Original Research Article

In-silico analysis of Newcastle disease virus strains from outbreaks in Zaria and Kano of Nigeria

ABSTRACT

Introduction: Newcastle Disease (ND) is the major constraint to poultry production in developing country like Nigeria. Numerous outbreak cases of ND have being reported frequently even in vaccinated farms. The large antigenic disparity of the Newcastle Disease Virus (NDV) and potential for significant evolutionary divergence between field strains and widely used vaccinations are thought to be the root of the issue.

Aim/Objectives:A cross-sectional studies was carried-out to identify a discrepancy between thevaccinal strains and circulating NDV field strains in Zaria and Kano so as to provide solution for the control of Newcastle disease.

Materials and Methods: In this study,molecular characterization and pathotyping of the field strains of NDV and strains of ND vaccines wascarried out to evaluate their relatedness. Using RT-PCR with primers targeting partial fusion gene, 19 out of the (n= 120) samples purposefully collected for the detection of NDV in Zaria(n= 44) and Kano (n= 76) were positives. Twelve of the 19 positive amplicons were successfully sequenced and analyzed.

Results: Only few number of the study's sample have similarity with some of the vaccine strains.

Conclusion: This study has raised concerns about the methods used to prevent NDV in Nigeria.

Recommendation: There is need to develop a field genotype-matched vaccines in the country and continual outbreak surveillance for effective management of the disease.

Keywords: Newcastle disease virus, strain, sequencing, phylogentic analysis, pathotyping, Nigeria

1. INTRODUCTION

The most lucrative birds in terms of contributions to the world economy are poultry. Theyare the most valuable breeds of avian species that added value to food security. With frequent disease outbreaks such as Newcastle disease that have devastating effects in developing nations like Nigeria; the continent's fourth-largest commercial producer of poultry, the industry is currently facing significant difficulties [1].The RNA virus known as Newcastle Disease Virus (NDV) or avian paramyxovirus type 1 (APMV-1) causes Newcastle disease and is characterized by enclosed, linear, single-stranded, non-segmented, negative sense genomes.[2,3]. The avian paramyxovirus serotype 1(APMV-1) belong to the genus *Orthoavulavirus (previously known as Avulavirus)* of family*Paramyxoviridae and the* order *Mononegavirales*[4]. The NDV strains can be classified as class I (I genotype) or class II (XV genotypes) based on genomic size and the nucleotide sequences of the F and L genes [5].

Pathotyping of NDV is essential for a better understanding of the epizootiology of the disease in addition to virus detection in an outbreak[6].This is even more crucial in light of recent reports of the virus's growing genetic diversity and widespread distribution in wild birds and avian species other than poultry [7,8].

In Nigeria, cases of Newcastle disease tend to increase around the time of the dry harmattan season [9]. The most common sickness of domestic and foreign birds in Zaria, according to reports, is Newcastle disease[9].According to a survey, wild birds in Kano were a factor in the spread of NDV to backyard chickens and industrial poultry farms [10]. Aldous *et al*[11] has previously suggested that the genotyping of NDV isolates should be integrated into reference laboratories' diagnostic virus characterization.This type of analysis will allow for quick epidemiological evaluation of the origins and propagation of the viruses causing ND outbreaks so that efficient control measures may be implemented [11].

Undoubtedly, the co-circulation of numerous NDV strains in some states of Nigeria is a significant factor that could contribute to the establishment of novel virulent isolates in the nation.[12]. Molecular characterization of field isolates of NDV in Nigeria is currently poorly understood due to a lack of data[13]. There is an urgent need to review the present Newcastle disease control strategies in Nigeria given the significant evolutionary divergence between the commonly used vaccinations and the NDV strains prevalent in the nation[12].The objective of

this study was to molecularly characterize Newcastle disease viruses isolated in Nigeria molecularly and to compare them to ND vaccine strains as well as other virulent strains.

2. MATERIALSANDMETHODS

2.1 StudyLocation

Kano state is located in Northwestern, Nigeria. The region is approximately between longitudes8°45Eand12°05Elatitudes10°30Nand13°02 NandassuchisapartoftheSudano-Sahelian zone of Nigeria. It is situated in the Sahelian geographic region, south of the Sahara. Kano is thecommercial nerve center of Northern Nigeria and is the second largest city in Nigeria [14]. Zaria isthe second largest city in Kaduna State, Nigeria. It is located between longitude $7^{o}36^{\text{‟}}$ to $7^{o}42^{\text{‟}}$ E and latitude $11^{o}00^{\text{‟}}$ to $11^{o}10^{\text{‟}}$ Nofthe equator[15].

