MOLECULAR CHARACTERISATION OF NEWCASTLE DISEASE VIRUS FROM DIFFERENT AGRO-ECOLOGICAL ZONES IN GHANA

BY:

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DEDICATION

I dedicate this work first and foremost to God Almighty for giving me strength, wisdom and the financial resources to enable me to complete it.

To my parents, Mr. and Mrs. Adotevi for their prayers, support and encouragement throughout my study period.

To my siblings also, who assisted me in every way possible.

DECLARATION OF ORIGINALITY

I hereby declare that this thesis which is submitted to the Department of Animal Science, College of Basic and Applied Sciences, University of Ghana, for the award of Master of Philosophy in Animal Science degree is the result of my investigation. This thesis has not been submitted or presented for another degree elsewhere, either in part or in whole, except for other people's work which was duly cited and acknowledged.

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Although Newcastle disease is reported to be endemic in Ghana, little information has been documented on the molecular epidemiology and the genotype distribution of the Newcastle disease viruses (NDVs) in the country. In this study, a total of four NDV isolates were sequenced and analysed. NDVs recovered were from outbreaks in backyard and commercial poultry farms between 2018 and 2019. A full genome sequence of all four isolates was performed. A commonly used region of the virus genome that spans nucleotide 61 to nucleotide 374 of the Fusion protein, including the cleavage site was targeted. Based on sequence analysis, two of the sequences were classified under Genotype XIV and the other two classified under Genotype XVIII. Phylogenetic analysis, amino acid sequence determination of the F0 cleavage site as well as pairwise distance analysis of the full fusion protein gene sequences were done. Results showed close genetic similarities between circulating strains within the West African sub-region. The emergence and identification of different genotypes could give an insight into the high rate of mutations occurring in NDV strains in Ghana or importation and transmission of strains from other countries, which also raises concerns about the efficacy of current NDV control measures in the country. The amino acid sequence identity of the Ghanaian strains and the NDV- I2 vaccine strain ranged from 82 – 83%. The genetic relatedness of some of the Ghanaian NDV strains to the NDV I2 vaccine strain makes the isolated Ghanaian strains prime candidates for the production of an NDV vaccine. Thus, there is the need for continuous surveillance and characterization of NDV in Ghana to monitor the emergence of new genotypes within the Ghanaian poultry industry.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Poultry production is the quickest developing segment of global animal meat production, with developing and transitional countries playing a leading role (Assa, 2012). Indeed, poultry farming is considered to be chief among the backup occupations of farmers to enhance their wages since it guarantees quick returns, requires the least space and minimum investments and can be performed by ordinary farmers (Rojendran and Mohanty, 2003).

In Ghana, the poultry sector is an essential source of income and livelihood for numerous individuals and families, especially within rural communities. The major sources of animal protein in the Ghanaian diet are chicken eggs and meat and their importance cannot be overlooked. According to the Ministry of Food and Agriculture, the poultry sector in the country has experienced tremendous growth in the last few decades with the establishment of commercial farms that have hundreds of thousands of broilers and layers for meat and egg production, respectively (MOFA, 2016).

The poultry industry in Ghana is made up of a rural sector and a commercial sector of which the rural sector is more common than the commercial. Production systems within rural poultry are characterized by little to no input supply as regards feed and veterinary health care consequently resulting in low productivity. The industry functions as being more economic and social than nutrition among the producers who are mainly the poor and economically vulnerable category, and includes mainly include women and children with whom they serve such capacities as a store of

significant worth, methods for raising capital for progressively capital-intensive ventures and cementing of cultural and social ties (Alders and Spradbrow, 2001; Smith, 2013).

In Ghana, the population of poultry is said to be more than 73 million (MoFA/SRID, 2011) This number, though seemingly large, still indicates an opportunity for improvement as the demand for animal protein is on the rise, and current numbers are unable to meet the current demand. Despite the importance of poultry rearing, several challenges have been identified as constraints to the poultry industry in Ghana. Chief among them are management, lack of capital, heat stress and sanitation and diseases. Notable among the diseases afflicting the industry are Avian Influenza (AI), Infectious Bursal Disease (IBD), Fowl Pox (FP), Salmonellosis, Gumboro, Chronic Respiratory Disease (CRD) and Newcastle Disease (ND) which is said to be most economically important (Atuahene *et al.,* 2010). Of the major diseases affecting poultry in Ghana between the years 2007 and 2012, it was recorded that Newcastle disease had the highest incidence and caused the most mortalities within the period under review (Veterinary Services Directorate, 2013).

Newcastle disease is an acute infection of the Newcastle disease virus which affects about 250 species of birds of which chickens are the most susceptible (OIE, 2012; Kang *et al.,* 2016). It can also be referred to as pseudo fowl pest, Ranikhet and avian pneumoencephalitis (OIE, 2012). The virulent strain of Avian Paramyxovirus Type 1 (APMV-1) is the cause of the disease. The International Office of Epizooties (OIE, 2012) has categorized it as a List A disease. The disease has remained widespread in both endemic and epidemic types all over the globe since its first formal report on poultry in Java, Indonesia (Kranevald, 1926) and Newcastle-upon-Tyne (Doyle, 1927) (Brown *et al.,* 1999).

The first occurrence of ND in Africa goes back to the 1930s and 1940 and ever since then, the disease has become endemic in many parts of the continent (Abolnik *et al.,* 2017). In West Africa,

the first incidence was recorded in the 1950s and despite several efforts to control the disease, there continue to be serologically high prevalence rates in the sub-region (Snoeck *et al.*, 2009**)**. The disease usually presents as a respiratory disease although other clinical signs such as diarrhoea, depression or nervous manifestations can be observed (OIE, 2012).

The Newcastle Disease Virus is a negative sense, single-stranded, enveloped RNA virus, with a genome of approximately 15.2kb in length comprising six genes coding approximately six structural proteins and other non-structural proteins (Kang *et al.,* 2016). Although all the strains belong to one serotype, the virus is divided into two wide groups, Class I and Class II. Class I members are known to have one genotype whereas, in Class II, the disease is sub-divided into sub-genotype I to XVIII, all expected to be virulent in poultry (Dimitrov et al. 2016; Bello et al. 2018).

In Africa, most of the virulent strains isolated belong to Class II with a lot of the genotypes belonging to V and VII. In West Africa specifically, most of the isolates are in genotypes VII and most recently XIV and XV (Dimitrov *et al.,* 2019).

In commercial poultry production, control strategies have a multifaceted approach which mainly includes biosecurity at the farm level and vaccination methods (Alexander *et al.,* 2004). However, despite the adoption of these strategies, there are still outbreaks that occur in many African countries including Ghana**.**

In Ghana, the I-2 thermostable vaccine is the most predominant vaccine being used in the control of ND. This however has not offered much improvement in the disease incidence in the country based on the figures from VSD in 2013. Viral shedding in poultry and vaccination strategies not being as effective under varying environmental conditions are the reasons that have been implicated in the cause of vaccine inefficiency in the prevention of Newcastle Disease (Dimitrov

et al., 2016). Viral shedding typically takes place over the incubation period and the virus can last for up to several weeks in the environment. Weak biosecurity measures can then cause further spread through contaminated food, water, equipment and human clothing (OIE, 2012).

1.2 Justification

Studies around the Newcastle disease have been mainly centred on the immunological properties of the virus and the response it generates within the infected birds rather than the genomic properties (Ashraf and Shah, 2014).

As vaccination and improved biosecurity measures are the main control strategies for the disease, extensive use of vaccines makes the condition for genetic modifications more favourable in pathogenic strains. In more recent times, the number of viruses that are being reported has increased (Dimitrov *et al.,* 2016b). Between 2013 and 2015, an estimate of 60 countries had reported ND outbreaks yearly with novel genotypes being discovered. The expansion in the number of genotypes indicates the diversity of the virulent NDV strains is enlarging, presupposing that perhaps vaccination might be a factor of this occurrence. Moreover, vaccine strains presently used (primarily genotypes I and II) are not less than 30 years old and were found to be genetically remote (18.3%–26.6% nuclear distance) from virulent strains of NDV (Dimitrov *et al.,* 2016b). Therefore, the vaccine's effectiveness in preventing viral discharge from even the vaccinated birds is decreased with such a large genetic distance between the modern NDV strains and the vaccine (Miller *et al.,* 2007; Miller *et al.,* 2009). In West Africa, as of 2016, just four complete genome sequences of the ND virus which are prevalent in the geographical location are available in sequence repositories (Shittu *et al.,* 2016a). The limited information on the circulating strains in western Africa is a challenge for efficient control policies to be developed. (Shittu *et al.,* 2016b). Obtaining additional sequences will facilitate the comprehension of the evolutionary dynamics of

the prevalent viral strains which will then aid in the development of effective controls of the disease. Despite the trans-border movements of poultry and other poultry products which are known to happen both legally and illegally across the countries and regions in West Africa (Ganar *et al.*, 2017).

Currently, there is little available information concerning the genotypes circulating within the West African sub-region unlike other NDV genotypes prevalent in most parts of the world. Genotypes XIV, XVII and XVIII have been determined to be the causative genotypes of the current ND outbreaks being experienced all over West Africa. These genotypes have only been isolated from outbreaks within the sub-region and are yet to be detected elsewhere, indicating that they might be indigenous to the region (Cattoli *et al.*, 2009; de Almeida *et al.*, 2009; Snoeck *et al.*, 2009; Solomon *et al.*, 2012a; Solomon, 2012b; Van Borm *et al.*, 2012; Snoeck *et al.*, 2013)

Here, a greater understanding of the epidemiological interactions between the circulating NDV strains, their genetic diversity and features, and global distribution is now essential for the development of new vaccines and other control strategies. (Dimitrov *et al.*, 2016a).

Research is, therefore, necessary in the following areas:

- isolation and molecular characterisation of NDV strains;
- a full genome-sequence analysis of distinct NDV isolates for further studies of epidemiology, vaccine production and developmental origins (Ashraf and Shah, 2014).

1.3 Hypothesis

There are no genetic variations among NDV strains circulating in the different agro-ecological zones of Ghana.

1.4 Objectives

- To sequence, the full genome of Newcastle disease virus strains obtained from chickens in the Guinea Savannah, Forest and Coastal Savannah zones of Ghana.
- To determine if there are genetic variations in the F gene among NDV isolates.
- To determine the genetic distance between isolates from Ghana and the vaccine strain

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Newcastle Disease

The World Organisation for Animal Health (OIE) defines Newcastle disease (ND) as a Newcastle Disease Virus (NDV) infection based on either the intracerebral pathogenicity index (ICPI) in day-old chicks or the correlation of multiple basic amino acids on the cleavage site (OIE, 2012).

It is a highly contagious and often severe disease found worldwide that affects birds including domestic poultry caused by a virus belonging to the family of paramyxoviruses (OIE, 2012). It is currently known to be one of the most important diseases of poultry causing high morbidity and mortality in birds.

ND poses a global issue that expresses itself predominantly as an acute respiratory disease; depression, nervous manifestations, or diarrhoea as the predominant clinical signs of infection (CFSPH, 2016).

The severity of the disease depends on the virulence of the infective virus and the susceptibility of the host. Newcastle disease in its highly pathogenic form is a disease listed in the World Organisation for Animal Health (OIE) Terrestrial Animal Health Code as a List A disease and must be reported to the OIE (OIE Terrestrial Animal Health Code) (OIE, 2012) which may result in trade restrictions (Miller, MSD manual, 2019).

