Original Article

Sahel Journal of Veterinary Sciences

Sahel J. Vet. Sci. Vol. 22, No. 2, Pp 20-26 (2025)
https://doi.org/10.54058/3jp1f639

Article History
Received: 02-05-2025
Revised: 26-05-2025
Accepted: 26-06-2025
Published: 16-07-2025

Molecular Characterization of Newcastle Disease Virus Isolates from Live Bird Markets and Commercial Farms in Zaria, Nigeria, based on Partial Fusion Gene

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ABSTRACT

This study focused on characterizing Newcastle Disease Virus (NDV) isolates previously obtained from live bird markets and commercial poultry farms in Zaria and environs. Ten NDV isolates, six from local chickens in live bird markets and four from exotic chickens, were isolated between November 2014 and January 2015. The isolates were collected for molecular study. They were amplified using RT-PCR, followed by sequencing of partial F gene. Eight of the ten isolates were successfully sequenced. The results indicated that all the NDV sequences were virulent based on their cleavage site motifs within the F gene. Phylogenetic analysis revealed significant divergence (>20%) between these strains and commonly used vaccine strains like La Sota, V4, and Komarov. Notably, all analyzed NDVs grouped within sub-genotype XIVb during phylogenetic clustering, displaying shared amino acid substitutions across different positions relative to other members of this sub-genotype. These findings offer insights into potential reasons behind NDV vaccine failures by highlighting genetic differences among circulating strains versus those used in vaccines- a rationale warranting further investigation through challenge studies to improve vaccine efficacy against emerging NDV variants.

Key words: Live Bird Markets; Newcastle Disease Virus; Sequence analysis; Sub-genotype XIVb

INTRODUCTION

Newcastle Disease Virus (NDV), classified as *Avian Orthoavulavirus type-1* (AOaV-1) and formerly known as *Avian Paramyxovirus type-1* (APMV-1), is a significant pathogen affecting avian populations worldwide due to its substantial genetic and antigenic variability across strains (Xu *et al*., 2017; Lu *et.al.,* 2022). This variability often leads to vaccine failures, as vaccines may not effectively protect against diverse circulating strains, particularly those with significant genetic divergence from commonly used vaccine strains like La Sota and Komarov (Hu *et al*., 2022). Recent study has highlighted the continuous evolution of NDV strains, such as those in genotype VII, which exhibit increasing pathogenicity and genetic divergence from traditional vaccine types (Ibrahim *et al*., 2024).

The economic impact of Newcastle disease on poultry industries globally is substantial. Outbreaks can lead to severe losses in both commercial and backyard flocks due to high mortality rates among infected birds. Effective vaccination strategies are crucial for controlling the spread of NDV; however, these efforts are often hindered by the virus's ability to evolve into new genotypes that may evade immune responses elicited by existing vaccines (Bello *et al.,* 2018a).

Live bird markets play a critical role in the epidemiology of NDV due to their potential for facilitating viral transmission among different bird species. The close proximity of various avian species within these markets creates an environment conducive to viral exchange and mutation (Ekiri *et al*., 2025). Consequently, understanding the genetic characteristics of NDV isolates from such settings is essential for developing targeted vaccination programs that can address emerging strain diversity effectively (Doan *et al*., 2022). In Nigeria, poultry farming is an important sector contributing significantly to food security and economic stability at both local and national levels. However, outbreaks of Newcastle disease pose a persistent threat to this industry's sustainability (Sadiq and Mohammed, 2017).

Therefore, characterizing isolates from live bird markets and commercial farms in Zaria will provide valuable insights into circulating strain dynamics within this region. This study aims to molecularly characterize NDV isolates collected from live bird markets and commercial poultry farms in Zaria and its environs. By examining the genetic diversity among these isolates, particularly focusing on their fusion gene sequences and comparing them with established vaccine strains like La Sota or

Komarov, this research seeks to elucidate potential reasons behind observed vaccine failures.

MATERIALS AND METHOD

RNA Extraction, Purity and Concentration

Ten Newcastle Disease Virus (NDV) isolates, collected between November 2014 and January 2015 from local chickens in live bird markets and exotic chickens from commercial poultry farms in Zaria and its environs, Kaduna State, Nigeria, were subjected to molecular analysis. The viral RNAs were extracted using the QIAgen RNeasy Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol at the Regional Laboratory for Avian Influenza and other Transboundary Animal Diseases unit, Virology Division, National Veterinary Research Institute Vom, Plateau State, Nigeria.

