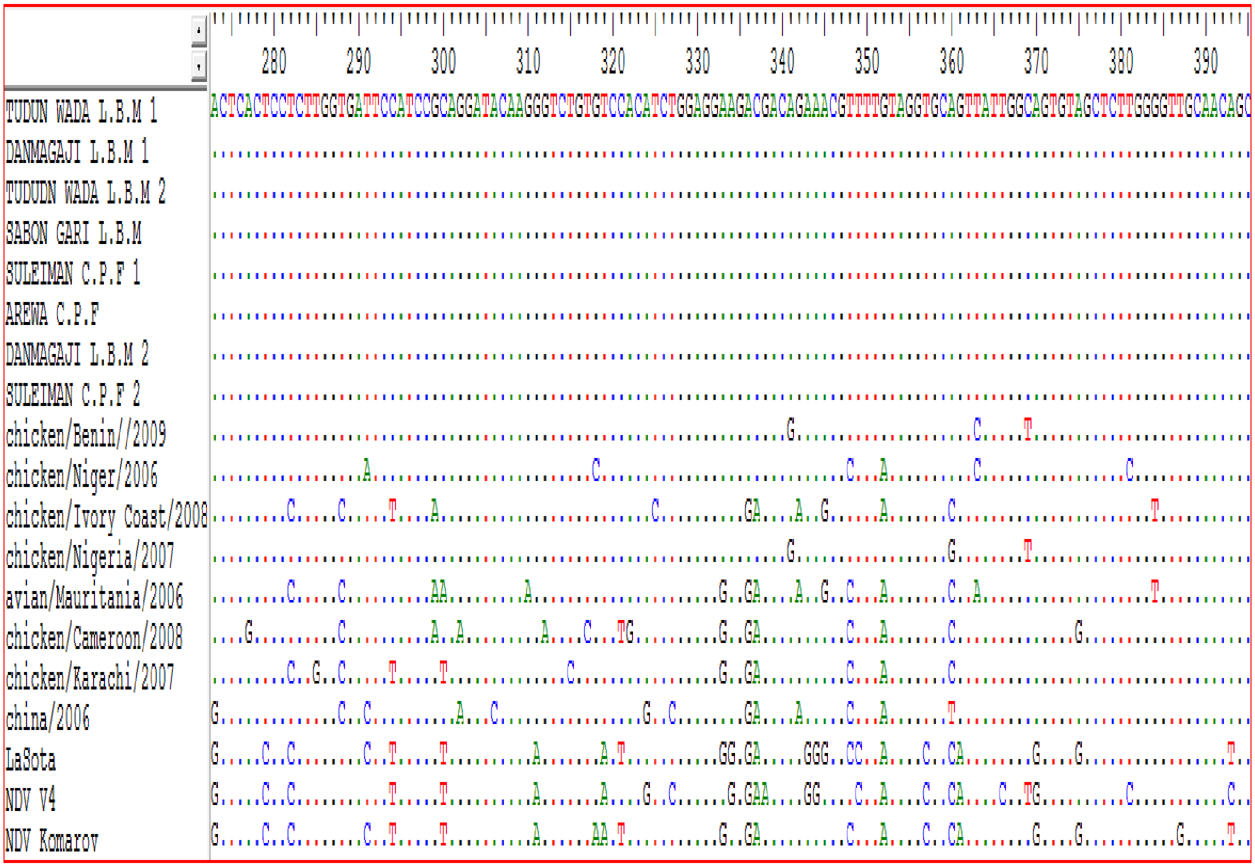
1/1

Appendix XI:Nucleotide sequence alignment (419bp) of the eight Nigerian (Zaria) NDV strains from commercial and local chickens including reference strains from gene bank

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DANMAGAJI L.B.M 1
TUDUDN WADA L.B.M 2
SABON GARI L.B.M
SULEIMAN C.P.F 1
AREWA C.P.F
DANMAGAJI L.B.M 2
SULEIMAN C.P.F 2
chicken/Benin//2009
chicken/Niger/2006
chicken/Ivory Coast/2008
chicken/Nigeria/2007
avian/Mauritania/2006
chicken/Cameroon/2008
chicken/Karachi/2007
china/2006
LaSota
NDV V4
NDV Komarov

Appendix XII: Result of first passage of M-gene positive NDV samples in to embryonated ND-free chicken eggs

Samples I.D	Embryonic mortality	HA result	Comment
NGR/ZRA CK/02	+	+	NDV isolated
NGR/ZRA CK/05	+	+	NDV isolated
NGR/ZRA CK/06	+	+	NDV isolated
NGR/ZRA CK/08	+	+	NDV isolated