2.1.1 Transmission

ND is usually transmitted through inhalation or ingestion by the birds mostly after direct contact with infected or carrier birds (OIE, 2012). The virus is usually shed in the faeces and respiratory

secretions of infected birds (CFSPH, 2016) and this can happen during the incubation stage, the clinical period and even during convalescence but for limited periods. The virus can also occur in eggs laid during clinical illness and in all parts of the carcass during infections with acute velogenic Newcastle disease virus (Miller, MSD manual, 2019). However, the information available on the survival of the virus is highly variable, as it is influenced by a variety of factors, such as humidity, temperature and exposure to light, in addition to the diagnostic methods used for detection of the virus (CFSPH, 2016).

The disease is found throughout the world is currently controlled in Canada, the United States and some European countries. However, because wild birds can sometimes carry the virus without succumbing to the disease, there is a possibility of outbreaks occurring anywhere poultry is raised (OIE Terrestrial Manual, 2008).

2.1.2 Clinical Signs and Symptoms

The onset of the disease is rapid with clinical signs showing 2-12 days post-infection (Miller, MSD manual, 2019). Clinical signs of the disease depend on several factors which include but are not limited to the strain of the virus, the species of the bird affected and the age of the bird (younger birds are usually more susceptible than older birds). In some cases, highly virulent infections can result in a high number of deaths without showing any clinical signs (OIE, 2012).

Observed signs in the bird include respiratory distress (coughing, gasping, sneezing and rales) usually predominant in lentogenic NDV infections. (Alexander, 2001). Nervous signs including tremors, paralysis, torticollis and clonic spasm are usually characteristic of neurotropic velogenic NDV infections (Miller, MSD manual 2019).

The characteristic signs of the most virulent form of viscerotropic velogenic NDV infection are respiratory distress in the bird accompanied by watery greenish diarrhoea, depression and swelling of the head and neck tissues. In layer birds, there might be a total or partial cessation of egg production. Mortality and morbidity are high particularly with velogenic NDV infections.

Viscerotropic velogenic Newcastle disease usually produces remarkable lesions. On serous membranes, petechiae may be observed; haemorrhages of the proventricular mucosa and intestinal serosa are accompanied by multifocal, necrotic haemorrhagic areas on the mucosal surface of the intestine, especially at lymphoid foci such as caecal tonsils. Splenic necrosis and haemorrhage and oedema around the thymus may also be seen (Miller, MSD manual 2019).

2.1.3 Diagnosis

The clinical signs presented by the disease are similar to that of highly pathogenic avian influenza and as such diagnosis is confirmed by laboratory testing (OIE, 2012).

The recommended method of diagnosis is generally the isolation of the virus from oropharyngeal swabs and tissues of infected birds by inoculation of the allantoic cavity of 9-11-day-old SPF embryonic chicken eggs. Infection confirmation is then done by the recovery of a haemagglutinating virus inhibited by NDV antiserum or by diagnostic PCR methods to detect NDV RNA (Miller, MSD manual 2019).

2.1.4 Treatment and Prevention

The disease currently has no cure and as such prophylactic vaccination is practised in most countries.

Enhanced biosecurity measures can aid in the protection of flock against Newcastle disease Biosecurity steps include bird sanitation, feed and water sources, reducing movement on and off the farm, and disinfecting vehicles and equipment entering the farm. Pests, such as insects and rodents, should also be tracked. If necessary, workers can shower and change into dedicated work clothes (CFSPH, 2016).

Vaccines are the preferred method of control for the disease and are widely used in areas where the circulating strains have been determined to be velogenic. Vaccination is known to prevent clinical signs in birds and also reduce viral shedding and transmission.

2.2 Newcastle Disease Virus

2.2.3 Virion Structure and Organisation

Newcastle disease virus (NDV) is known to be an enveloped, single-stranded non-segmented negative-sense RNA virus (Seal *et al*, 2000). The virus is an avian paramyxovirus type 1 (APMV-1), belonging to genus Avulavirus, family *Paramyxoviridae* and order Mononegavirales (Alexander and Senne, 2008). The ND virus genome is approximately 15.2kb long and follows the rule of six for paramyxovirus genomes by encoding six proteins in its genome (Kolakofsky *et al.*, 1998). The virus is known to have a molecular weight of 5.25.7x 10^6 Daltons (Kapczynski *et al.*, 2013).

There are six genes in the order; 3‘-NP-P-M-F-HN-L-5‘contained in the NDV genome that code for the six major polypeptides (nucleoprotein, phosphoprotein, matrix, fusion, haemagglutinin-neuraminidase and the large protein, respectively) (Lamb and Kolakofsky, 2002). Present are also two non-structural proteins V and W which are usually a result of differential initiation or transcriptional editing of the P gene mRNA (Qin *et al.*, 2008).

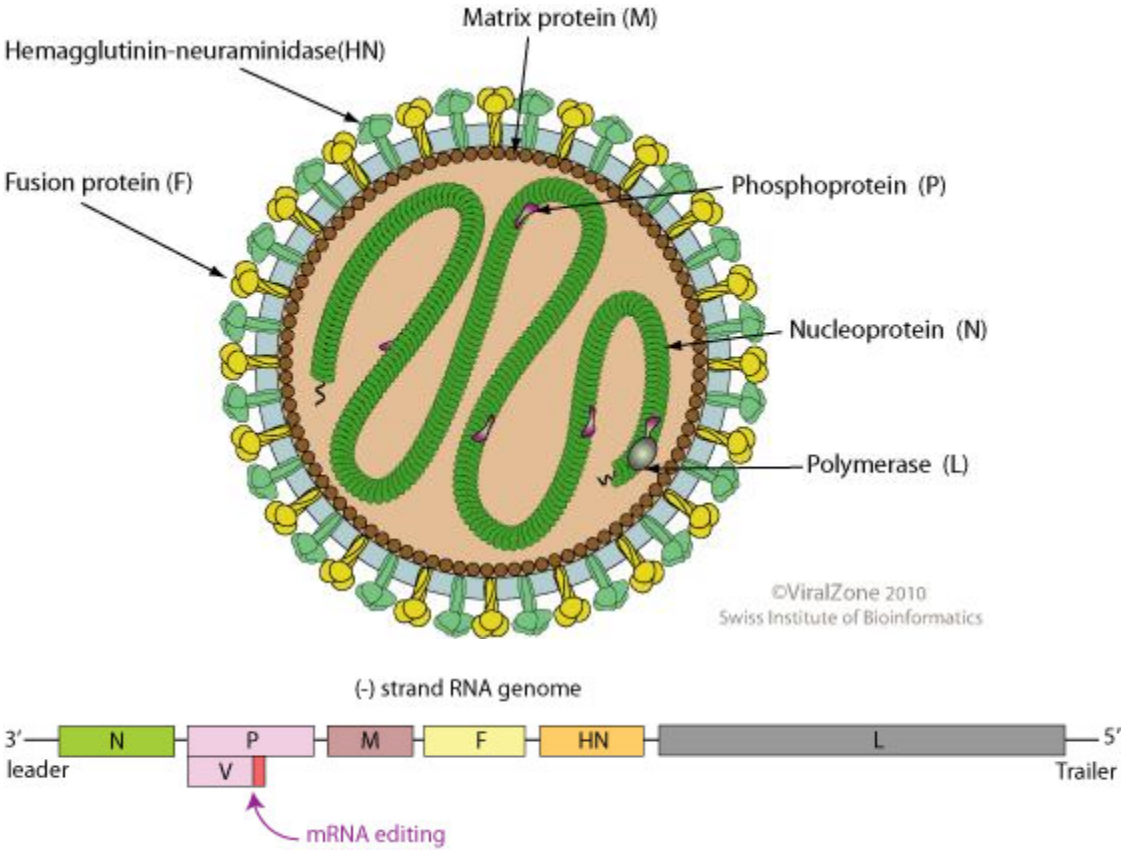

Fig *1*: *Negative-stranded RNA linear genome, about 15kb in size encodes for six structural proteins (Swiss Institute of Bioinformatics, 2010)*

The genome contains 55 nucleotides long extra cistronic region known as 'leader' at its 3' end and its 5' end, 114 nucleotide long regions known as 'trailer'. The leader and trailer are very important for viral genome transcription and replication. There are some conserved transcriptional control sequences present at the beginning and end of each gene which is known as gene start (GS) and gene end (GE), respectively. The GS acts as the transcriptional promoter and GE acts as the

transcriptional terminator. Between the genes, intergenic regions (IGS) are present. The length of these IGSs varies from 1-47 nucleotides (Phale, 2018).

2.2.4 Virus Entry, Replication and Assembly

Same as infections with others in the subfamily *Paramyxovirinae*, NDV infection is caused by the recognition and the binding of the virion to the sialylglyco-conjugates present on the host cell surface followed by the fusion of the viral lipid surface with the host cell membrane (Knipe *et al.*, 2007, Connaris *et al.*, 2002).

The primary adsorption of the virus to the target cell, with sialic acid residues or cell surface proteins acting as receptors, is usually promoted by the attachment protein. The fusion of the viral membrane with the host cell membrane is mediated by the F protein. Initially synthesized as nonfusogenic precursors of F0 paramyxovirus F protein is a trimeric type I integral membrane protection, which requires subsequent cleavage into the F1+F2 fusogenic disulphide related heterodimer (Everet *et al.*, 2009).

After successful entry into the cell, the cytoplasm of the host cell is the site for transcription of the viral genome which is initiated by the N, L and P proteins (MacLachlan *et al.*, 2017). Large numbers of messenger RNA transcripts are produced from genes closer to the promoter region than the terminator region and occur as a result of RNA dependent RNA polymerase transcribing the leader RNA and each of the viral genes into the different 5' capped and 3' polyadenylated mRNAs along the way (Whelan *et al.*, 2004)

The P of Paramyxoviridae is known to encode three to seven P/V/C proteins. This gene is essential for viral replication, however, the functions of the proteins produced by alternative transcription and translation are yet to be fully defined (Fenner's Veterinary Virology, 5th Edition). Replication begins when a sufficient number of viral proteins are produced and transcription stops. It produces

a full-length anti genome of negative-sense RNA in conjunction with the N protein (Lamb and Parks, 2007).

The nucleocapsids form a helix, accompanied by the incorporation of the P and L proteins, in the cytoplasm of the host cell and with an initial attachment of the N protein to the RNA. These nucleocapsids are conveyed to the plasma membrane and linked to the M protein via the F and HN surface glycoproteins. During the process of budding from the host cell, the viral envelope is formed on the virion particle (Lamb and Parks, 2007).

2.2.5 Proteins of ND Viral genome

The Genome encodes for at least eight proteins namely NP, P, M, F, HN, L, V and W.

2.2.5.1 Nucleocapsid Protein

The Nucleocapsid protein (NP) of NDV stains negative in electron microscopy. This is a flexible helical structure of approximately 18nm in diameter and 1µm in length. The essential subunit of the structures is a single 489 residue polypeptide which is around 53 kDa in molecular weight (Kho *et al.,* 2001). Located inside the central channel is the viral RNA which is encircled by 2200 to 2600 NP subunits (Choppin andCompans, 1975) which protect the viral RNA from nuclease activities.

The role the NP in conjunction with the L and P proteins plays in replication and transcription of RNA and the processes involved are yet to be fully understood (Yussof and Tang, 2001). The NP protein is also known to interact with the Matrix Protein during viral assembly (Blumberg *et al.,* 1981).

2.2.5.2 Phosphoprotein and Phosphoprotein gene-editing proteins

Newcastle Disease Virus P gene sequence analysis reveals that the phosphoprotein is rich in serine and threonine residues and serve as possible sites of phosphorylation (Lamb and Kolakofsky, 1996). Nucleotide sequence analysis also revealed that the protein comprises 395 amino acids with its molecular weight being calculated as -42kDa ((McGinnes *et al.,* 1988; Steward *et al.,* 1993).