RNA concentration (in ng/µl) and purity were analyzed using a spectrophotometer (Eppendorf, Hamburg, Germany). The method involved measuring the absorbance of diluted RNA samples at wavelengths of 260/230 nm (target ratio: 2.0–2.2) and 260/280 nm (target ratio: 1.8–2.1). Nucleic acid quantification was performed by applying the Beer-Lambert law, which establishes a linear relationship between absorbance and concentration.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Sequencing

A one-step RT-PCR was performed for the amplification of an NDV fragment spanning positions M610-F581 including the crucial F0 cleavage site within both Matrix and Fusion genes respectively using specific primers:

- Forward Primer:

M610 5′-CTGTACAATCTTGCGCTCAATGTC-3′

Reverse Primer:

F581 5′-CTGCCACTGCTAAGTTGTGATAATCC-3′

The RT-PCR reaction mixtures consisted of nuclease-free water (11.5 µL), 5x PCR buffer (5 µL), dNTPs (0.5 µL), Avian Myeloblastosis Reverse Transcriptase enzyme (0.5 µL), RNase inhibitor (0.5 µL), RNA template (5 µL), and primers M610 and F581 at 1 µL each. The cycling conditions began with a reverse transcription at 50°C for 30 minutes followed by initial denaturation at 95°C for 15 minutes. Subsequent amplification involved 35 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and elongation at 72°C for 1 minute, with a final extension of 7 minutes at 72°C. The amplified products were resolved on a 1.5% agarose gel stained with ethidium bromide under an electric field of 100 V applied for 30 minutes. Bands were then visualized using a gel documentation system. Sanger's sequencing was conducted at Inqaba Biotechnology Pretoria, South Africa, following standard protocol.

Data Analysis

Nucleotide sequences were edited using BioEdit software (v7.2.5). Subsequently, these sequences were aligned with those of reference vaccine strains and representatives from various genotypes/sub-genotypes (JX546245, JX039390, FJ772484, FJ772466, FJ772455, GU182331, GQ245799, JX524403, AY845400 and KT445901) retrieved from GenBank. The aligned sequences were translated into amino acids, and a phylogenetic tree was constructed using the Neighbor-Joining method. Evolutionary distances were computed using the Kimura 2-Parameter model, with results expressed as base substitutions per site.

RESULTS

Amplification of Newcastle Disease Virus

A 1181 base pair (bp) DNA fragment spanning from position 610 of the matrix gene to position 581 of the fusion gene, including the F0 cleavage site, was amplified for each of the ten isolates. The results showed that all the ten isolates were positive for NDV (Figure 1).

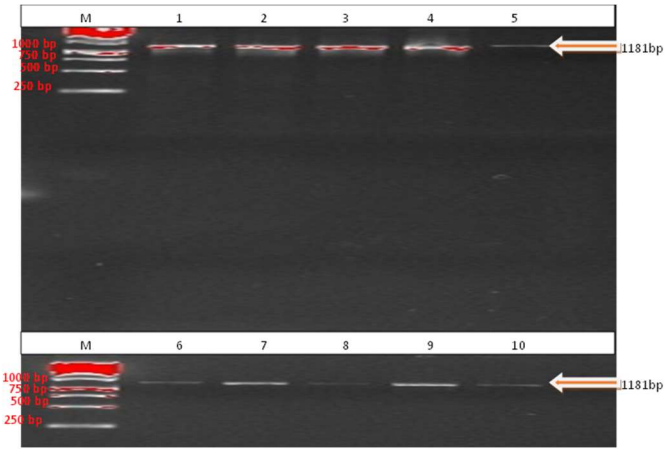

Figure 1: RT-PCR product bands (Lanes 1-10) of partially amplified Newcastle disease virus position 610 of M-gene region to position 581of F-gene region from isolates obtained from chickens in commercial poultry farms and live bird markets. The expected amplicon size is 1181 bp. M represents 1kb plus ladder.

Sequencing and Analysis of Amino Acids at the F0 Cleavage Site

Eight out of the ten NDV isolates were successfully sequenced, and the sequences have since been deposited in GenBank with the following assigned accession numbers: KX168401-KX168408. The analyzed deduced amino acid motif at the F-gene cleavage site revealed that all the NDVs from this study contained multiple basic amino acids at the cleavage site, a characteristic typical of virulent strains. All the strains exhibited a sequence motif ^{112}RRQKR^{116}- F^{117} (Figure 2).

