Analysis of Amino Acid Changes in the Fusion Protein of Virulent Newcastle Disease Virus from Vaccinated Poultry in Nigerian Isolates

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1. Introduction

Newcastle disease (ND) is a globally reported viral disease affecting over 200 species of birds [1] primarily controlled by vaccination [2]. It is an Office International des Épizooties (OIE) notifiable disease [1]; however, only few countries report its incidence to OIE, especially in developing countries where the disease is enzootic [3] among vaccinated and unvaccinated poultry. ND has received extensive attention because of its ability to spread, high mortality, vaccine failure, and other economic losses associated. The aetiology of ND is virulent strain of Avian paramyxovirus type-1 (APMV-1) also known as Newcastle disease virus (NDV) of the genus Orthoavulavirus belonging to the family Paramyxoviridae and order Mononegavirales [1]. It is a negative sense, non-segmented, and single-stranded enveloped RNA virus [4].
NDV fusion (F) glycoprotein mediates fusion between the viral and host cellular membranes [5, 6]. It is synthesized as an unreactive F0 precursor, containing 1662 nucleotides (nt) coding for 553 amino acids (aa) with an approximate 55kDa weight [7, 8]. F0 is proteolytically cleaved by specific host cellular proteases at the peptide bond between residues 116 and 117 forming two disulphide linked polypeptides, F1 and F2 which are 48–54 kDa and 10–16 kDa, respectively [9, 10]. There are several domains that have been identified throughout the length of these polypeptides important for viral fusion activity [11].

Although Newcastle disease (ND) is said to be enzootic in Nigeria, little information exists on the molecular epidemiology and the lineage distribution of the Newcastle disease virus (NDV) in the country [3] and there is paucity on reports of virulent Newcastle disease virus (vNDV) strains obtained from dead/sick vaccinated animals in the outpatient veterinary clinic. The importance of detection and pathotyping of NDV in understanding the epizootiology of the virus in any region cannot be overemphasized [12] especially with the growing need for evaluation of the efficacy of existing ND vaccines [13]. Presently, two live attenuated monovalent vaccines—LaSota and Komarov—are commercially available for the control of ND in intensively reared poultry in Nigeria, but there are reports of ND outbreak among vaccinated flocks. Knowledge of the increasingly evolving genetic variation of vNDV is important for developing genetically matched vaccines which can prevent ND vaccine failure and maybe future outbreaks in the country.

The aim of this study was to isolate and characterize Newcastle disease virus (NDV) full fusion (F) gene detected from dead chicken of vaccinated flock presented to a veterinary clinic for post-mortem examination in Kano State, Nigeria, during March and April, 2020. Details of amino acid mutation were noted, documented, and compared with previously reported vNDV isolates from Nigeria, West Africa, and commercially available NDV vaccine strains.

2. Materials and Methods

2.1. Animal Ethics Declaration. This study did not include the use of live chickens. International and national guidelines for the care and use of animals were followed by experts at the veterinary clinic during post-mortem examinations and sample collection.

2.2. Sample Collection. Pooled organ samples (proventriculus, spleen, and small intestine) and swabs (cloacal and tracheal) were collected in viral transport medium (VTM) from one hundred chicken cadavers with history of vaccination against NDV that was reported to a veterinary clinic located in Kano State metropolis during March-April, 2020 from flocks presenting with respiratory discomfort, weakness, greenish diarrhoea, anorexia, high mortality and morbidity, and drop in egg production in layers characteristic of ND. The samples were labelled and transported immediately on ice and stored at −4°C until analysis was conducted.

Post-mortem examination was conducted on all specimens, and characteristic lesions were noted.

2.3. NDV Total Viral RNA Extraction, Reverse Transcription Polymerase Chain Reaction, and F-Gene Sequencing. All experiments were carried out according to standard protocol. Viral RNA was extracted directly from pooled organ samples and swabs using the Quick-RNA™ Viral Kit (Zymo Research, USA) as specified in the product manual. Fifty samples were processed successfully.

NDV M-gene was detected using protocols described [14]. Two overlapping fragments covering 1662 bp of the full F-gene were amplified using two pairs of primers (Table 1). One-step RT-PCR was performed using One Taq one-step RT-PCR Kit (New England BioLabs Inc). cDNA synthesis was achieved at 50°C for 30 minutes followed by incubation at 94°C for 15 minutes. RT-PCR was performed with 40 cycles of denaturing at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 68°C for 2 minutes with final extension at 68°C for 10 minutes, and PCR products were maintained at 4°C. Electrophoresis of PCR products was done on 1% ethidium bromide stained with 1.5% agarose gel at 90 volts and 120 Amps for 35 minutes and compared with a 100 bp DNA ladder, and amplified products were visualized under ultraviolet (UV) illumination using gel documentation system-image capture (Biometra, Germany).

The amplicons were sequenced by Macrogen Ltd (Netherlands), and sequences obtained were submitted on the NCBI database and assigned accession numbers OK491971–OK491977.

2.4. Phylogenetic Tree Construction and Evolutionary Distance Analysis. Nucleotide sequence alignment, editing, and analysis were done using the Bioedit software (7.2.5). Nucleotide sequence similarity and molecular phylogenetic analysis was computed on MEGA (11.0.). Inferred evolutionary F-gene sequence of the study isolates, some reported NDV isolates, and known vaccine strains was conducted by the maximum likelihood method based on JTT matrix-based method using 1000 bootstrap replicates.

3. Results

3.1. Characteristic Pathological Lesions on Organs Seen during Post-Mortem Examination. 3.2. Molecular Detection of NDV. The overall M-gene detection rate by RT-PCR was 54% (27/50). A 121-bp fragment was amplified, and products of electrophoresis were visualized by ultraviolet (UV) trans-illumination.