2.2Category of the Selected Areas:

2.2.1 LiveBird Market

Samples from Kano were primarily obtained from the live bird market at Sabon-gari, Fagge LGA Kano state, Nigeria. The Sabon-gari live-bird market is a key location in Kano where all varieties of domesticated, semi-domesticated, and wild birds are kept in cages (each holding around 50 birds) or tiny rooms by individual vendors for sale. The local birds are typically sourced by the bird traders from a variety of locations, including the nearby villages in the states of Kano, Jigawa, and Katsina. To obtain the indigenous birds, they occasionally travel up to the Niger Republic.

2.2.2 Veterinary Teaching Hospital

The samples from Zaria were obtained at the Ahmadu Bello University Veterinary Teaching Hospital (ABUVTH), Zaria, Kaduna state, Nigeria. The ABUVTH collects samples from carcasses brought in for postmortem investigation at the hospital or from farms where a disease outbreak is suspected.

2.3Category of Birds Sampled

Improved chickens, local chickens, ducks, pigeons, mallard ducks, turkeys, guinea fowl, and geese are among the birds that were tasted at the Sabon-gari live bird market. Every sample taken at the ABUVTH, Zaria, came from exotic chickens.

2.3.1 Inclusion Criteria for Sampling

Only domesticated and partially domesticated birds are considered throughout the study period.

2.4Sample Collection

Between December 2018 and March 2019, a total of 120 samples were purposefully gathered at the ABUVTH in Zaria, Kaduna state, and the Sabon-gari live bird market in Kano. ABVTH provided forty-four (44) samples, while Sabon-gari Live Bird Market provided seventy-six (76) samples(Table 1).The samples were properly labeled with a designated sample I.D, site of swab and date of sample collection.The sample were transported in virus transport medium (Hanks' transport medium) containing 2000 U/ml of penicillin, 2 mg/ml of streptomycin, 0.05 mg/ml of gentamycin and 100 U/ml of mycostatin in cold flask and stored in a refrigerator at -20 0C in Ahmadu Bello University Veterinary Teaching Hospital, Zaria. The total samples where then transported together to Laboratoire Central de L'Elevage (LABOCEL), Niamey, Niger Republic and stored at -80 °C before the NDV detection process.

Table1:numberof birdssampled forthe study

Sourceofsample	Typeof bird	Cloacalswab	Trachealswab	Location
LBM	Improvedchickens	10	10	Kano
LBM	Localchickens	7	7	Kano
LBM	Ducks	6	6	Kano
LBM	Pigeons	6	6	Kano
LBM	Mallardducks	3	3	Kano
LBM	Turkeys	2	2	Kano
LBM	Guineafowls	2	2	Kano
LBM	Geese	2	2	Kano
ABUVTH	Improvedchickens	22	22	Zaria
Total		60	60	

2.5Molecular Detection of Newcastle Disease Viruses

2.5.1 ViralRNAextraction

RNA was extracted from 100 µl of each sample suspension using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions at the molecular biology laboratory of the Laboratoire Central de L'Elevage (LABOCEL), Niamey, Niger Republic.

2.5.2 OnestepReverseTranscriptase-PolymeraseChainReaction(RT-PCR)

The following primers were used in a one-step RT-PCR to amplify the 300 base pair F-gene fragment that was reported at the IZSVe for the genotyping and pathotyping of APMV-1 [16]: Forward: NOH-For 5' TACACCTCATCCCAGACAGG 3' and Reverse: NOH-Rev 5' AGTCGGAGGATGTTGGCAGC 3'.