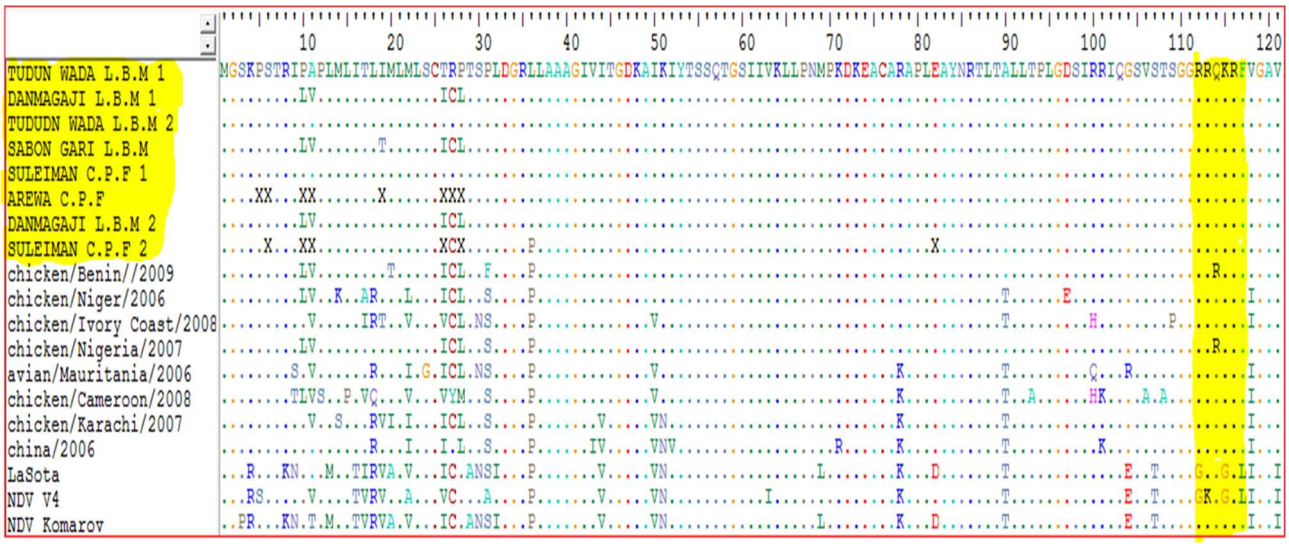

Figure 2: The pathotypes of Newcastle Disease Virus (NDV) isolates based on the amino acid motif of the partial F-gene. Isolates highlighted in yellow at the top left corner are from this study, whereas unhighlighted isolates at the left were reference strains retrieved from the Genbank. The yellow section on the right indicates amino acid motif positions 112-117, which define the pathotype of NDV. Additionally, several amino acid substitutions are visible.

Substitutions of Some Amino Acid Residues

Based on analysis of deduced amino acid sequences for partial F-gene fragments from eight strains in this study, alongside representatives from different genotypes, several amino acid substitutions were identified:

- R→Q^{114} substitution: Shared among all NDV strains from this study and other sequences retrieved from GenBank when compared with the two representatives (accession numbers: **JX546245, from Benin Republic and JQ039390, from Nigeria**) of sub-genotype XIVb.
- R→K^{4} substitution: Present in all NDV strains from this study and referenced sequences compared to vaccine strains La Sota, V4, and Komarov.
- S→P^{5} substitution: Shared between NDV strains from this study, La Sota and Komarov when compared with V4 vaccine strain
- T→I^{16} substitution: Common in all NDV strains from this study and representatives of sub-genotype XIVb compared to La Sota, V4, and Komarov.
- V→I^{63} substitution: Unique to V4 vaccine strain in comparison to NDV strains from this study
- D→E^{82} substitution: Unique among study strains when compared with La Sota and V4 vaccine stains.
- T→S^{107} substitution: Shared by all study strains with sub-genotype XIVb representatives against La Sota, V4, and Komarov.

Three specific isolates [Danmagaji1 (02NGZCK015), Sabon Gari (04NGZCK015) and Danmagaji2 (09NGZCK015)] shared unique substitutions: P→L^{10} and A→V^{11} when compared with the two representative sequences of sub-genotype XIVb from Benin Republic (accession no: **JX546245) and Nigeria (accession no: JQ039390);** while the five other live bird market isolates had distinct L→P^{10} , V→A^{11} , I→T^{26} , C→R^{27} , and L → P^{28} substitutions compared to sub-genotype XIVb strains.

Phylogenetic Analysis

The topology of the tree indicates formation of a major cluster between sequences from this study and representatives of sub-genotype XIVb from Katsina State, Nigeria (accession no: **JQ039390)**, and Benin Republic (accession no: **JX546245**) retrieved from GenBank. Furthermore, three distinct sub-clusters can be identified: representatives of sub-genotype XIVb; strain in this study from a commercial poultry farm; and a major sub-cluster that includes all NDV strains obtained from live bird markets (Figure 3).