The P protein association with the L and the NP protein form an active complex that is involved in genome replication and transcription (Hamaguchi *et al.,* 1983, 1985). By inserting 1-4 non-template Guanine nucleotides at specific position 484, transcriptional modification of the mRNA encoding the P protein at the editing site (476-CUAAAAAGGGCCCA-489) allows for potential translation of two non-structural proteins V and W (Steward *et al.,* 1993; Locke *et al.,* 2000).

The V protein contains a highly conserved motif resembling a zinc finger binding protein (Steward *et al.,* 1995) and a cysteine-rich C terminal region, which are probably involved in the replication and pathogenesis of the virus (Mebatsion *et al.,* 2001).

2.2.5.3 The Matrix (M) Protein

The Matrix protein is the most abundant protein inside the virion particle. Sequencing of the M gene of many strains of NDV and its translated products consist of 364 amino acids with a calculated molecular weight of approximately 40kDa (Chambers *et al.,* 1986; Seal et al., 2000).

It is a hydrophobic protein with many basic residues. The M protein is believed to play an important role in viral assembly by interaction with the nucleocapsid, lipid bilayer and surface glycoproteins exposed on the inner membrane surface (Yusoff and Tan, 2001).

2.2.5.4 The Large (L) Protein

This is the largest structural protein of the Newcastle Disease virus. It is made up of 2204 amino acids with a molecular weight of approximately 249kDa (Yusoff *et al.,* 1987). As of the year 2000,

only the L genes of the NDV strains Beaudette C and B1 were fully sequenced. Even though it is the largest protein, it has the least abundance in the virion core (about 50 copies per virion particle) (Tordo *et al.,* 1988). The protein possesses 5' and 3' poly (A) polymerase activities on the nascent viral mRNA.

2.2.5.5 The HN protein

The HN protein is one of the major antigenic determinants of the Newcastle disease virus.

The gene is made up of about 2000 nucleotides that carry an open reading frame encoding 517, 577, 581 or 616 amino acids (Sakaguchi *et al.,* 1989; Tan *et al.,* 1995). The HN protein is a type II integral membrane protein with a single hydrophobic domain at the N-terminal region. This glycoprotein possesses both HA and NA activities (Scheid and Choppin, 1974).

The neuraminidase activity of the virus is responsible for the binding of the virus to sialic acid from the virion surface as well as the infected host cell membrane (Miller *et al*., 2013).

Absorption of the virus to specific receptors on red blood cells forming a lattice framework between the cells causes haemagglutination (Kimball, 1990). The NA and HA association on the same glycoprotein which is characteristic of NDV is in contrast with the distribution of these activities in *Orthomyxoviridae*. Yusoff and others in 1988 stated that monoclonal antibodies which recognize amino acids 454, 456, 457 and 460 are known to inhibit neuraminidase activity.

2.2.5.6 The Fusion (F) Protein

The F protein is a type I integral membrane protein. It is also one of the main immunogenic determinants of the Newcastle disease virus. The F protein is known to be 1792 nucleotides long encoding 553 amino acids long precursor polypeptide with a calculated molecular weight of approximately 55kDa (Chamber *et al.,* 1986b).

At the peptide bonds of residues 116 and 117, the precursor is cleaved proteolytically to generate two disulphide linked polypeptides F1 and F2 by special cellular proteases (Goton *et al.,* 1992; Ogasawara *et al.,* 1992). This cleavability of the F0 is a major determinant for virulence.

Amino acid sequence analysis of Fgene reveals a conserved hydrophobic region of about 20 residues located at the amino-terminus of the F1 fragment (Lamb and Kolakofsky, 1996).

Before interaction with the host cell, the fusion protein is believed to be in a metastable conformation. Several 3-dimensional structures of the F protein in this form are available (Wilson *et al.,* 1981; Chen *et al*., 1998; Rosenthal *et al.,* 1998). The protein then undergoes conformational changes that expose the peptides resulting in the embedding of the viral particle in the host cell membrane (Baker *et al.,* 1999). It mediates the penetration by the virus by pH-dependent fusion between the envelope and the host cell membrane for the viral nucleocapsid to enter the host cell cytoplasm (Peeples, 2001).

2.3 Molecular Basis for Pathogenicity

Pathogenicity and virulence are most often used interchangeably however they are very different with regards to NDV. Pathogenicity is defined as the ability of an organism to cause disease or damage in its host (Studdert *et al.,* 2011, Casadevall and Pirofski, 2001). This is usually influenced by several factors spanning from both the host and the organism in question and the environment as well. Virulence however is defined as the measure of the degree of pathogenicity of an organism. It's a quantitative factor that can be measured in the laboratory.

In the course of NDV replication, the F protein which is synthesized as a non-functional Fo precursor is thus cleaved into the F1 and F2 polypeptides by host proteases such as trypsin which belong to the Furin family. This process causes viral particles to become infectious. When the cleavage of the virulent F0 is mediated by host proteases, it would cause damage to vital organs.

As such virulent viruses can then replicate in a wide range of host tissues and organs causing a systemic infection (Umali *et al.*, 1993; Wang *et al.*, 2015). However, the F0 molecule of less virulent strains leads to growth restrictions as it is less sensitive and would only grow on specific host types (Umali *et al.*, 1993).

Many studies confirm that several basic amino acids are present at the cleavage site in virulent virus strains (Phale, 2018). The degree of pathogenicity is closely related to the amino acid sequence motif present at the cleavage site. Most pathogenic strains of NDV (velogens and mesogens) infecting chickens have the amino acid sequence 112 R/K-R-Q-K/R-R116 at the C-terminus region of the F2 protein and F (phenylalanine) at residue 117 of the N-terminus of the F1 protein; whereas viruses of low virulence have the amino acid motif 112 G/E-K/R-Q-G/E-R116 in the same region and L (leucine) at residue 117 (Morrison, 2003).

However, some NDV strains of pigeon origin [PPMV-1] examined have the amino acid sequence 112G-R-Q-K-R-F117 and are pathogenic for several Avian species and have high ICPI values (Collins *et al.*, 1994). Therefore at least a pair of basic amino acids at residues 116 and 115 plus phenylalanine at residue 117 and basic amino acid [R] at residue 113 are necessary for the virus to be virulent in chickens.

While multiple specific amino acid cleavage site sequences of the F0 protein appear to be necessary for NDVs to be virulent in chickens, other factors associated with other virus genes and proteins have been shown to cause virulence variation. It has been shown that the HN protein can affect the virulence of viruses using reverse genetic techniques (Huang *et al.*, 2004; Romer-Oberdorfer *et al.*, 2006). Likewise, it has been shown that the V protein is an alpha alpha-interferon antagonist and has an important effect on virus virulence (Huang *et al.*, 2003). Numerous PPMV-1 viruses have been isolated which have shown a low intracerebral pathogenicity index (ICPI)

(<0.7) despite numerous base amino acid cleavage patterns values and the difference(s) responsible for this decreased virulence has not been identified yet. (Alexander, 2011).

2.4 NDV Classification and Geographic Distribution

NDV has traditionally been categorized according to five pathotypes that refer to the clinical signs found in completely susceptible infected chickens (Cann, 1997; Brown *et al.,* 1999). Asymptomatic enteric NDV - the presence of avirulent viruses in the intestinal tract, lentogenic NDV low virulence viruses which cause mild or inapparent respiratory infection, mesogenic NDV - Low mortality, acute respiratory disease, and nervous symptoms in some moderately virulent virus-infected birds, neurotropic velogenic NDV (NVNDV) – High mortality respiratory and neurological symptoms caused by highly virulent neurotropic viruses and viscerotropic velogenic NDV (VVNDV) Responsible for acute lethal infection triggered by extremely virulent viscerotropic viruses in which haemorrhagic lesions are generally seen in the gastrointestinal (GI) tract (Cann, 1997; Brown *et al.,* 1999).

Antigenic and genetic diversity is recognized in the Newcastle disease virus even though they all belong to a single serotype (Alexander *et al.,* 1997; Aldous *et al.,* 2003; Kim *et al.,* 2007a). Genetically, various schemes have been concurrently used to classify NDV in the past. The first system is the one in which Aldous and colleagues group the virus into six lineages and 13 sublineages (Aldous *et al.,* 2003; Snoeck *et al.,* 2009). Later, an additional lineage and seven more sublineages were proposed and added (Snoeck *et al.,* 2009; Cattoli *et al.,* 2010).

The other NDV taxonomy scheme proposed by Ballagi-Pordány et al. in 1996 and subsequently endorsed by Czeglédi et al groups NDV isolates into two separate classes, namely Class I and

Class II, with each class having different genotypes further divided into subgenotypes (Czeglédi *et al.*, 2006).

There have been conflicts and confusion generated by these schemes of classification, and as such, it was necessary to develop unified criteria for NDV taxonomy. Diel *et al.* (2010) suggested adopting the genotype-based classification in 2010 after a comprehensive study of these two schemes not only because it is the most commonly used, but also because it provides a greater correlation between developmental distances between the intergenetic communities and their phylogenetic relationships

Based on this new criterion, phylogenetic topology which used the complete instead of the partial F gene coding sequences was used to classify a new genotype. Besides this, a minimum of four isolates should be obtained from epidemiologically different events and must form a phylogenetic cluster not less than 60% as the bootstrap value. In addition, there should be an average interpopulation distance of ≥ 10 between the isolates. A mean evolutionary distance of 3-10% will however be used to define a new subgenotype within a group (Diel *et al.*, 2012).

NDV isolates then were broadly classified into class I and class II based on these objective criteria. All Class I isolates are grouped under a single genotype and three sub-genotypes based on their high genetic relatedness which is almost 96%. These isolates were mostly obtained from wild and domesticated birds in Africa, Asia, Europe, and America. All isolates belonging to this class are thought to be of low virulence in chicken except the isolate that caused the devastating outbreak in the early 1990s in Northern Ireland (Aldous *et al.*, 2003).

Fan *et al*. (2015) divided Class I into nine genotypes while Class II was divided into ten based on the isolated sequences over some time. Class I viruses are mostly lentogenic and are usually isolated from waterfowl, domestic poultry and shorebirds (Fan *et al.,* 2015).

In recent literature, however, Class II isolates have been categorized into genotypes I to XVIII with a majority of the genotypes being divided into subgenotypes (Snoeck *et al.,* 2013a; Snoeck *et al.,* 2013b; He *et al.,* 2018). Viruses in Class II have been isolated mainly from domestic fowls and wild birds found in North and South America, Africa, Asia and Europe (Dimitrov *et al.,* 2016). In 2016, Dimitrov *et al.* substantiated that Class II viruses contain varying virulence strains which span from the highly virulent strains which have been the cause of epidemics experienced in different parts of the world to the vaccine strains which are usually mostly used in disease control (Dimitrov *et al.,* 2016).

Class II viruses are known to be distributed worldwide with the genotypes V, VI, VII, and VIII being the predominant genotypes. The first identification of Newcastle Disease in North America was in the United States in 1944 (Beaudette *et al.,* 1948) even though it was previously reported to have been present (Dimitrov *et al.,* 2019). Viruses of various genotypes have been isolated in the United States since they were first identified (Dimitrov *et al.,* 2016; Brown and Bevins, 2017). In wild birds acting as natural reservoirs for the virus, several Class I and Class II viruses are virulently inferior (Goldhaft, 1980; Ramey *et al.,* 2013, 2017). There have only been two major disease outbreaks that plagued commercial poultry in the US in the last 50 years before 2018. First was a genotype VI predecessor of the virulent viscerotropic pathotype of the disease. The next major outbreak occurred in Southern California between the years 2002 and 2003 (Nolen, 2003b). The virulent NDVs of genotype V and subgenotype Vb triggered this epidemic, which

was more strongly associated with outbreaks in Honduras and Mexico from 1996 to 2000 (Perderson *et al*. 2004).