Using25µlfinalreactionvolume,reactionmixturescontaining13.4µlRNasefreewater,5µlPCRbuffer5X,0.5µldNTPs,0.5µlOnestepRT-PCREnzymeMix,0.1µlRNaseInhibitor,5µlofRNAtemplate and 1µl each of the primers. The amplification was carried out in a 96-well thermal cycler (Applied Biosystems 2720), which was programmed to begin at 50°C and run for 30 minutes. Initial denaturation then took place for 15 minutes at a temperature of 94 °C. At the cycling temperatures of 94 °C for 30 seconds, 55 °C for 1 minute, and 68 °C for 1 minute, 35 cycles of denaturation, annealing, and elongation of the templates were completed. Finalextensionofthetemplateswasachievedat68°C for7minutes.Theproductswereelectrophoresedat 80Vfor 40minutes in1.5%agarosegel withTris-acetateEDTA(TAE)andbandswerevisualizedunderanultraviolettransilluminator(ENDURO^{TM}GDSlabnet,aplagen).

2.5.3 PurificationofthePCRProduct

The positive samples were purified using a Wizard SV Gel and PCR Clean-up system (Promega). The purified amplicons were stored at -20°C before sending them to LGC Genomics, Berlin, Germany for sequencing.

2.6Sequence Data Analyses

2.6.1 Editing of the Sequences

The software program BioEdit (v7.1.11) was used to edit nucleotide sequences. Afterwards, sequences were exported in Fast Adaptive Shrinkage Thresholding Algorithm (FASTA)

format to notepad after being validated for base calling and length editing.

2.6.2 SequenceAlignment

To find closely similar sequences to include in multiple sequence alignments, the sequences in FASTA format from the notepad were blasted for homology searches (http://www.ncbi.nlm.nih.gov/blast).For comparison, representative pathotypes were also pulled from the GenBank. Numerous sequence alignments were performed usingClustalW in MEGA7software.All thesequences weretrimmed tothelength of thestudysamples.

2.7PhylogeneticAnalyses

The phylogenetic tree was built using both the maximum-likelihood approach and the neighbor joining tree separately. The evolutionary distances, which are measured in the units of the number of base substitutions per site, were calculated using the Tamura 3-parameter technique. Twelve sample sequences and twenty-seven sequences retrieved from the GenBank were used in the analysis. Evolutionary analyses wereconductedin MolecularEvolutionary GeneticAnalysis7 (MEGA 7; https://www.megasoftware.net) [17]. The analysis involved four vaccine virus sequences [18] and 10 sequences retrieved from GenBank, including NDV LaSota (JF950510), NDV Hitchner-B1 (AF309418), NDV Komarov (KT445901), NDV Beaudette C (X04719).

2.7.1 EvolutionaryDistanceAnalyses

Homologybetweenstrainswasinferredfrom amatrixrelationshiptableanddatawerepresentedinfigures.

3. RESULTS AND DISCUSSION

3.1 ResultofMolecularDetectionofNDV

For each of the 120 samples, a 300 base pair (bp) DNA fragment was amplified using reverse transcription polymerase chain reaction (RT-PCR). The findings revealed that the Newcastle Disease Virus was present in 19 samples.The result of thepartial F gene amplification of 120 samples shows that only 19 (15.8%) of the field samples were positive.Outofthe 19positivesamples, 8(42.1%)were fromcloacalswabsand11(57.9%) from the tracheal swabs. Among the 44 samples from Zaria, 10 (22.7%) showed positiveresultwhile only 9

(11.8%)werepositiveout of the76 samples from LBM Kano but only 12 positive samples were sequenced and provided with accession number (Table 2).

Table2:sequencedsamplesamongthepositives

S/N	Accessionnumber	SampleI.D(Fstrands)	Location
1	MN339518	NDV_NIGKD1	Zaria
2	MN339519	NDV_NIGKD2	Zaria
3	MN339520	NDV_NIGKN3	Kano
4	MN339521	NDV_NIGKN4	Kano
5	MN339522	NDV_NIGKN5	Kano
6	MN339523	NDV_NIGKN6	Kano
7	MN339524	NDV_NIGKN7	Kano
8	MN339525	NDV_NIGKN8	Kano
9	MN339526	NDV_NIGKN9	Kano
10	MN339527	NDV_NIGKN10	Kano
11	MN339528	NDV_NIGKD11	Zaria
12	MN339529	NDV_NIGKN12	Kano