F-gene amplification was successfully carried out in seven samples, and products of electrophoresis were visualized by UV trans-illumination (Figure 1). 1662 bp nucleotide sequences obtained were deposited in the GenBank (details are given in Table 2).

3.3. Phylogenetic Tree and Evolutionary Distance Analysis. Phylogenetic tree was constructed (Figure 2) using MEGA (11.0). The genetic relatedness of the study isolates, reference
KU665482.1 LaSota.71.IR/2016, eight commercially available vaccine strains and NDV F-gene sequences obtained from GenBank database was inferred by phylogenetic analysis. All study isolates clustered around the newly classified genotype XIV(sub-genotype XIVb) in class II which is widely reported in Nigeria.

Nucleotide blast analysis shows a 99% nucleotide identity to virulent NDV MT543153 isolated in 2019 from backyard poultry in Niger (a country to the north border of Nigeria). The evolutionary divergence as nucleotide (nt) homology and amino acid (aa) homology between study isolates and commercially available vaccines is shown Tables 3 and 4.

3.4. Molecular Characterization and Mutational Analysis of the Functional and Antigenic Domains. The complete translated 553 fusion protein amino acid sequences obtained from the study isolates were used to compare their functional and antigenic domains relative to nine vaccine strains using KU665482.1 LaSota.71.IR/2016 as reference, six vNDV strains previously reported from Nigeria, and two vNDV strains isolated from West Africa (Tables 5–10). Notable substitutions around these regions were observed. Interestingly, among the research isolates, these substitutions were sometimes observed differently. Numbering system of amino acid (aa) was used to name the detected aa substitutions with respect to observed genetic variations.

Along the hypervariable region (residues 1–31), 14 substitutions leading to P4K, P4E, A11V, A11E, L15Q, L28P, and A29T mutations were observed (Figure 3 (a), Tables 5 and 7). In comparison to the LaSota reference strain KU665482, all isolates displayed a S31P of the signal peptide. Eight transmembrane domains have been reported[18] at residues 14–27, 15–25, 118–131, 120–128, 266–269, 429–432, 499–525, and 501–523. Compared to the LaSota strain, this region is highly non-conserved in all isolates except for 266–269 with no amino acid substitution (Figure 3 (a)). The major epitopes involved in virus neutralization are conserved in all residues except for one amino acid substitution Lys AAG to Arg AGA (K78R) of the A2 neutralizing epitope identified in all isolates (Figure 3 (a), Tables 5 and 7). However, nucleotide (nt) substitution occurred even in the conserved epitopes (Supplementary material (Available (here)) which shows disposition of these sites to aa mutation.

All seven isolates share the characteristic virulent motif 112-R-R-K-R/F117 at the F0 cleavage site indicating that they are velogenic NDV strains (Figure 3 (a)). G112R, Q114R, and G115K have been observed in study isolates (Tables 5, 7, and 8). Along the fusion peptide region (117–142), five aa substitutions, L117F, I118V, I121V, within the study isolates.

Table 1: Primers used for sequence of full F-gene of NDV.

| Primer name     | Direction | Primer Location | Product size | References  |
|-----------------|-----------|-----------------|--------------|-------------|
| NDV-F4217F      | Forward   | 5′-TGCGGAGTGTGAAAGTCACTCATT-3′ | 4217-4239 | 1240 bp     | JF966385.1   |
| NDV-F5457R      | Reverse   | 5′-TGCTGAGGCAAACCCTTTG-3′ | 5438-5457  |             |              |
| NDV-F5296F      | Forward   | 5′-ATTTGAGCGCGCTTGATCAGG-3′ | 5296-5317  | 999 bp      |              |
| NDV-F6295R      | Reverse   | 5′-CGTTTCTACCCGCTGTACTGCTCTT-3′ | 6272-6295 |             |              |

Characteristic pathological lesions on organs seen during post mortem examination

Table 2: Sample collected, sequence ID, and corresponding accession number.

| Sample collection ID | Sequence ID | Isolated specimen voucher | Isolated from | Accession number |
|---------------------|-------------|---------------------------|---------------|-----------------|
| PCR/014/200320      | Seq 14      | F gene KN 14              | Broiler       | OK491971 Avian Orthoavulavirus 1 Isolate KN 14 |
| PCR/036/250320      | Seq 36      | F gene KN 36              | Broiler       | OK491972 Avian Orthoavulavirus 1 Isolate KN 36 |
| PCR/048/300320      | Seq 48      | F gene KN 48              | Broiler       | OK491973 Avian Orthoavulavirus 1 Isolate KN 48 |
| PCR/055/310320      | Seq 55      | F gene KN 55              | Broiler       | OK491974 Avian Orthoavulavirus 1 Isolate KN 55 |
| PCR/056/310320      | Seq 56      | F gene KN 56              | Broiler       | OK491975 Avian Orthoavulavirus 1 Isolate KN 56 |
| PCR/071/020420      | Seq 71      | F gene KN 71              | Layer         | OK491976 Avian Orthoavulavirus 1 Isolate KN 71 |
| PCR/075/030420      | Seq 75      | F gene KN 75              | Layer         | OK491977 Avian Orthoavulavirus 1 Isolate KN 75 |

Details of sample ID, collection dates, isolates of study, source, and accession numbers as assigned by NCBI.
G124S, and I135V, are seen (Tables 5, 8, and 9). L117F and I118V are expected in the virulent furin-like molecule. In addition, the F protein has six highly conserved potential N-linked glycosylation sites Ng1–Ng6 [18] with sequence Asn (Asparagine)-X-Ser(Serine)/Tr(Treonine) (N-X-S/T) where X is any aa except proline and aspartate [19,20]. Amino acids at these sites were used and conserved in all NDV isolates of this research at residues 85NRT, 191NNT, 366NTS, 447NIS, 471NNS, and 541NNT. Hence, there was no loss of glycosylation site though there was one substitution compared to the LaSota strain at residue 191NKT (Figure 3(a)). However, nt substitutions occurred in different patterns at these regions among study isolates which resulted in same sense mutation.