In May 2018, an NDV-positive backyard swab sample was screened in California after the sudden epidemic which killed more than 400 birds. A study was therefore undertaken with California isolated viruses in 2002 (CA02) and 2018 (CA18) in 2019 and Belize in 2008 (BE08) to determine genetic characteristics by Dimitrov *et al*. (2019). The F gene was sequenced and the evolutionary distance of these isolates was determined. The consensus of all 3 isolates anticipated a site of the cleavage fusion protein, which contained three fundamental amino acids in 113–116 positions and a residual of phenylalanine at 117 (113RQKR↓F117). Based on the OIE assessment criteria for virulence of NDV isolates, such a site pattern is especially important for virulent viruses (OIE, 2012). The full fusion gene coding sequences of the studied isolates were further analysed to establish the evolutionary distances between these isolates and between other viruses. In Honduras, the virus that caused the NDV California (CA18) outbreak in 2007 (98.2% of the nucleotide identity), BE08 and CA02 (97.9% and 97.4%, respectively), was found to be genetically closer to class II Vb NDV isolated from chicken in 2018-2019. The mean genetic distance for CA18 over the past 20 years was 4.2% (from 3.2% to 5.9%) compared to seven other Vb sub-genotype viruses isolated from the U.S. in Amazon parrots and chickens in Mexico. The average evolutionary distance between the Vb subgenotype and the other Vc, Va and Vd subgenotypes, 6.5%, 10.2%, and 11.2% respectively, was also less compared to that observed in chickens from Honduras (2000) and Nicaragua (2001) and parrots from the US in the 1980s, with mean nucleotide distances of 7.8% (from 6.0% to 9.1%) (Dimitrov *et al.,* 2019).

In South America, the disease is known to be more endemic. Class II viruses have been isolated from Peru (NDV-Peru/08) which was classified as being velogenic. Initially, the pathogenicity of

NDV-Peru/08 was assessed by fusion (F) protein cleavage site sequencing and by the standard ICPI test. The F protein cleavage site sequence indicated the presence of three basic amino acid residues at positions 113, 115 and 116 and phenylalanine at position 117 (112R-R-Q-K-R-F117). An ICPI of 1.78 (Diel et al., 2011), typical of velogenic NDV strains, was the result of intracerebral inoculation of NDV-Peru/08 in day-old chicks. (Diel *et al.,* 2012).

In 1946, NDV was first reported in Mexico. Highly related velogenic NDV have been isolated from different geographical regions in Mexico. Most of the recent NDV isolates from Mexico belong to class II, genotype V (Merino *et al.,* 2009; Perozo *et al.,* 2008; Absalon *et al.,* 2012) with subgenotype Vb (Diel *et al.,* 2012), and have a divergence of approximately 16% in the amino acid sequence compared with those of the genotype II vaccines. Phylogenetic analysis from a study by Garcia *et al.,* 2013 showed slight divergence among the same genotype virus indicating that the virus is continuously evolving (Garcia *et al.,* 2013). The disparity between virulent genotype V viruses and vaccine strains that promote viral shedding, as well as the persistence of NDV in backyard poultry and wild birds, may explain why velogenic NDV caused intermittent outbreaks in the Mexican poultry industry.

In Pakistan, velogenic NDV which was closely related to genotype VII was isolated from apparently healthy backyard poultry in 2010. This genotype is known to be the predominant genotype that circulates in Asian countries. A study done by Karachi shows that there are at least 2 different genotypes (VI and VII) circulating in Pakistan (Munir *et al.,* 2012).

HN genes have been engineered to specifically differentiate NDV genotypes and predict the actual pathogenicity of isolates since the length of the HN protein varies and the pathogenicity of the cleavage site is not the sole determinant. All these facts suggest that without showing clinical signs, rural poultry can harbour virulent NDV strains and thus act as quiet carriers and pose a potential

danger to commercial poultry. This finding, however, also shows the resistance of local breeds and their ability to harbour the virulent strains of the virus. Nevertheless, immuno-compromising management practices and the existence of secondary infections such as avian influenza and other bacterial and viral pathogens can lead to an illness that requires further research in rural birds.

Genotype VII is known to be connected with Asian, African and Middle Eastern outbreaks. In Ethiopia, viruses of genotype VII (Fentie *et al*., 2013) and genotype VI (de Almeida *et al.*, 2013; Chaka *et al.*, 2013) were identified and isolated from a few village chicken specimens (Damena *et al.*, 2016).

Genotypes XI and XIV have recorded the largest genetic distance between each other (28.9%) and 12.8% and 26.6% when the rest of the genotypes were compared to each other (Dimitrov *et al*., 2016). In only restricted geographical areas – Madagascar (XI) and Nigeria, Benin and Mali (XIV) over the last 10 years, viruses of these two genotypes have been isolated (Snoeck *et al*., 2009). Virulence of NDV has been shown to have a multigenic feature like influenza viruses that primarily come from HN, V and L NDV proteins. For example, the Beaudette C (BC) strain of NDV can demonstrate the essential role of L protein in pathogenicity, which increases virulence if it is carried by the L-protein in LaSota. The V-deficient viruses tend to slow down and have impaired growth in cell culture, which indicates V-protein virus participation by downregulation of the host immune response. Applying reverse genetics, it has been concluded that when the HN protein of BC strain was replaced with that of LaSota, the virulence of BC was reduced and the reverse was true for the LaSota strain. In addition, it was demonstrated that the function of 5'UTR in the HN gene was crucial to the virus pathogenicity. There was a Y526Q substitution at the receptor-binding site of the HN protein of Chicken/BYP/Pakistan/2010. This substitution caused a reduction in pathogenicity both in vivo and in vitro. This finding may explain the decreased or

attenuated pathogenicity in rural poultry birds of Chicken/BYP/Pakistan/2010 (Munir *et al.*, 2012). It should be recognised, however, that the isolate recovered pathogenicity when chicken embryos were contaminated, and this might require more studies to look at this mechanism at the molecular level (Munir *et al.*, 2012).

2.4.1 Newcastle Disease Virus Distribution in Africa and West Africa

ND outbreaks in Africa are mostly thought to be frequent, severe, and generally under-reported, as the monitoring and reporting system for animal diseases is inefficient. Within the continent, several genotypes within the Class II have been isolated including but not limited to IV in Sudan (Aldous *et al.*, 2003), V in East and West Africa (Ballagry-Pordany *et al.*,1996), VI and VII in South Africa (Abolnik *et al.*, 2004; Abolnik *et al.*, 2008). Most recently genotypes XIV and XVIII were discovered in West Africa (Cattoli *et al.*, 2010; de Almeida *et al.*, 2013; Samuel *et al.*, 2013). XIV genotypes were isolated from chickens and turkeys in Nigeria while XVIII is more widely distributed after being isolated from birds in Ivory Coast, Mali and Mauritania (Dimitrov *et al.*, 2016).

2.5 Vaccination

There is currently no known available treatment for Newcastle Disease and due to this, vaccination and strict biosecurity measures are the best way to control the disease and virus spread. At the moment, several live and attenuated vaccines are in use for the control of the disease all over the world.

The first NDV live vaccine was developed in the late 1940s after the discovery of the LaSota and Hitchner B1 strains which led to the commercialization of the vaccine then beginning in the 1960s

(Mayers *et al.*, 2017). Most of the strains used as live vaccines belong to the avirulent or lentogenic pathotype. Table 1 below shows the various known NDV vaccines in circulation around the world

Table 1: Known NDV vaccines in circulation globally

Virus strain	Pathotype	Vaccine type	Origin
Ulster 2C	Avirulent	Live or inactivated	Ireland
V4	Avirulent	Live or inactivated	Australia
V4-HR	Avirulent	Live (Thermostable)	Australia
I-2	Avirulent	Live (Thermostable)	Australia
F	Lentogenic	Live or inactivated	India
Hitchner B1	Lentogenic	Live or inactivated	USA
LaSota	Lentogenic	Live or inactivated	USA
Mukteswar	Mesogenic	Live (Booster)	India
Komarov	Mesogenic	Live (Booster)	Israel
AG68	Velogenic	Inactivated	Iraq

Source: EURL Meeting, APHA (2018)

Most chickens are usually vaccinated to prevent being infected by the virus. Despite this, vaccinated birds are still able to succumb to the disease resulting in respiratory distress and reduction in laying performance of layer birds (Cho *et al.*, 2008; Bwala *et al.*, 2012). Vaccination, however, has been instrumental to the drop in NDV infections around the world and is still the most important method of control for the disease (Albiston and Gorrie, 1942).

Sterilizing immunity which is the main objective behind vaccination of flock has however not been fully achieved with the current NDV vaccines. In the best case, the vaccines can stimulate an immune reaction which lowers the probability or eliminates the presence of clinical disease and death in the bird as well as reduces the amount of NDV that is shed (Kapcyzynski *et al.*, 2013). The vaccine also then raises the infective dose of the virus within the bird (Miller *et al.*, 2009).

An effective immunity program should offer protection to sub-optimally or non-vaccinated animals in a flock that is well-vaccinated, consequently resulting in herd immunity (Marangon and Busani, 2006). However, this result can only be obtained when hemagglutination inhibition antibody titres are higher than eight in 85% of the flock that has been vaccinated twice (Kapczynski *et al.*, 2013). The findings from the field reports show the challenge to velogenic NDV will be survived only by birds with HI titres above 16 after numerous vaccinations as more than 60% of the flock succumbed with lower titre values (Kapczynski and King, 2005). In general, HI concentrations of 32 or greater are considered protective (Allan *et al.*, 1978).

Velogenic NDV is controlled by frequent vaccination which is used to provide immunological protection against the disease in all the major poultry businesses. Our knowledge of NDV protection is based mainly on the antibody production directed towards the viral proteins that are associated with attachment and fusion. However, we are unable to fully understand the avian immune reaction to NDV. Although NDV exists as a single serotype, the latest velogenic NDV isolates show that the vaccine viruses found in the 1950s could lose efficacy in fresh 21st-century viruses. A renewed investigation into immunity from NDV in poultry is especially necessary and one of the problems currently lies in identifying the molecular processes of innate immunity which contributes to increased immunity against infection and a reduction in transmission and shedding of the virus and transmission. In addition, while undeniable, CMI's contributions to the general protection against NDV remain mainly undefined. It is necessary to further characterize the cell kinds and epitopes concerned so that fresh vaccines can benefit from this insight. In this genomic era, where the complete chicken genome is available, it has become possible to investigate and test the structure and function of the components of the avian immune system participating in NDV protective immunity. In light of recurrent outbreaks, a better understanding of the avian immune

response to the virus infection continues to be a major concern in developing better control strategies (Kapczynski *et al.*, 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study sites and sample collection

Thirty (30) suspected samples of Newcastle disease virus were collected from several sites all over the country covering three different agro-ecological zones as indicated in Figure 2.