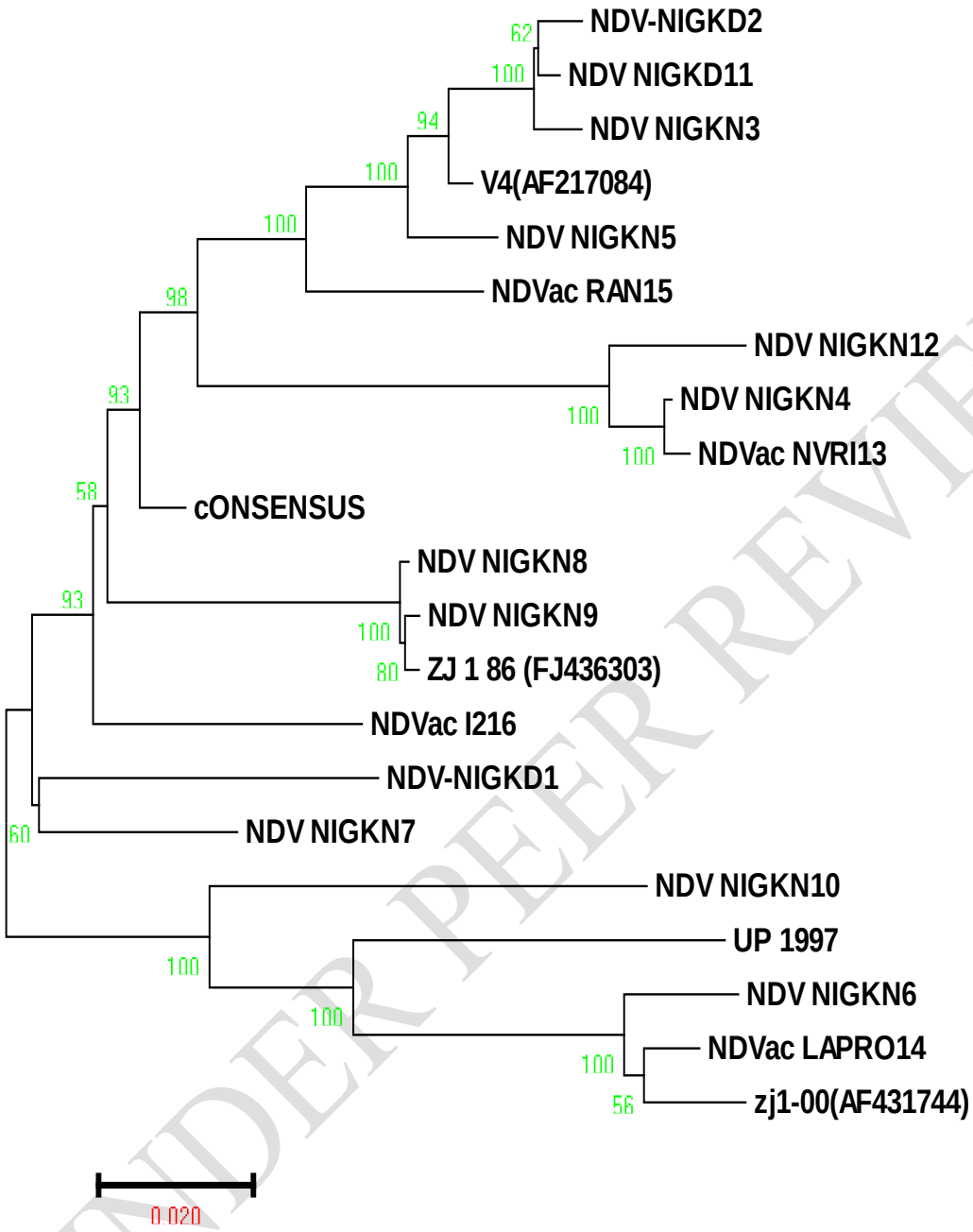

Figure1:evolutionaryrelationshipsoftaxabetweenthesamples andNDvaccines

The evolutionary history was inferred using the Neighbor-Joining method [32]. The optimal treewith the sum of branch length = 0.57935104 is shown. The percentage 2f replicate trees in whichthe associated taxa clustered together in the bootstrap test (400 replicates) are shown next to thebranches [33]. The tree is drawn to scale, with branch lengths in the same units as those of theevolutionarydistancesusedtoinferthephylogenetictree.Theevolutionarydistanceswerecomputed using the Tamura 3-parameter method [34] and are in the units of the number of basesubstitutions per site. The analysis involved 21 nucleotide sequences. Codon positions includedwere 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted inMEGA7[17].