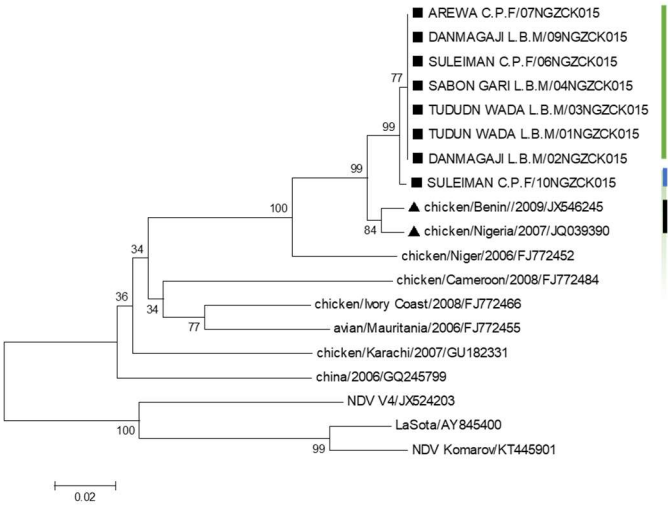

Figure 3: Neighbour joining tree based on a variable region (nucleotide 47 to 419) of the partial F-gene of Newcastle disease virus characterized in this study with representative strains of different genotypes/sub-genotypes. Strains from this study were depicted with ■.

Genetic Distance Similarities and Divergence

Sequence homology among the isolates studied ranged from 97.6% to 100%, with a maximum divergence of 2.4% for one commercial farm isolate (10NGZCK015). Mean divergence between three specific isolates from this study (02NGZCK015, 04NGZCK015, 09NGZCK015) compared to sequences from sub-genotype XIVb was 2.5%. Other five isolates had a mean divergence of 4.2% when compared with the two reference sub-genotypes. Compared against vaccine strains, mean similarity was approximately between 77% (La Sota and Komarov) and 79% (V4) indicating significant divergence averaging around 22–23% (Table 1).

	TWADA	DMGJI	TWADA	SGARI	SLMAN	AREWA	DMGJI	SLMAN	Benin	Niger	ICost	Nigra	Mtani	Croon	Kraci	China	LSota	NDVV4	Kmrov
TWAD	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
Mtan	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
Chin	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

Table 1: Percentage nucleotides sequence identity matrix between sequences from this study (colored yellow) and other reference strains. The internal similarity among sequences within this study is highlighted in green. Representatives of sub-genotype XIVb and vaccine strains are colored turquoise, while their similarity results when compared with sequences from this study are highlighted in red. **NOTE**: TWADA= Tudun wada; DMAGJI= Danmagaji; SGARI= Sabongari; SLMAN= Suleiman; Icost= Ivory coast; Nigra=Nigeria Mtani= Mauritania; Croon= Cameroon; Kraci= Karachi; LSota= LaSota; Kmrov= Kamarov

DISCUSSION

The identification of the protein sequence motif ^{112}RRQKR^{116}-F^{117} at the F-gene cleavage site in this study is a characteristic of virulent Newcastle disease virus (NDV) strains. This motif is crucial for understanding the pathogenicity of NDV, as it plays a significant role in the virus's ability to infect host cells. Research has shown that NDV isolates from various outbreaks consistently display this virulent motif. For instance, a study analyzing isolates from Ethiopia confirmed the presence of the ^{112}RRQKR^{116}-F^{117} sequence in virulent strains circulating in local poultry markets, indicating its role in ongoing outbreaks and economic impacts on poultry farming (Mulisa *et al*., 2014). Wang *et al.* (2017) reported that the virulent Fcs motifs, such as "RRQKRF," demonstrate enhanced fusogenic activity, which correlates with higher pathogenicity in host species. Further investigations into the F protein's cleavage site have classified various NDV isolates based on their amino acid sequences.

The presence of sub-genotype XIVb across all strains recovered in Zaria, along with high sequence similarities between commercial farms and live bird markets (ranging from 97.9% to 98.5%), suggests horizontal transmission facilitated by human interaction and movement between these settings (Hamisu *et al*., 2017). Bello *et al.* (2018b) confirm that NDV sub-genotype XIVb is prevalent in Nigeria. In addition, this sub-genotype has been identified in various domestic bird species, indicating its widespread circulation within poultry populations (Eid *et al*., 2022). Recently, Umar *et al*. (2024) reported that all virulent NDV strains identified in chickens and pigeons from Bauchi, Nigeria are of sub-genotype XIVb (XIV.2). The identification of high sequence similarity among strains from different sources suggests a common lineage and potential for transmission across different environments, such as farms and markets (Moustapha *et al*., 2023). This spread can occur through direct contact, fomites, or airborne transmission, exacerbated by poor biosecurity practices (Hamisu *et al*., 2017). Environmental factors like the harmattan wind may also play a role in virus dissemination (Aliyu *et al.,* 2015).