Cysteine residues are important in the connection between F1 and F2 sub-units to maintain the F protein structure. Cysteine residues are conserved at positions 25, 27, 76, 199, 338, 347, 362, 370, 394, 399, 401, 424, 514, and 523 of the F protein in most NDV isolates [20]. Amino acids are used and conserved in all the isolates except for a unique point cysteine (C) to Serine substitution at residue 394 in OK491977 leading to loss of one cysteine residue. Several nt substitutions were observed in the region which resulted in same sense mutation (Figure 3(b), Tables 6 and 9).

Table 3: Percentage nucleotide identity between study isolates, traditional vaccine strains, and some isolates reported from West Africa.

| NDV isolate                                      | Nucleotide homology (%) |
|--------------------------------------------------|-------------------------|
|                                                  | OK491971 | OK491972 | OK491973 | OK491974 | OK491975 | OK491976 | OK491977 |
| KU665482.1 LaSota.71.IR/2016                     | 81.58    | 81.70    | 81.50    | 81.44    | 81.44    | 81.26    | 81.20    |
| KT445901.1 Avian orthoavulavirus1 strain Komarov | 82.10    | 82.22    | 82.04    | 81.86    | 81.86    | 81.74    | 81.65    |
| JF950509.1 Newcastle disease virus strain Mukteswar | 83.02    | 83.14    | 83.35    | 83.29    | 83.17    | 82.87    | 82.99    |
| JN872154.1 Avian orthoavulavirus 1 isolate       | 82.14    | 82.26    | 82.18    | 81.99    | 81.99    | 81.81    | 81.75    |
| JN872151.1 Avian orthoavulavirus 1 isolate       | 81.82    | 81.94    | 81.74    | 81.68    | 81.68    | 81.50    | 81.44    |
| JX316216.1 Newcastle disease virus strain R2B      | 82.43    | 82.56    | 82.50    | 82.20    | 82.20    | 82.08    | 82.17    |
| EU289028.1 Newcastle disease virus strain VG/GA   | 81.56    | 81.68    | 81.44    | 81.38    | 81.38    | 81.20    | 81.14    |
| JN872152.1 Avian orthoavulavirus 1 isolate Ulster | 84.14    | 84.39    | 84.21    | 84.27    | 84.03    | 83.97    | 83.67    |
| KC987036.1 Newcastle disease virus strain F        | 81.77    | 82.02    | 81.74    | 81.56    | 81.68    | 81.44    | 81.47    |
| KU058680.1 NDV/Duck/Nigeria/903/KUDU-113/1992     | 90.78    | 91.14    | 91.08    | 91.14    | 91.02    | 91.02    | 90.78    |
| HP969167.1 NDV/Turkey/Nigeria/NIE 10-082/2011     | 91.51    | 91.87    | 91.69    | 91.75    | 91.87    | 91.57    | 91.40    |
| HP969143.1 NDV/Chicken/Nigeria/NIE 09-1597/2009   | 95.79    | 96.15    | 95.97    | 95.91    | 96.03    | 95.85    | 95.55    |
| FJ772449.1 NDV/Avian-913-33Nigeria-2006           | 88.76    | 89.00    | 88.86    | 88.80    | 88.67    | 88.80    | 88.49    |
| JX390609.1 NDV/Chicken/Togo/A KO18/2009           | 88.49    | 88.73    | 88.55    | 88.61    | 88.49    | 88.55    | 88.19    |
| HP969175.1 NDV/Chicken/Nigeria/NIE 10-306/2011    | 88.34    | 88.58    | 88.37    | 88.31    | 88.31    | 88.25    | 88.03    |
| JP966385.1 NDV 2008_Mali_ML007_08                 | 88.76    | 89.00    | 88.86    | 88.80    | 88.67    | 88.80    | 88.49    |
| MT543153.1 NDV/Chicken/Niger/89/2019             | 98.43    | 98.68    | 98.31    | 98.68    | 98.68    | 98.13    | 98.13    |

Values calculated from the complete F-gene sequences. Align sequence nucleotide blast showing homology of appropriate % query cover on NCBI blastn suite.

Figure 2: DNA Ladder, band showing 999 bp: Lane 1-5, 8, and 11 positive samples, Lane 6 and 10 negative samples, lane 7 positive control, and lane 9 negative control.
Table 4: Estimates of evolutionary divergence (%) between sequences calculated using aa substitutions between study isolates, traditional vaccine strains, and some isolates reported from West Africa.