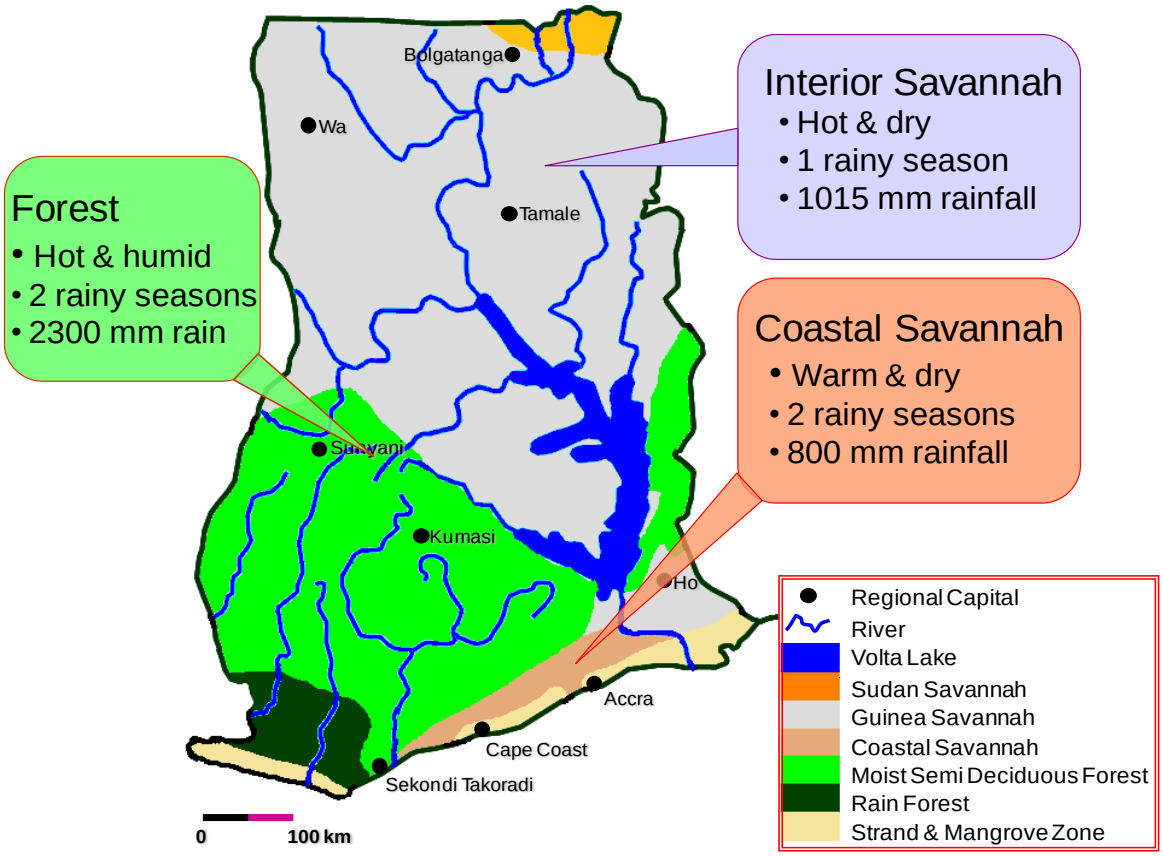

Figure 2: Agro-Ecological Zones in Ghana (google.com)

All the samples were obtained from birds exhibiting clinical symptoms of Newcastle Disease.

The inclusive criteria were chickens exhibiting sneezing and rales, gasping, coughing, tremor, muscle spasms, paralysis, torticollis (twisting of the neck), watery, greenish or white diarrhoea, swelling of the tissues and ruffled feathers (OIE, 2012).

Birds were first tested for avian influenza using the FluDETECT® Avian (Zoetis, USA). This was done to eliminate the differential diagnosis for the above clinical symptoms. All the birds tested negative and were euthanized, brain and trachea tissue samples were harvested, collected and stored on ice. Tissue and swab samples were then stored in the -80˚C freezer at the Molecular Genetics Laboratory of the Animal Science Department for further analysis.

3.2 Isolation of Newcastle Disease Virus in the Laboratory

Tissue samples were crushed with a mortar and pestle and homogenized with a virus transport medium made up of Phosphate Buffer Saline and glycerol in a 1:1 ratio. 250mg of Penicillin, 200mg of streptomycin and 250mg of gentamicin were also added to the solution. The Virus transport medium was then mixed with the crushed tissue sample and centrifuged at maximum speed for two minutes.

Nine to eleven day-old embryonated chicken eggs were candled, marked and the inoculation site disinfected with 70% ethanol. Four eggs were inoculated per sample. For inoculation of the virus, a hypodermic syringe (1 mL) was employed with a needle. The needle was used to inject 0.2 ml of the virus into the allantoic cavity through a pinhole in the eggshell passing through the chorioallantoic membrane. Glue was used to carefully seal the hole and the eggs were incubated again for 48-72 hours at 37.2˚Celcius (C).

Eggs that showed no signs of development and that had died were discarded. The inoculated eggs were incubated at a constant temperature of 37 ºC, with relative humidity maintained at 50- 55%

in the incubator. The eggs were candled daily. The inoculated eggs were incubated for 2-3 days and allowed to chill overnight before harvesting the allantoic fluid.

The harvested allantoic fluid was checked for the presence of haemagglutinin. A positive result indicated the presence of the Newcastle disease virus. The harvested allantoic fluid was divided into aliquots and stored at -80˚C.

3.3 Virus detection by hemagglutination

3.3.1 5% Chicken RBC Suspension Preparation

Blood was drawn from a healthy chicken into EDTA tubes to prevent coagulation. Alsever's Solution was mixed with the blood in a 15ml centrifuge tube with a ratio of 1:1. The mixture was centrifuged at 1500 rpm for 5 minutes at 4º C. The supernatant was discarded leaving the pellet in the tube 10ml of PBS was then added to the pellet and centrifuged at the same speed and timed for a total of 3-4 washes until the supernatant was clear and then was discarded. 0.5ml of RBC left at the bottom of the tube was aspirated and dispensed into a new 15ml tube. 9.5 ml of PBS was added to the tube for a 5% suspension in PBS and stored at 4º C.

3.2.2 Detection of viruses by hemagglutination Test

A drop of allantoic fluid was mixed with another drop of RBC on a clean and sterile slide. The ability of the allantoic fluid to cause hemagglutination in the chicken RBCs indicated positive results for virus growth.

The negative control was represented by mixing a drop of RBC with another drop of Phosphate Buffer Saline (PBS). The reaction was left for a few minutes and photos were taken for documentation.

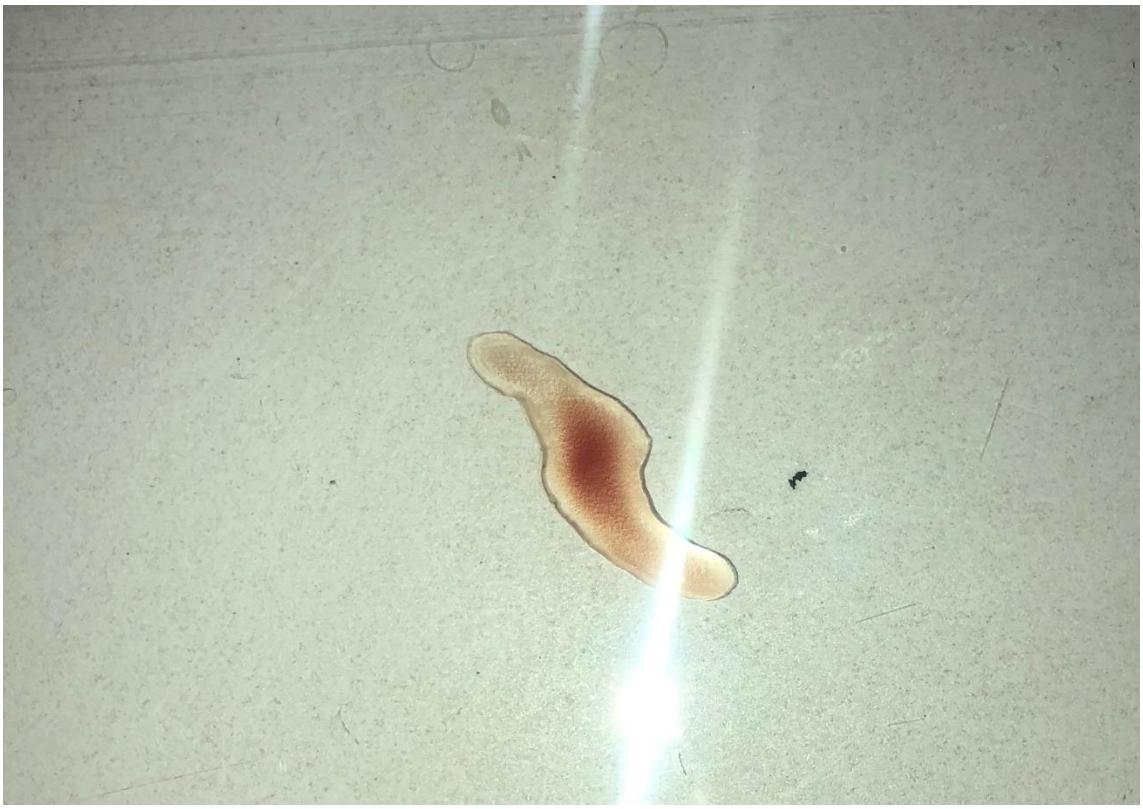

Figure 3: *Haemagglutination Activity demonstrated by samples to confirm the presence of NDV*

3.4. Isolation of total RNA from infective allantoic fluid

Frozen NDV containing allantoic was allowed to thaw at room temperature. 130 µl of Lysis Binding Solution containing Carrier RNA and isopropanol was added to 50 µl of the sample in nuclease-free Eppendorf tubes. The tubes containing the mixture were vortexed at maximum speed for one minute. Bead mix was prepared using 10 µl of RNA binding beads and 10 µl of Binding enhancer per sample and the tubes vortexed at maximum speed.

The tubes were placed on a magnetic stand to capture the RNA binding beads for 10 minutes. When the capture was complete the RNA binding beads formed pellets against the magnets in the magnetic stand.

150µl of Wash one solution (isopropanol added) was added to each sample and the tubes were vortexed for five minutes. The RNA binding beads were placed again on the magnetic stand for approximately five minutes until the mixture became clear which indicated that the capture was complete. The supernatant was carefully discarded without disturbing the beads. This step was repeated as the samples have to be washed twice with the Wash one solution.

The same volume of Wash two solution (100% ethanol added) (150µl) was added to each sample and vortexed for five minutes. The RNA binding beads were captured as in the previous washes. The supernatant again was carefully aspirated and the beads were removed from the stand before washing again with the same procedure as the first. All the supernatant was discarded to prevent inhibition of downstream application such as Reverse transcription PCR and to obtain pure RNA.

The beads were then shaken for 5 minutes to dry them and to allow any remaining alcohol to evaporate.

Elution buffer (40µl) was added to each sample and vortexed for 5 minutes at maximum speed. The RNA beads were again captured after standing on the magnet however the supernatant which contained the RNA was transferred into another nuclease-free tube and stored at -80º C.

3.5 Diagnostic Real-Time PCR for Pathotype Detection

A PCR master mix for both the F and the M gene for diagnostic PCR was prepared on ice. A volume of 7.25µl of the master mix was distributed into each well of the PCR plate. 5.25µl of the

sample was also added to each well and mixed thoroughly to give a final volume of 12.5µl per well. The plate containing the samples was centrifuged at 4,400rpm at 4ºC for five minutes.

After centrifugation, real-time PCR was conducted with QuantStudio™ Design and Analysis Applied Biosystems, Europe under the following cycling conditions;

45° C for 10 min	Reverse transcription
95° C for 5 minutes	Initial denaturation
40 cycles of	
95° C for 5 sec	Denaturation
65° C for 5-sec Annealing	

3.6 Genome Sequencing

Sequencing of extracted RNA was performed at the Reference Laboratory for Avian Influenza, Animal and Plant Health Agency, Weybridge, United Kingdom.