Nonetheless, the prevalence of ND in this study (15.8%) was higher than the prevalence reported by Hamisu *et al* (12.6%) [13]. The findings of this study indicate a prevalence of ND in Zaria of 22.7%, which is nearly identical to the 29.1% reported incidence of ND in Zaria [19].

The difference between these outcomes was likely caused by the fact that the samples for this study came from Zaria and were primarily collected based on the history and postmortem lesions of Newcastle disease as presented to the ABUVTH, while Hamisu *et al* [13] collected samples from chickens with apparent clinical signs of the disease in LBM and commercial poultry farms.

The 10% seroprevalence of ND in Kano reported by Adamu *et al* [10] for the previous six years is consistent with the 11.8% prevalence of ND from LBM Kano. Only one positive sample for LBM Kano was from a local chicken (MN339529 in Table 2); all the rest were from the improved chickens. This may be due to the high number of samples (20) taken from the enhanced chickens compared to the local chickens (14), ducks (12), pigeons (12), mallard ducks (6),turkeys (4), guinea fowls (4) and geese (4). Furthermore, since the other birds are not typically vaccinated, it is possible that the enhanced chickens were shedding the vaccine virus as a result of their past immunization before being sold. High percentage of positive samples from the trachealswabs in this study is in line with the study which shows that NDV is more likely found in the trachea than inthe cloaca of the birds [13, 20] and this is because the major route of the spread of NDV is viaaerosol,andthereforethereishightendencyofvirusconcentrationinthetrachea.

The 12 sequenced samples (Table 2),four reference ND vaccines (NDVac_NVRI13, NDVac_LAPRO14, NDVac_RAN15, NDVac_I216) and five reference sequences were used to create the evolutionary relationship depicted in Figure 1. It is possible that the NDV_NGKN3 from Kano and NDV_NGKD11 from Zaria are the same derivation of the vaccine strain reported by Solomon [21] due to their high sequence similarity (100%) and 94% homology with V4 AF217084 and this might be as a result of the vaccine being used in Kano and Zaria poultry farms. There is considerable homology between the mesogenic vaccine stains NDVac_RAN15 from India and NDV_NGKN5 from Kano. The Kano strains of NDV_NGKN4andNDV_NGKN12 are 100% identical to the Nigerian vaccine strain NDVac_NVRI13.The NDVac_NVRI13 vaccinal strain and the strains from Kano and Zaria listed above have a close evolutionary relationship, as evidenced by their low interpopulation evolutionary distance. Because the bootstrap value (56%) is below the threshold (70%) for a credible evolutional association, it is possible that the velogenic vaccine strain AF431744.3 (ZJ1) from China and the lentogenic vaccine strain NDVac_LAPRO from Hungary do not share any evolutionary relationships.