| NDV isolate                                      | Amino acid homology |
|--------------------------------------------------|---------------------|
|                                                  | OK491971  | OK491972  | OK491973  | OK491974  | OK491975  | OK491976  | OK491977  |
| KU665482.1 LaSota.71.IR/2016                    | 0.208      | 0.207     | 0.207     | 0.208     | 0.208     | 0.210     | 0.210     |
| KT445901.1 Avian orthoavulavirus1 strain Komarov| 0.203      | 0.202     | 0.201     | 0.203     | 0.203     | 0.205     | 0.205     |
| JP950509.1 Newcastle disease virus strain Mukteswar | 0.193     | 0.191     | 0.188     | 0.188     | 0.190     | 0.193     | 0.091     |
| JN872154.1 Avian orthoavulavirus 1 isolate Beaudette C | 0.204     | 0.202     | 0.202     | 0.204     | 0.204     | 0.206     | 0.206     |
| JN872151.1 Avian orthoavulavirus 1 isolate Hitchner | 0.205     | 0.204     | 0.204     | 0.205     | 0.205     | 0.208     | 0.207     |
| JX316216.1 Newcastle disease virus strain R2B     | 0.202      | 0.200     | 0.198     | 0.202     | 0.202     | 0.203     | 0.202     |
| EU289028.1 Newcastle disease virus strain VG/GA | 0.209      | 0.207     | 0.208     | 0.209     | 0.209     | 0.211     | 0.211     |
| JN872152.1 Avian orthoavulivirus 1 isolate Ulster | 0.178      | 0.175     | 0.176     | 0.175     | 0.178     | 0.179     | 0.182     |
| KC987036.1 Newcastle disease virus strain F       | 0.207      | 0.204     | 0.204     | 0.207     | 0.205     | 0.208     | 0.207     |
| KU058680.1 NDV/Duck/Nigeria/903/KUDU-113/1992     | 0.090      | 0.093     | 0.094     | 0.093     | 0.095     | 0.095     | 0.097     |
| HF969167.1 NDV/Turkey/Nigeria/NIE 10082/2011      | 0.089      | 0.085     | 0.087     | 0.086     | 0.085     | 0.088     | 0.089     |
| HF969143.1 NDV/Chicken/Nigeria/903/1597/2009      | 0.043      | 0.039     | 0.041     | 0.042     | 0.041     | 0.043     | 0.045     |
| FJ772449.1 NDV/Avian-91333-Nigeria-2006           | 0.121      | 0.118     | 0.119     | 0.120     | 0.121     | 0.120     | 0.123     |
| JX390609.1 NDV/Chicken/Togo/AKO18/2009           | 0.123      | 0.120     | 0.122     | 0.122     | 0.123     | 0.122     | 0.126     |
| HF969175.1 NDV/Chicken/Nigeria/NIE 10-306/2011   | 0.126      | 0.124     | 0.124     | 0.125     | 0.125     | 0.126     | 0.128     |
| JF966385.1 NDV/2008_Mali_ML007_08                 | 0.121      | 0.118     | 0.119     | 0.120     | 0.121     | 0.120     | 0.122     |
| MT543135.1 NDV/Chicken/Niger/89/2019             | 0.016      | 0.013     | 0.017     | 0.013     | 0.019     | 0.018     | 0.095     |

The number of amino acid substitutions per site between sequences is shown. Analyses were conducted using the Poisson correction model [17]. This analysis involved 24 amino acid sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [16].

Table 5: Amino acid changes of fusion protein of vNDV study isolates compared with vaccine strains and strains previously reported in Nigeria and West Africa.

| ID virus isolate                                      | Amino acid at indicated position on fusion protein |
|-------------------------------------------------------|---------------------------------------------------|
|                                                       | Hypervariable region/ signal peptide                | AGR | Cleavage site | Fusion peptide |
|                                                       | 4 11 15 29 31 78 112 133 114 115 116 117 118 121 124 135 |     |              |               |
| *KU665482.1 LaSota.71.IR/2016                         | R A L P A S K G R Q G R L I I G I                 |     |              |               |
| KT445901.1 Avian orthoavulavirus 1 strain Komarov†    | — T — — — — R — — K — F — — —              |     |              |               |
| JF950509.1 Newcastle disease virus strain Mukteswar†  | — V — L T — — R — — R — F — — S —            |     |              |               |
| JN872154.1 Avian orthoavulavirus 1 isolate Beaudette C| — V — — — — R — — K — F — — —              |     |              |               |
| JN872151.1 Avian orthoavulavirus 1 isolate Hitchner† | — T — — — — R — — K — F — — —            |     |              |               |
| JX316216.1 Newcastle disease virus strain R2B†        | — T — — — — R — — K — F — — —            |     |              |               |
| EU289028.1 Newcastle disease virus strain VG/GA†     | — — — — — R — — K — F — — —            |     |              |               |
| JN872152.1 Avian orthoavulavirus 1 isolate Ulster†   | — V — — T — — K — — — — —               |     |              |               |
| KC987036.1 Newcastle disease virus strain F†         | — — — — — R — — K — F — — —            |     |              |               |
| OK491971 Avian orthoavulavirus 1 isolate KN 14†      | K V — L — P R R R — R K — F V V S V        |     |              |               |
| OK491972 Avian orthoavulavirus 1 isolate KN 36†      | K V — — T P R R R — R K — F V — — V        |     |              |               |
| OK491973 Avian orthoavulavirus 1 isolate KN 49†      | K V — L T P R R R — R K — F V — — V        |     |              |               |
| OK491974 Avian orthoavulavirus 1 isolate KN 55†      | K V — L T P R R R — R K — F V — — V        |     |              |               |
| OK491975 Avian orthoavulavirus 1 isolate KN 56†      | K V — L — P R R R — R K — F V — — V        |     |              |               |
| OK491976 Avian orthoavulavirus 1 isolate KN 71†      | E E Q L T P R R R — R K — F V — — V        |     |              |               |
| OK491977 Avian orthoavulavirus 1 isolate KN 75†      | K V — L T P R R R — R K — F V — — V        |     |              |               |
| KU058680.1 NDV/Duck/Nigeria/903/KUDU-113/1992‡       | K — — — — L T — — R — — K — F — — —        |     |              |               |
| HF969167.1 NDV/Turkey/Nigeria/NIE 10-082/2011‡       | K V — L T — — R R — — K — F — — —        |     |              |               |
| HF969143.1 NDV/Chicken/Nigeria/NIE 09-1597/2009‡    | I V — L T — — R R — — K — F — — V        |     |              |               |
| FJ772449.1 NDV/Avian-9133-Nigeria-2006‡              | K V P M T — — — — — — K — F — — —        |     |              |               |