3.7 Sequence Mapping and Annotation

Sequenced data were mapped and annotated with Geneious Prime Software version 2020.2(https.www.geneious.com).

3.8 Sequence Alignment

Multiple sequence alignments were prepared using CLUSTALW. The preliminary phylogenetic trees were reconstructed using the Unweighted-pair group method with arithmetic mean algorithm (UPGMA), Minimum Evolution (ME) and Maximum Parsimony in MEGA X software (Tamura *et al.*, 2007). The robustness of the groupings was assessed by bootstrap resampling of 1,000 replicates. A distance matrix was generated using the maximum composite likelihood method. The

final Phylogenetic tree was constructed using the Neighbourhood joining method due to its popularity and robustness.

CHAPTER FOUR

4.0 RESULTS

4.1 Virus Isolation

Out of the 30 samples collected, 22 of the samples tested positive after isolation of the virus from tissue samples of dead birds and swab samples from disease birds using 9-11days embryonated chicken eggs. Table 2 shows the samples that tested positive for NDV and their locations.

Table 2: NDV positive samples and their locations in Ghana

ID Generated	Sampled tissue	Location	Agro-Ecological Zone	Positive/Negative
1	Brain	Accra	Coastal Savannah	+
2	Brain	Pokuase	Coastal Savannah	+
3	Brain	La	Coastal Savannah	+
4	Brain	Bojuase	Forest	+
5	Brain	Northern	Interior Savannah	+
6	Trachea	Ablekuma	Coastal Savannah	+
7	Trachea	Kasoa	Coastal Savannah	+
8	Brain	Pokuase	Coastal Savannah	+
9	Oropharygeal swabs	Akim Oda	Forest	+
10	Brain	Kumasi	Forest	+
11	Brain	Kasoa	Coastal Savannah	+
12	Brain and Trachea	Northern	Interior Savannah	+
13	Brain and Trachea	Upper West	Interior Savannah	+
14	Brain and Trachea	Akim Oda	Forest	+
15	Brain and Trachea	Upper West	Interior Savannah	+
16	Brain and Trachea	Denu	Coastal Savannah	+
17	Oropharygeal swabs	Dodowa	Forest	+
18	Brain/Trachea	Pokuase	Coastal Savannah	+
19	Brain and Trachea	Upper West	Interior Savannah	+
20	Brain and Trachea	Kumasi Tafo	Forest	+
21	Brain and Trachea	Upper West	Interior Savannah	+
22	Brain	Northern	Interior Savannah	+
23	Brain and Trachea	Volta Region	Coastal Savannah	-
24	Brain and Proventriculus	Upper West	Interior Savannah	-
25	Brain and Trachea	Kumasi Tafo	Forest	-

26	Trachea and Proventriculus	Upper West	Interior Savannah	-
27	Trachea and Proventriculus	Upper West	Interior Savannah	-
28	Trachea and Proventriculus	Upper West	Interior Savannah	-
29	Brain and Trachea	Kumasi Tafo	Forest	-
30	Brain and Trachea	Volta Region	Coastal Savannah	-

4.2 Geographic Distribution of the Sequences

The whole genome of four isolates was successfully sequenced (samples 4, 5, 17 and 21) (Table 1). The isolates were obtained from chickens showing clinical signs of ND in Bojuase, Dodowa, Tamale and Wa. Both the Tamale and Wa isolates were obtained from non-commercial birds isolated between 2018 and 2019. The isolates from Bojuase and Dodowa were obtained from commercially bred poultry. Three of the sequences originated from tissue samples while one was derived from oropharyngeal/cloacal swabs. Table 3 below are the sequence lengths of the samples sequenced.

Table 3: Samples and their full genome sequence lengths

Sample	Sequence Length(bp)
4	15,192
5	15,198
17	15,196
21	15,198

4.3 Genome Mapping of Sequenced Isolates

Genome maps were obtained after mapping and annotation to closely related sequences in GenBank. Sample 4 and 17 were mapped to an isolate from Nigeria with accession number KY19990. Sample 5 and 21 were mapped to an isolate from Mali with accession number JF966387.

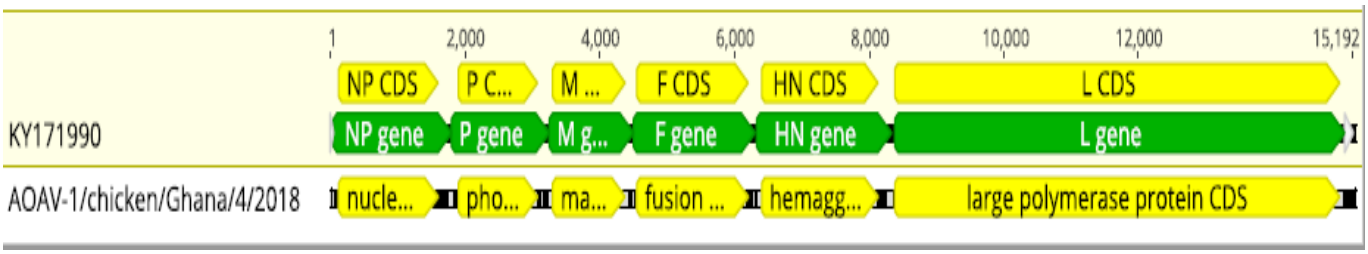

Figure 4: *Gene map of Sample 4 mapped to sequence with accession number KY171990*

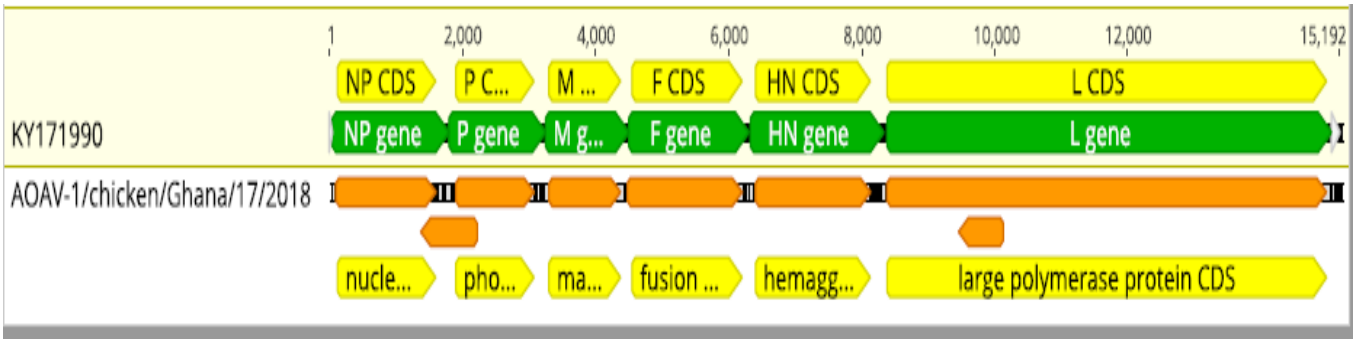

Figure 5: *Gene map of Sample 17 mapped to sequence with accession number KY171990*

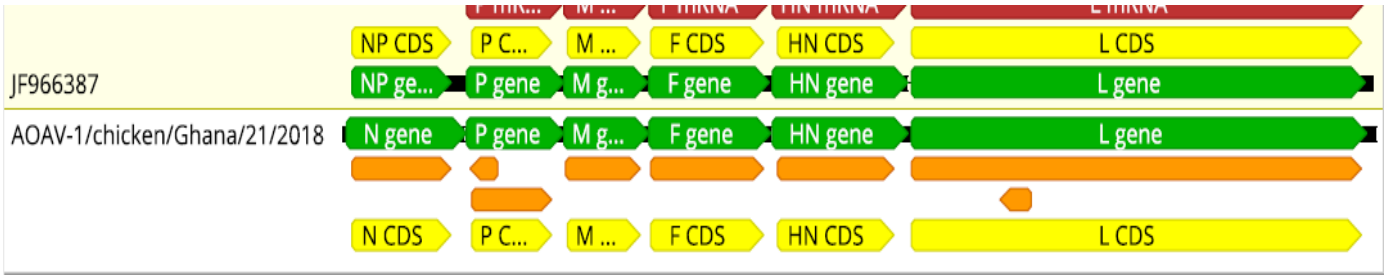

Figure 6: *Gene map of Sample 21 mapped to sequence with accession number JF966387*

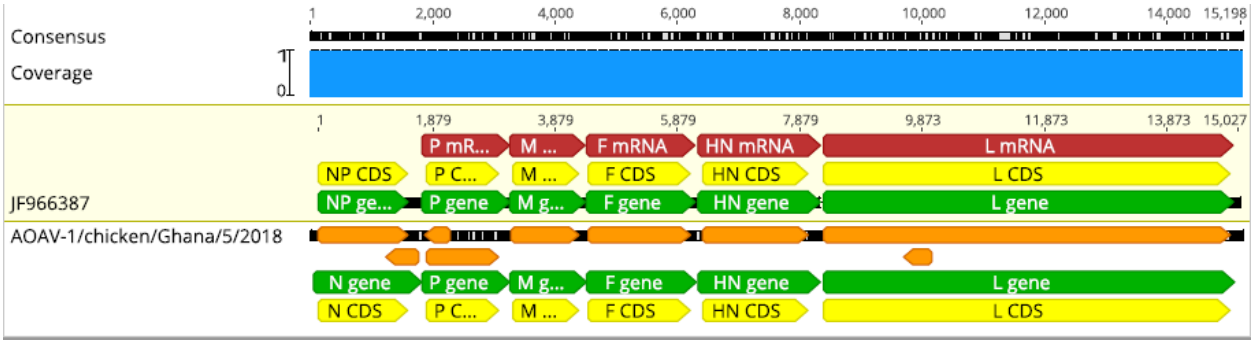

Figure 7: *Gene map of Sample 5 mapped to sequence with accession number JF966387*

4.4 Phylogenetic classification

Phylogenetic analysis of the complete genome, F and HN genes were performed using the neighbourhood joining method.

The four isolates were clustered differently with 4 and 17 being grouped and 5 and 21 also being clustered together. Results based on a BLAST search showed samples 4 and 17 is closely related to isolates in Genotype XIV (96% identity) while samples 5 and 21 belong to Genotype XVIII (97% identity).