All NDV strains from this study clustered with representatives of sub-genotype XIVb from Nigeria and Benin Republic isolated earlier. The extensive trans-border trade activities at border towns like Kamba could facilitate the introduction of NDV from neighboring countries into Nigeria's poultry market (Amoia *et al.,* 2024). As reported by Abraham-Oyiguh *et al*., (2014), local poultry vendors likely contribute to spreading NDV between neighboring states in Nigeria.

Despite vaccination efforts using La Sota strain vaccines from various countries, vaccine-related strains were not detected among commercial farms or live bird markets in this study. Consequently, phylogenetic analyses revealed significant divergence (>20%) between field isolates and vaccine strains, but this has clinical consequences. Studies have shown that low similarity (<90%) between field and vaccine strains correlates with increased viral shedding (Miller *et al*., 2013).

African NDV strains often share specific residues on the F gene (Snoeck *et al*., 2013); however, variations observed in this study may influence immune evasion and vaccine effectiveness without necessarily altering the pathotype characteristics of the virus (Liu *et al*., 2015). Several studies have reported that amino acid substitutions in NDV surface proteins (F and HN) can affect antigenic sites, thermostability, and immune recognition, which are critical for viral evolution, pathogenicity, and vaccine efficacy (Selim *et al.,* 2018; Mase *et al.,* 2021; Babaeimarzangou *et al.,* 2023). The unique substitutions observed in these isolates may represent ongoing adaptation to host immune pressure or environmental conditions in live bird markets and commercial farms in Zaria. Specifically, S→P^{5} substitution may indicate a conserved adaptation among certain NDV lineages. Proline substitutions (e.g., S315P in HN) have been associated with increased thermostability and potentially altered virulence or immune response (Babaeimarzangou *et al.,* 2023). Generally, amino acids substitutions, especially those involving proline and cysteine, may influence the structural and antigenic properties of NDV proteins, potentially affecting viral fitness, immune evasion, or vaccine response. For instance, loss of cysteine residues in the F protein has been observed in some NDV isolates and may impact antigenic structure and function (Selim *et al.,* 2018).

Genotype XIV includes two sub-genotypes: XIVa and XIVb, both highly virulent and predominantly found in domestic birds across Africa (Snoeck *et al*., 2013). Studies have highlighted the genetic diversity of NDV across Africa, including genotypes XVII and XVIII found primarily in West and Central Africa (Megahed *et al*., 2020). The persistence of genotype XIVb suggests its adaptation to local conditions or inadequate control measures against its spread within Nigeria's poultry sector (Shittu *et al*., 2016).

Live bird markets remain critical sites for NDV transmission due to their role as hubs for diverse avian species interactions under minimal biosecurity conditions (Sun *et al*., 2022). This underscores the need for enhanced surveillance strategies focusing on these areas to prevent outbreaks that can spill over into commercial farms.

Conclusion

The findings of this study showed that all the NDV isolates that were successfully sequenced are virulent strains due to the presence of multiple basic amino acids at the F0 cleavage site, specifically the motif ^{11}2RRQKR116-F^{117}. Furthermore, the study identified several amino acid substitutions that distinguish the isolates from vaccine strains like La Sota and V4. While significant divergence (22–23%) was observed between the isolates and vaccine strains, the sequence homology among the isolates themselves was notably high, ranging from 97.6% to 100%. The study further indicated a close relationship between isolate from this study and

representatives of sub-genotype XIVb. There is the need for regular surveillance programs in poultry farms and live bird markets to monitor NDV strains. This should include RT-PCR testing for early detection and characterization of emerging virulent strains and to identify specific mutations associated with virulence. This will aid in understanding the evolutionary dynamics of NDV and inform vaccine development strategies. Given the observed genetic divergence from existing vaccine strains, it is crucial to develop a genotype-matched vaccine based on circulating virulent strains in order to enhance the effectiveness of the vaccines against ND outbreaks.

Acknowledgement

The authors wish to thanks the technical support received from the staff of Regional Laboratory for Avian Influenza and other Transboundary Animal Diseases unit, Virology Division, National Veterinary Research Institute Vom, Plateau State, Nigeria.

Conflict of Interest

The authors have no conflict of interest to declare.

Author's Contribution

TMH, HMK, KAM and LS designed the work; TMH collected the samples and carried out the laboratory work; YSM reviewed the manuscript.

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