The signal peptide is AGR, the cleavage site is K, and the fusion peptide positions are 4, 11, 15, 29, 31, 78, 112, 133, 114, 115, 116, 117, 118, 121, 124, and 135.
Table 5: Continued.

| ID virus isolate                  | Amino acid at indicated position on fusion protein | Hypervariable region/ signal peptide | AGR | Cleavage site | Fusion peptide |
|----------------------------------|--------------------------------------------------|--------------------------------------|-----|--------------|---------------|
| JX390609.1 NDV/Chicken/Togo/AKO18/2009 | K V — L T — R R — R K — F — — — — | 4 11 15 28 29 31 78 112 113 114 115 116 117 118 121 124 135 | | | |
| HF969175.1 NDV/Chicken/Nigeria/NIE 10–306/2011 | K V P M T — — R R K — F — — — V | | | | |
| JF966385.1 NDV 2008 Mali ML007-08 | K V — L T P R R — K F V — — V | | | | |
| MT543153.1 NDV/Chicken/Niger/89/2019 | K V — L T P R R — K F V — — V | | | | |

Variable positions along functional sites in the fusion protein showing point mutation compared with nine commercially available vaccine strains using LaSota KU665482.1 as reference. Mutation patterns vary in some instances among research isolates. *Reference strain. †Vaccine strain. ‡Study isolates.

Nigerian isolates. —, no change in the aa compared with the reference; AGR, antigenic region; R, arginine; K, lysine; E, glutamic acid; I, isoleucine; A, alanine; T, threonine; V, valine; L, leucine; Q, glutamine; P, proline; M, methionine; G, glycine; F, phenylalanine; S, serine.

Table 6: Amino acid changes along fusion protein of vNDV study isolates compared with vaccine strains and strains previously reported in Nigeria and West Africa.

| ID virus isolate                  | Amino acid at indicated position on fusion protein | Conserved cysteine residue |
|----------------------------------|--------------------------------------------------|--------------------------|
| *KU665482.1 LaSota.71.IR/2016†  | K N S I T N C N E R K T| 145 272 278 285 288 297 394 476 479 482 486 494 498 |
| KT445901.1 Avian orthoavulavirus 1 strain Komarov† | | |
| JF950509.1 Newcastle disease virus strain Mukteswar† | N — — — — — — — — — — | |
| JN872154.1 Avian orthoavulavirus 1 isolate Beaudette C‡ | — — — — — — — — — — | |
| JN872151.1 Avian orthoavulavirus 1 isolate Hitchiner‡ | — — — — — — — — — — | |
| JX316216.1 Newcastle disease virus strain R2B‡ | — — — — — — — — — — | |
| EU289028.1 Newcastle disease virus strain VG/GA‡ | — — — — — — — — — — | |
| JN872152.1 Avian orthoavulavirus 1 isolate Ulster‡ | N — — — — — — — — — — | |
| KC987036.1 Newcastle disease virus strain F‡ | — — — — — — — — S — — | |
| OK491971 Avian orthoavulavirus 1 isolate KN 14‡ | N Y P — N — — — A N R S | |
| OK491972 Avian orthoavulavirus 1 isolate KN 36‡ | N Y P — N — — — A N R S | |
| OK491973 Avian orthoavulavirus 1 isolate KN 48‡ | N Y P K N K — — — A N R S | |
| OK491974 Avian orthoavulavirus 1 isolate KN 55‡ | N Y P — N — — — A N R S | |
| OK491975 Avian orthoavulavirus 1 isolate KN 56‡ | N Y P K N — — — A N R S | |
| OK491976 Avian orthoavulavirus 1 isolate KN 71‡ | N Y P — N — — — T D A N R S | |
| OK491977 Avian orthoavulavirus 1 isolate KN 75‡ | N Y P — N — — — A N R S | |
| KU058680.1 NDV/Duck/Nigeria/903/KUDU-113/1992‡ | N Y — — N — — — A S R — | |
| HF969167.1 NDV/Turkey/Nigeria/NIE 10–082/2011‡ | N Y P — N — — — A N R A | |
| HF969143.1 NDV/Chicken/Nigeria/NIE 09–1597/2009‡ | N Y P — N — — — A D R A | |
| FJ772449.1 NDV/Avian-913-33-Nigeria-2006‡ | N Y — — N — — — A S R — | |
| JX390609.1 NDV/Chicken/Togo/AKO18/2009 | N Y P — N — — — A S R — | |
The three heptad repeat regions HRa (143–185), HRb (268–299), and HRc (471–500) in the isolates [19] displayed 1, 5, and 6 aa substitutions, respectively, compared to the Ku665482.1 LaSota.71.1R/2016 reference strain. These are K145N at HRa; N272Y, S278P, I285K, T288N, and N297K at HRb and N476T, N479D, E482A, R486N, K494R, and T498S at HRc. Notably, substitution I285K was seen only in OK491973 and OK491975. N297K was seen in isolate OK491976 only. N476T was observed only in isolate OK491976. Interestingly, T498S was observed in all study isolates and MT543153 NDV/chicken/Niger/89/2019 but not in any other isolate included in the phylogenetic tree analysis even those isolated previously from Nigeria or Africa (Figure 3(b), Tables 6, 9, and 10).