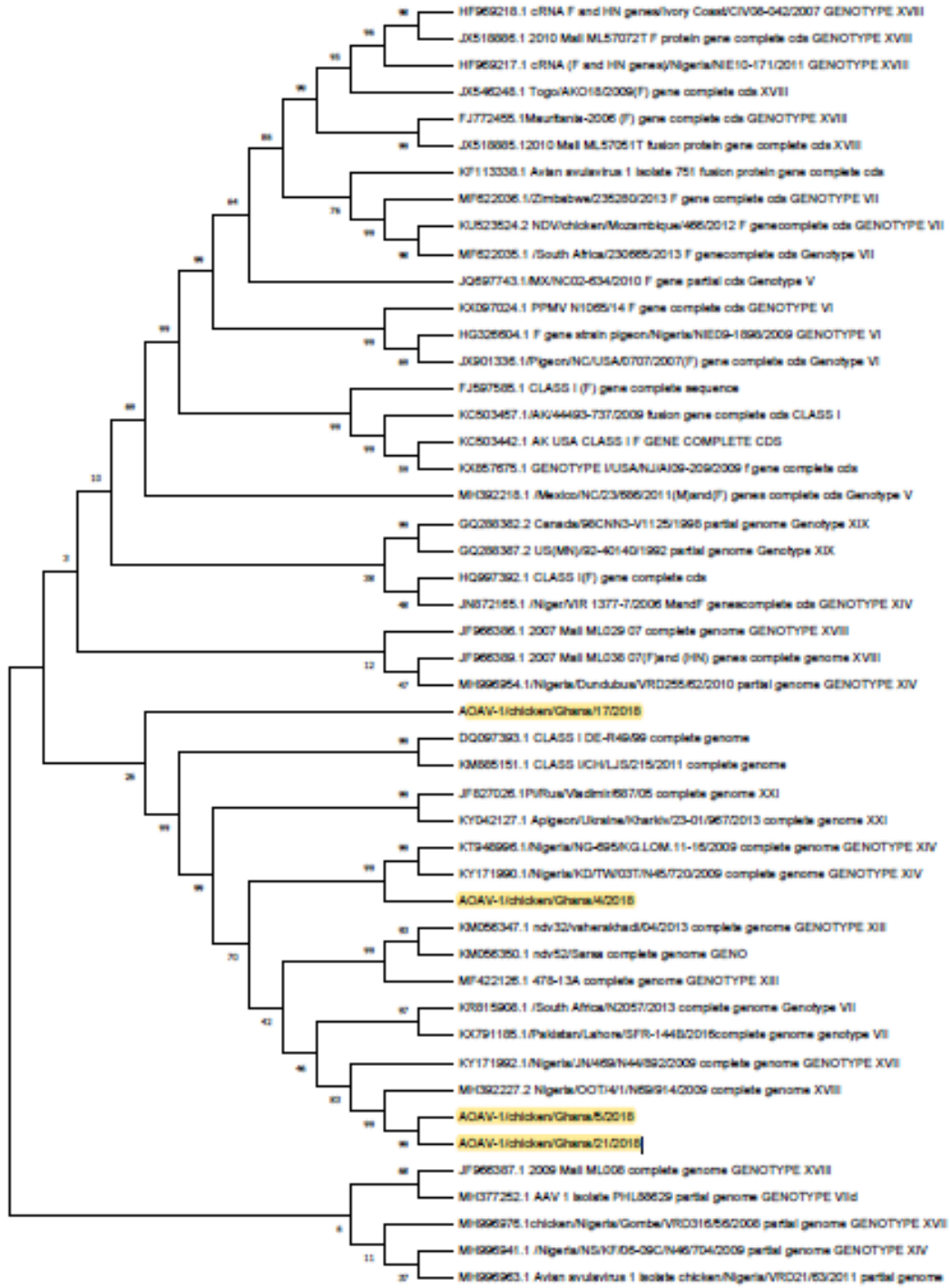

Figure 8. Phylogenetic analysis of Newcastle class II isolates using the whole genome with the neighbourhood-joining method 1000 bootstrap replicates. Of the 50 sequences used, 4 were collected from diseased chickens from Ghana (coloured) and 4 represent Class I genotypes and the

remaining represent important Class II genotypes as described by Dimitrov *et al.*, 2019. The Roman numerals on the right represent the genotypes the isolates belong to.

4.4.1 Genotype XVIII isolates

Isolates belonging to Genotype XVIII originated from Wa in the Upper West Region and Tamale in the Northern region of Ghana. These locations are within the Interior Savanah agro-ecological zone. The nucleotide identity between the two isolates is 98.62%. Our Genotype XVII sequences are most similar to isolates from Togo (accession no. JX390609) and Nigeria (accession no. MH392227) to which they shared between 97.8 and 97.5% nucleotide identity respectively.

4.4.2 Genotype XIV isolates

Isolates belonging to Genotype XIV originated from Bojuase and Dodowa in the Greater Accra region of Ghana. These locations are within the Coastal Savannah and Forest agro-ecological zones respectively. The nucleotide identity between the two isolates is 98.96%. Our Genotype XIV sequences are most similar to isolates from Nigeria (KY171993) and Mali (JF966386) to which they shared between 98.8 and 94.6% nucleotide identity respectively.

4.5 Analysis of the amino acid sequence at the F proteolytic cleavage site

Analysis of the amino acid residues of the 4 NDV strains was done. The amplified F protein gene coding sequence for each strain was 1,662 nucleotides, directing the synthesis of a protein predicted to be 553 amino acids in length. The five potential asparagine-linked glycosylation sites (positions 85, 191, 366, 447 and 471) and 10 cysteine residues (positions 76,199, 338, 347, 362, 370, 394, 399, 401 and 424) were all conserved in all the samples (Chambers *et al.*, 1986; McGinnes and Morrison, 1986).

Deduced amino acid pattern at the F gene cleavage site was analysed for pathotypes, showing that all four of the isolated viruses contained a pair of dibasic amino acids at the cleavage site, characteristic of virulent strains. Of the four isolates characterised as virulent, 2 exhibited the sequence motif ^{112}RRQKR^{116}-F (Samples 4 and 17) and 2 exhibited the motif ^{112}RRKKR^{116}- F (Sample 5 and 21). It was noted that the 2 isolated which were clustered together in the same genotypes exhibited the same motif sequence.

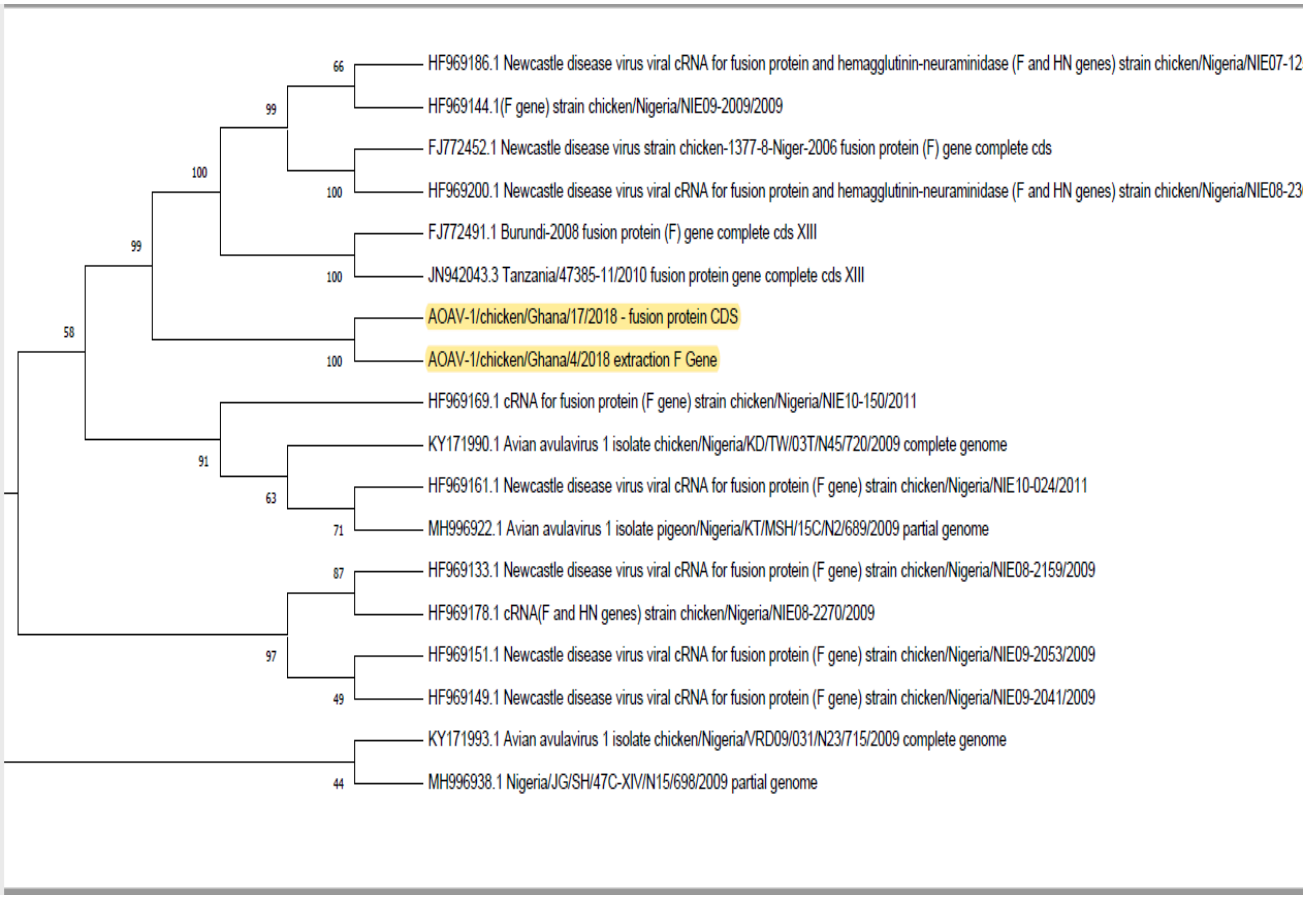

Figure 9: The above figure shows the phylogenetic analysis of Newcastle disease virus genotype XIV isolates of the fusion gene with the neighbourhood-joining method with 1000 bootstrap replicates. Of the 18 sequences used, 2 were collected from diseased chickens (coloured), 14 represent other genotype XIV isolates, and 2 are outliers (from genotype XIII).

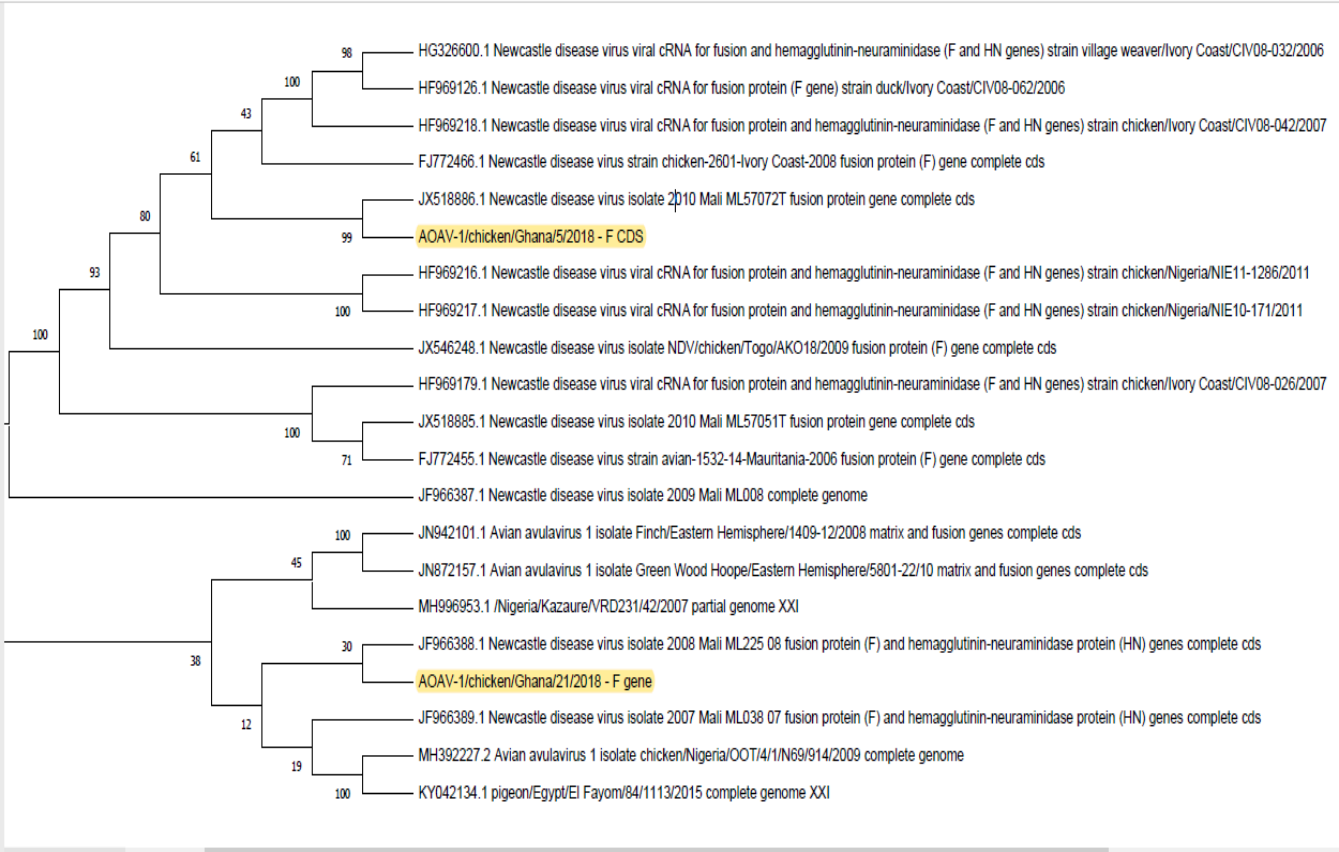

Figure 10: The above figure shows the phylogenetic analysis of Newcastle disease virus genotype XVIII isolates of the fusion gene with the neighbourhood-joining method with 1000 bootstrap replicates. Of the 21 sequences used, 2 were collected from diseased chickens (coloured), 17 represent other genotype XVIII isolates, and 2 are outliers (from genotype XXI).