4. Discussion

NDV management in Africa is complicated [21]. The economic impact of ND among vaccinated and unvaccinated
Table 8: Point mutation pattern along fusion protein of study isolates compared to reference LaSota strain KU665482.1.

| ID virus isolate          | Nucleotide at indicated position along the fusion gene | Cleavage site | Fusion peptide |
|---------------------------|-------------------------------------------------------|---------------|----------------|
| * KU665482.1 LaSota.71.IR/2016† | CAG  GGC  CGC  CTT | 340–342 343–345 346–348 349–351 | ATA  ATT  GGT  ATA |
| OK491971 Avian orthoavulavirus 1 isolate KN 14† | CGG  AAG  CGT  TTT | (Q114R) (G115K) (R116) | (L117F) GTG  GTT  AGT  GTA |
| OK491972 Avian orthoavulavirus 1 isolate KN 36† | CGG  AAG  CGT  TTT | (Q114R) (G115K) (R116) | (L117F) GTG  GTT  AGT  GTA |
| OK491973 Avian orthoavulavirus 1 isolate KN 48† | CGG  AAG  CGT  TTT | (Q114R) (G115K) (R116) | (L117F) GTG  GTT  AGT  GTA |
| OK491974 Avian orthoavulavirus 1 isolate KN 55† | CGG  AAG  CGT  TTT | (Q114R) (G115K) (R116) | (L117F) GTG  GTT  AGT  GTA |
| OK491975 Avian orthoavulavirus 1 isolate KN 56† | CGG  AAG  CGT  TTT | (Q114R) (G115K) (R116) | (L117F) GTG  GTT  AGT  GTA |
| OK491976 Avian orthoavulavirus 1 isolate KN 71† | CGG  AAG  CGT  TTT | (Q114R) (G115K) (R116) | (L117F) GTG  GTT  AGT  GTA |
| OK491977 Avian orthoavulavirus 1 isolate KN 75† | CGG  AAG  CGT  TTT | (Q114R) (G115K) (R116) | (L117F) GTG  GTT  AGT  GTA |

Variable positions along functional sites in the fusion protein showing nucleotide substitution compared with LaSota KU665482.1 vaccine strain as reference. Not all substitution resulted in mutation because of degeneracy of amino acid. Italics positions show substitution site. †Reference strain. Vaccine strain.

Table 9: Point mutation pattern along fusion protein of study isolates compared to reference LaSota strain KU665482.1.

| ID virus isolate          | Nucleotide at indicated position along the fusion gene | HRa | HRb | Conserved cysteine residue |
|---------------------------|-------------------------------------------------------|-----|-----|----------------------------|
| * KU665482.1 LaSota.71.IR/2016† | 145  272  278 | 285  288  297 | 394 | 1180–1182  TGC |
| OK491971 Avian orthoavulavirus 1 isolate KN 14† | 433–435 814–816 832–834 | 862–864 862–864 889–891 | AAA  ACT  AAT | TGGC |
| OK491972 Avian orthoavulavirus 1 isolate KN 36† | (K145N) (N272Y) (S278P) | AAA  ACT  AAT | AAA (T288N) | TGC |
| OK491973 Avian orthoavulavirus 1 isolate KN 48† | (K145N) (N272Y) (S278P) | AAA  AAA  AAA | AAA (I285K) (T288N) (N297K) | TGC |
| OK491974 Avian orthoavulavirus 1 isolate KN 55† | (K145N) (N272Y) (S278P) | AAA  AAA  AAA | AAA (I285K) (T288N) (N297K) | TGC |
| OK491975 Avian orthoavulavirus 1 isolate KN 56† | (K145N) (N272Y) (S278P) | AAA  AAA  AAA | AAA (I285K) (T288N) (N297K) | TGC |
| OK491977 Avian orthoavulavirus 1 isolate KN 75† | (K145N) (N272Y) (S278P) | AAA  AAA  AAA | AAA (I285K) (T288N) (N297K) | TGC |

Variable positions along functional sites in the fusion protein showing nucleotide substitution compared with LaSota KU665482.1 vaccine strain as reference. Not all substitution resulted in mutation because of degeneracy of amino acid. Italics positions show substitution site. †Reference strain. Vaccine strain.

A: adenine; G: guanine; C: cytosine; T: thymine.
The research also provides details of F-gene sequence of seven nary clinic in Nigeria for post-mortem examination. This dead chickens from vaccinated fock presented to a veterinary consultancy by poultry producers, to the best of our between circulating and vaccine strains [24]. Despite huge new genotypes [19, 21], and significant antigenic distance vaccinated focks [23], viral evolution and identification of vaccinated focks [22], sub-optimal protection levels among reports of vaccine failure and high mortality among vaccinated and non-vaccinated flocks [21]. Despite huge new genotypes [19, 21], and significant antigenic distance vaccinated focks [23], viral evolution and identification of vaccinated focks [22], sub-optimal protection levels among reports of vaccine failure and high mortality among vaccinated and non-vaccinated flocks [21].

The NDV isolates reported here originated from different poultry farms within and outside the metropolis of Kano State. Post-mortem lesions include haemorrhagic intestinal ulcers, haemorrhagic caecum tonsils, haemorrhagic and inflamed proventriculus, and haemorrhagic trachea, among others (Figure 4). These lesions agree with overt clinical signs reported by poultry handlers such as difficulty in breathing, greenish diarrhoea, weakness, and anorexia with wing and leg paralysis as the most common neurological symptoms among flocks. Haemorrhage at the tip of the proventriculus is highly suggestive of ND [26, 27]. Generally, distributed lesions suggest that the virus is able to infect and replicate in most organs, typical of vNDV as supported by the cleavage site motif of the isolates.

In a previous review report, phylogenetically, the Nigerian genotype XIVa isolates form a cluster with some strains in Niger Republic while genotype XIVb isolates tend to be more closely related to the 2009 isolates from Benin Republic [24]. They further stated that the isolates in genotype XIVa that share the highest nucleotide similarity with those from Niger Republic while genotype XIVb isolates tend to be more closely related to the 2009 isolates from Benin Republic [24]. They further stated that the isolates in genotype XIVa that share the highest nucleotide similarity with those from Niger Republic while genotype XIVb isolates tend to be more closely related to the 2009 isolates from Benin Republic [24]. They further stated that the isolates in genotype XIVa that share the highest nucleotide similarity with those from Niger Republic while genotype XIVb isolates tend to be more closely related to the 2009 isolates from Benin Republic [24].

### Table 10: Point mutation pattern along fusion protein of study isolates compared to reference LaSota strain KU665482.1.

| ID virus isolate | Nucleotide at indicated position along the fusion gene |
|-----------------|------------------------------------------------------|
|                 | Codon | 476 | 479 | 482 | 486 | 494 | 498 |
| KU665482.1 LaSota,71,IR/2016² | AAT | AAT | GAC | AGA | AAA | ACA |
| OK491971 Avian orthoavulavirus 1 isolate KN 14² | AAT | (N479D) | (E482A) | (R486N) | (K494R) | (T498S) |
| OK491972 Avian orthoavulavirus 1 isolate KN 36² | AAT | GAC | GCA | AAC | AGA | TCA |
| OK491973 Avian orthoavulavirus 1 isolate KN 48² | AAT | (N479D) | (E482A) | (R486N) | (K494R) | (T498S) |
| OK491974 Avian orthoavulavirus 1 isolate KN 55² | AAT | GAC | GCA | AAC | AGA | TCA |
| OK491975 Avian orthoavulavirus 1 isolate KN 56² | AAT | (N479D) | (E482A) | (R486N) | (K494R) | (T498S) |
| OK491976 Avian orthoavulavirus 1 isolate KN 71² | ACT | GAC | GCA | AAC | AGA | TCA |
| OK491977 Avian orthoavulavirus 1 isolate KN 75² | AAT | (N479D) | (E482A) | (R486N) | (K494R) | (T498S) |

Variable positions along functional sites in the fusion protein showing nucleotide substitution compared with LaSota KU665482.1 vaccine strain as reference. Not all substitution resulted in mutation because of degeneracy nature of amino acid. Italic positions show substitution site. * Reference strain. ‡ Vaccine strain. ² Study isolates. HR: heptad repeats; A: adenine; G: guanine; C: cytosine; T: thymine.
Figure 3: Molecular phylogenetic analysis based on the nucleotide sequences of the F-gene of study isolates, some reported NDV isolates and known vaccine strains using bootstrap consensus of 1000 replicates. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [15]. The tree with the highest log likelihood (-20685.06) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. This analysis involved 61 amino acid sequences. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [16].
Komarov vaccine strains in Kano State, Nigeria, the spread of this genotype to other regions of the continent may spread vaccine resistance as well.

The F protein is capable of provoking host immune response, and it is necessary for producing neutralizing antibodies against NDV induced by vaccines [19]. Mutations along this gene will impact antibody production which will be heterologous even at the level of F-gene [31]. Percentages of nucleotide identity and aa homology between the study isolates and commercially available vaccines ranges between 81.14 and 84.39% and 0.175–0.211 respectively (Tables 3 and 4). Compared to the commonly used vaccines in Kano State (LaSota and Komarov) with representative KU665482.1 LaSota.71,IR/2016 and KT445901.1 Avian orthoavulavirus 1 strain Komarov (Tables 3 and 4), range is 81.20–81.70%; 0.207–0.210 and 81.65–82.22; 0.201–0.205 respectively indicating considerable diversity [32]. LaSota is classified under genotype II, while most commonly reported wild vNDV strains in the 21st century are found among genotype VII [24]. Existing antigenic variations among West Africa strains and the LaSota vaccine may affect its protective efficacy to confer protection against all West African strains [33]; in cases where the LaSota vaccine provided protection against clinical disease, it did not prevent infection and viral shedding [33]. Although all NDV strains belong to one serotype, protection provided by genotype II vaccines against heterologous challenge has been recently under controversy [31] with several reports of LaSota vaccine failing to provide complete protection against morbidity and or mortality during experimental heterologous vNDV challenge necessitating the growing need for development of antigenically matched vaccines to circulating strains [12].

The fusion protein is a major target for the immune response, and immunity raised against this protein is effective in the neutralization of NDV infectivity [34]. In this report, several nt substitutions occur at specific and conserved antigenic sites which in some instances resulted in aa substitution (mutation) (Tables 7–10). The neutralizing infective strains and vaccine strains are among reported causes of ND vaccine failure [19]. Most commonly used ND vaccine strains including LaSota were developed in the 1950s and 1960s [32] and show considerable degree of genetic divergence from currently circulating vNDV wild strains [31]. LaSota is classified under genotype II, while most commonly reported wild vNDV strains in the 21st century are found among genotype VII [24]. Existing antigenic variations among West Africa strains and the LaSota vaccine may affect its protective efficacy to confer protection against all West African strains [33]; in cases where the LaSota vaccine provided protection against clinical disease, it did not prevent infection and viral shedding [33]. Although all NDV strains belong to one serotype, protection provided by genotype II vaccines against heterologous challenge has been recently under controversy [31] with several reports of LaSota vaccine failing to provide complete protection against morbidity and or mortality during experimental heterologous vNDV challenge necessitating the growing need for development of antigenically matched vaccines to circulating strains [12].