4.6 Analysis of the HN Gene Sequence

The HN gene was extracted from the whole genome sequence of isolates using Geneious Prime Software (https//www.geneious.com). The amino acid lengths of all isolates were 571aa which is known to be the length for virulent NDV. The sialic binding site (NRKSCS) was also conserved on all isolates between positions 234-239. A motif of PDEQDYQIR between positions 345 and

353 is known as the immunodominant area of the HN gene. There was also no substitution of E to K at position 347.

4.7 Comparison of Isolates with Vaccine Strains Used in Ghana

A BLAST of the vaccine strain in circulation with the sequenced samples showed a percentage identity of 82.69%, 82.91%, 83.21% and 83.06% for samples 4,17,5 and 21 respectively. Table 4 shows the percentage identity of the samples with the vaccine strains

Table 4: Percentage identity of samples sequenced to vaccine strain in use in Ghana

Sample ID	Percentage Identity to I2 Vaccine
4	82.96%
5	83.21%
17	82.91%
21	83.06%

CHAPTER FIVE

5.0 DISCUSSION

ND continues to be an endemic disease in Africa and causes huge losses in commercial and rural poultry farming most of which go unreported (Awan *et al.*, 1994). This was demonstrated by several outbreaks affecting several flocks during the study period and the farmers' unwillingness to report to the appropriate authorities.

It is suspected that in regions of the world where it is enzootic, virulent NDV strains are continuously evolving and that Africa is a major breeding ground for such viruses. NDV is a complex virus genetically and clinically, with multiple genotypes isolated around the globe. A recent study in Madagascar indicates that NDV strains are unique and more divergent from strains isolated in other parts of the world (Maminiaina *et al.*, 2010). Thus, understanding the diversity of NDV in Africa and specifically, West Africa is important to promote early detection and thus control strategies. This would also include the development of more efficient vaccines that do not only reduce mortality and morbidity in birds but reduce or eliminate viral load in shedding (Arthur *et al.*, 2012).

The four sequenced genomes were classified into two genotypes XIV and XVIII. These strains have been isolated from outbreaks within the West African sub-region. The four sequenced isolates conformed to the genome structure of Paramyxovirus encoding six genes the order NP-P-M-F-HN-L (Lamb and Kolakofsky, 2002). The lengths of the genomes are 15,192, 15,198, 15,156 and 15,196 for samples 4,5,17 and 18 respectively.

Phylogenetic classification grouped 2 of the sequences into genotypes XVIII and XIV. These genotypes are composed of strains predominantly isolated in the West Africa sub-region (Dimitrov

et al., 2019). It would be noted that the XIV sequences came from isolates in the southern part of Ghana and were obtained from foreign breeds of birds in commercial production units.

Genotype XIV isolates are said to be the most isolated strains in Nigeria, where both subgenotypes XIVa and XIVb have been recovered from domestic birds. Subgenotype XIVa isolates appear to be more genetically complex, with average evolutionary distances between intra-genotypes between 2.6% (Bello *et al.,* 2018). In particular, all genotype XIV isolates are so far confined to the West African subregion only, where they wrecked chaos in the regional poultry industry (Samuel *et al.,* 2012).

XVIII sequences were obtained from the northern part of the country (Interior Savanna zone) and were isolated from rural birds while XIV sequences were isolated from the Forest agro-ecological zone. This would suggest that the circulating genotypes differ per agro-ecological zones.

A limiting factor to this study was the low number of sequences obtained from the sample collection to further substantiate the above statement.

Studies by da Silva et al. (2020), however, confirmed the presence of genotype XVIII in the southern part of Ghana, obtained from an infected bird from Pokuase which falls within the Coastal Savanna region of Ghana. The isolate was also retrieved from a commercial poultry farm. This would infer that the distribution of ND strains is not restricted by the agro-ecological zone

The genetic analysis of the full sequences of the fusion protein gene showed that all the four Ghanaian NDV isolates have shown at least three basic amino acids in the C-terminus of the 112-116 residue F2 protein and the 117-residue phenylalanine; thus, all are considered to be virulent strains by OIE definition (OIE,2019).

Two out of the four isolates have an F-protein cleavage site of ^{112} RRQKR↓F^{117} which contains four basic amino acids. The isolates that possess this motif were classified under Genotype XIV. The remaining two sequences which were classified into Genotype XVIII have an F-protein cleavage site of ^{112}RRRKR↓F^{117} containing five basic amino acids. Motifs for virulent cleavage containing 5 basic amino acids have been identified in isolates from Mali and Nigeria (Almeida *et al.*,2009; Solomon *et al.*, 2012).

The virulent motif "RRQKRF" is said to be the most diverse among all other virulent cleavage sites. Also, recent research on the amino acid composition of the cleavage site of NDV F has shown that strains that possess Q at the third position in the cleavage site have an increased cell to cell spreading ability (Wang *et al.*, 2017).

The isolation of two different virulent motifs aligns with the study done by Samuel *et al.*, which demonstrated the co-circulation of viruses with different F-protein cleavage site sequences at the same time and place in West Africa (Samuel *et al.*, 2012).

HA and HI tests are known to be the conventional diagnostics for NDV identification (Triosanti *et al.*, 2018). In this study, an HA test was done after harvesting allantoic fluid to confirm the presence of the virus. This hemagglutination ability of the virus is a result of the properties of the HN protein which mediates viral entry into the host cell together with the F protein.

HN protein acts as an agent to identify a sialic acid receptor on the surface of targeted host cells during the attachment phase of NDV to a host cell. In addition, the protein in combination with the F protein activates a fusion mechanism of viruses in the target cell membranes. Theoretically, F protein cooperates with HN protein during the fusion process to execute a membrane fusion so that the virus can reach the surface of a host cell (Porotto *et al.*,2012; Heiden *et al.*, 2014).

It has been hypothesized that the HN gene can clearly identify the NDV genotypes and can truly predict the pathogenicity of the isolates since the length of the HN protein varies and the site of cleavage is not the only pathogenicity criterion (Sakaguchi *et al.*, 1989; Munir *et al.*, 2011). Nine different versions, namely 570 amino acids (570aa), 571aa, 572aa, 577aa, 578aa, 586aa, 582aa, and 616aa, have been identified and characterised in detail (Murulitharan *et al.*,2013).

The HN proteins had a length of 571 amino acids for each of the analysed viruses, which is the length most commonly observed for the virulent NDV isolates, and had a cysteine in the position that is necessary for disulphide-linked HN homodimers formation. A previous study showed that the amino acid cysteine in this role influences both the activity of attachment and the activity of fusion promotion and enhances the virulence and pathogenicity of NDV viruses (Romer-Oberdorfer *et al.*, 2006).

A known substitution on the HN at 347 from E to K is said to enable the virus to escape monoclonal antibody detection. In summary, mutations in different regions of the HN protein can result in changes in one or more of the HAd, NA, and fusion promotion activities as such the virulence of NDV is linked to the HN protein (Yan *et al.*, 2018).

On HN amino acid sequence analysis of the sequenced samples, it was observed that the asparagine-linked glycosylation sites were all conserved in all four samples.

Vaccination has been the major form of control for the virus in poultry. Further control of ND has been facilitated by strict biosecurity to complement vaccination, which prevents the virus from getting into contact with poultry (Miller and Koch, 2013). ND vaccines are widely used to decrease clinical illnesses from endemic infections with low virulence strains (Miller et al., 2007).

It has been demonstrated that genetic diversity in Avian Avulavirus-1 strains influences the efficacy of vaccination control, not necessarily in terms of clinical protection, but mainly in terms of virus shedding and subsequent spread of the infection (Miller *et al.,* 2007). For the production of new vaccines and vaccination strategies, a deeper understanding of the epidemiological ties between circulating NDVs, their genetic diversity and characteristics, and global distribution is essential (Dimitrov et al.,2017).

Miller *et al.* (2007) there was significantly higher virus shedding for strains that had 90% or lower percentage identity with the vaccine strains. In addition, the distinct mutations found in the African strains examined in this study were located in the neutralizing epitopes of the two main antigens (F and HN glycoprotein) targeted at the defensive immune response (Seal *et al.,* 2000), thus the scientific argument for further in-depth investigations into the antigenic properties of these isolates and thorough analyses of the effectiveness of existing vaccines and vaccination procedures in Africa is provided.

The amino acid sequence identities between I2 and the other isolated strains from Ghana ranged from 87% to 92% for the F gene, thus the sequences were more divergent. This supports the idea that the antigenic differences of NDV strains relative to vaccine strains can reduce vaccine efficacy (Dortmans, *et al.,* 2012).

The present investigation offers valuable information on the epidemiology, diagnosis and control of NDV in Africa and highlights the importance of promoting surveillance of transboundary animal diseases in developing countries. It provides proof of the need to promote the exchange of data and sequences and to continually track the efficacy of validated diagnostic tests. As shown by current and prior research (Xing *et al.,* 2008), failure to perform conventional tests, such as virus isolation, can pose serious risks for the correct diagnosis of infectious diseases.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has confirmed the circulation of more than one genotype of Newcastle Disease Virus in Ghana all within the Class II caste. The isolated samples were found to belong to GENOTYPES XIV and XVIII. These genotypes are known to be retrieved from isolates within the West African sub-region.

Comparison of the F gene for all the isolated sequences showed that the motif at the cleavage site differed across agro-ecological zones. Isolates from the Interior Savannah carried the motif between positions 112 and 117 while those from the Forest Zone had the motif. This further demonstrates the diversity of the NDV strains within the country.

It was observed that there exists a high genetic distance between the isolated strains and the vaccine strain being currently used in the country. Studies has shown that vaccines are effective against the disease by preventing the occurrence of clinical signs in the flock. However, they might not be able to reduce the viral load in the fluids of the infected animal and this does not entirely prevent transmission of the disease.

6.2 Recommendations

It is recommended that further studies be carried out on whether the strain that causes an outbreak in both rural and commercial farms is affected by the change in seasons.

Also, more characterisation projects are carried out to determine whether there are other genotypes present in the country. This will provide a basis for improving the current vaccine that is being used as prophylactic treatment.

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