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epitopes are important in forming antigenic epitopes, and aa substitution in this region induces the formation of neutralizing escape variants [20, 35–37]. The single point K78E mutation of the A2 antigenic epitope seen in all study isolates is due to AAA-AGA nt substitution at codon 232–234 (Table 7) which has been previously reported to induce escape mutation in NDV Beaudette-C clone against MAbs generated using LaSota strains [5]. The role of this mutation in genotype XIVb requires more attention. Though the scope of this research did not involve the generation of K78E mutants to test LaSota efficacy, it is notable from samples and data collected that though the flocks were vaccinated, ND is still reported. The fusion peptide domain between aa residues 117 to 142 is a conserved hydrophobic region located at the amino end of the F1 polypeptide and has been reported to insert into the target membrane to initiate membrane fusion [38, 39]. Phenylalanine (F) residue at position 117 has been reported as a major contributor to initiatemembranefusion[38,39].Phenylalanine(F)residue has been reported to insert into the target membrane to induce escape mutation in NDV Beaudette-C clone against MAbs generated using LaSota strains [5]. The role of this mutation in genotype XIVb requires more attention. Though the scope of this research did not involve the generation of K78E mutants to test LaSota efficacy, it is notable from samples and data collected that though the flocks were vaccinated, ND is still reported. The fusion peptide domain between aa residues 117 to 142 is a conserved hydrophobic region located at the amino end of the F1 polypeptide and has been reported to insert into the target membrane to initiate membrane fusion [38, 39]. Phenylalanine (F) residue at position 117 has been reported as a major contributor to initiatemembranefusion[38,39].Phenylalanine(F)residue has been reported to insert into the target membrane to induce escape mutation in NDV Beaudette-C clone against MAbs generated using LaSota strains [5].

The F-genes of all seven vNDV detected and characterized have not been established and require further study. Based on nucleotide analysis, all isolates show high degree of antigenic variability from commonly used LaSota and Komarov ND vaccines in Kano State and others commercially available. Although caution is warranted in considering genetic distance between NDV vaccines and the challenge virus as the sole cause of the reported cases of vaccine escape in field report, [51] the isolation and detection of vNDV genotype XIVb among vaccinated flock require further research and especially the role of the A2 antigenic epitope in inducing antibody escape mutation among that sub-genotype. Furthermore, the role of Genotype II NDV strain vaccine pressure on evolutionary change among Class XIVb vNDV require further evaluation. The role of Genotype II NDV strain vaccine pressure on evolutionary change among Class XIVb vNDV require further evaluation. In order to generate genetically matched vaccines for this region, ND surveillance and molecular analysis of circulating strains should be encouraged and reported.

**Data Availability**

The F-genes of all seven vNDV detected and characterized during this research are available with no restriction on NCBI database with their corresponding accession numbers.

**Disclosure**

This work was part of the PhD study of Olubukola O. Funsho-Sanni. This paper is available online as a preprint [52].

**Conflicts of Interest**

The authors have no conflicts of interest to declare that are relevant to the content of this article.

**Authors’ Contributions**

Olubukola O. Funsho-Sanni and Elijah E. Ella were responsible for conceptualization. Olubukola O. Funsho-Sanni was responsible for sample collection. Olubukola O. Funsho-Sanni and Lawal D. Rogo were responsible for methodology, molecular analysis, and investigation. Olufunsho S. Sanni and Olubukola O. Funsho-Sanni were the authors and reviewers.

5. Conclusion

Virulent NDV genotype XIVb was detected among vaccinated poultry in Kano State, Nigeria. Fusion gene of seven vNDV strains was successfully sequenced, and details are available on NCBI GenBank (OK491971-OK491977). Based on amino acid analysis, several mutations were seen and reported along the F-gene compared to commercially available vaccines. Whether these mutations individually or combined affect antigenicity of the virus remains to be established. Based on nucleotide analysis, all isolates show high degree of antigenic variability from commonly used LaSota and Komarov ND vaccines in Kano State and others commercially available. Although caution is warranted in considering genetic distance between NDV vaccines and the challenge virus as the sole cause of the reported cases of vaccine escape in field report, [51] the isolation and detection of vNDV genotype XIVb among vaccinated flock require further research and especially the role of the A2 antigenic epitope in inducing antibody escape mutation among that sub-genotype. Furthermore, the role of Genotype II NDV strain vaccine pressure on evolutionary change among Class XIVb vNDV require further evaluation. The role of Genotype II NDV strain vaccine pressure on evolutionary change among Class XIVb vNDV require further evaluation. In order to generate genetically matched vaccines for this region, ND surveillance and molecular analysis of circulating strains should be encouraged and reported.

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Olubukola O. Funsho-Sanni and Elijah E. Ella were responsible for conceptualization. Olubukola O. Funsho-Sanni was responsible for sample collection. Olubukola O. Funsho-Sanni and Lawal D. Rogo were responsible for methodology, molecular analysis, and investigation. Olufunsho S. Sanni and Olubukola O. Funsho-Sanni were the authors and reviewers.
responsible for data sourcing, figures, and tables. Olufunsho S. Sanni, Lawal D. Rogo, and Olubukola O. Funsho-Sanni were responsible for original draft preparation. Ismaila Shittu was responsible for F-gene primer design and protocol. Elijah E. Ella, Helen I. Inabo, and Sodangi A. Luka were responsible for supervision and validation. All authors were responsible for review and editing.

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Supplementary Materials

Sixty-one representative NDV F-gene sequences of different genotypes and sub-genotypes isolated from Nigeria and Africa downloaded from GenBank were used for phylogenetic tree and molecular analysis of the study isolates. The file is compatible with Bioedit or MEGA. (Supplementary Materials)

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