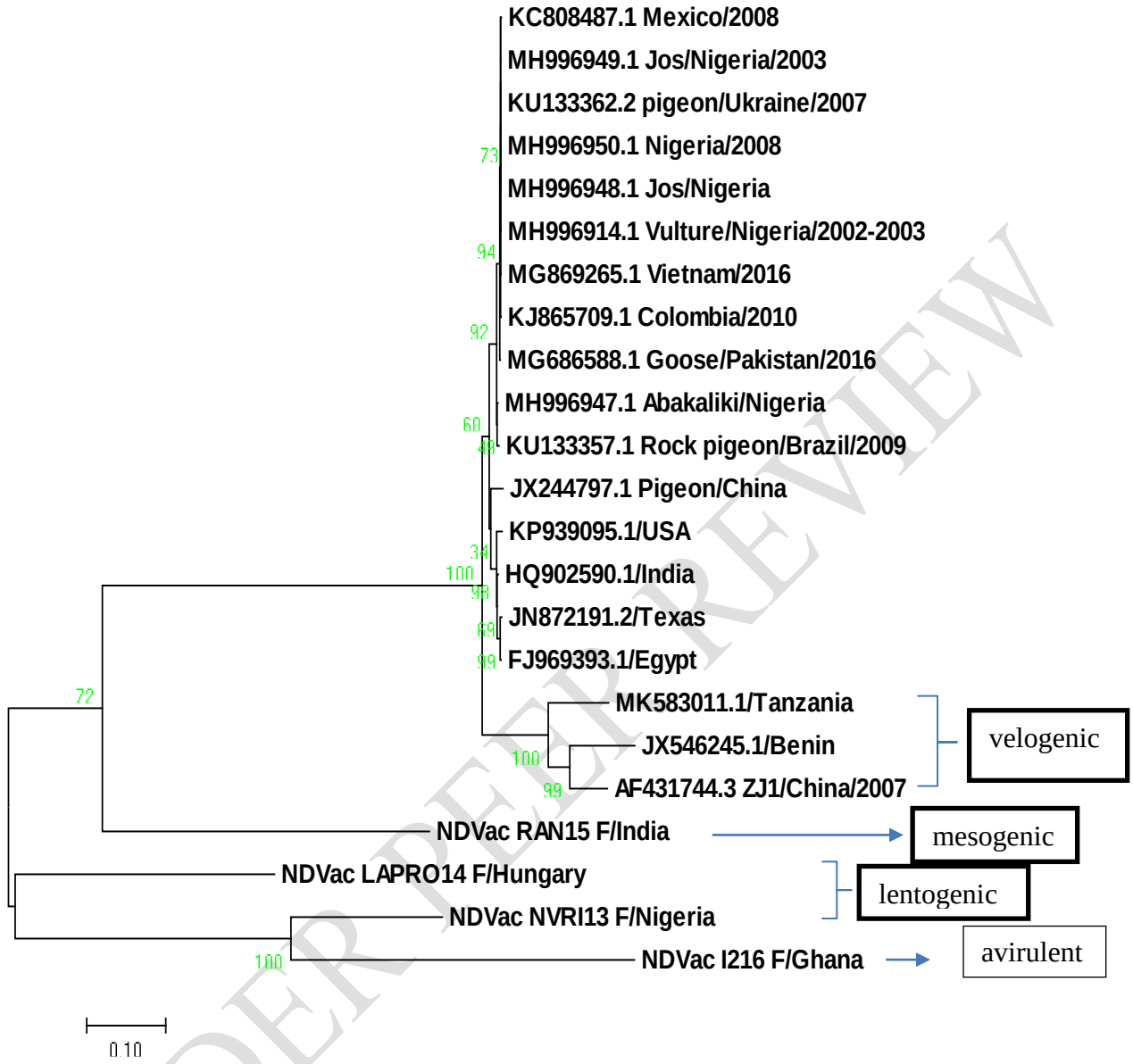

Figure 2: evolutionary relationships of taxa between four ND vaccines and other strains retrieved from the GenBank

The evolutionary history was inferred using the Neighbor-Joining method [32]. The optimal tree with the sum of branch length = 2.70632264 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (400 replicates) are shown next to the branches [33]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method [34] and are in the units of the number of base substitutions per site. The analysis involved 23 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1329 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [17].

Grimes [22] classified the NDV vaccine strains I-2 and V4 as avirulent, La Sota as lentogenic, and Mukteswar and Komarov as mesogenic pathotypes in the FAO Basic Laboratory Manual. Mesogenic vaccines, particularly when administered to chickens as a subsequent vaccination, have been found to provide the best protection against clinical disease and mortality as well as against virus shedding[23].Many field strains of NDV with severe clinical symptoms that afflict chickens in Nigeria are of the velogenic pathotype.[13, 24]. A Study conducted in Nigeria shows that the mesogenic vaccine, Komarov, ismore closely related to the velogenic virus under study than the lentogenic vaccine, La Sota [25]. The analysis of this study (Figure 2) revealed that JX546245 had an evolutionary relationship with highly pathogenic NDV strains from China (AF431744) with 99% similarity and interpopulation evolutionary similarity of 100% with that of Tanzania, MT15 (MK583011). Hamisu *et al* [13] had reported that some NDV strains from Zaria have similar homology with NDV strain from Benin Republic (JX546245) (MK583011).

A polybasic amino acid motif and phenylalanine at position 117 (^{112}RRQKR↓F^{117}) were found in the deduced amino acid sequence of the fusion protein cleavage site of MT15 from chicken in Tanzania, and the sequence also had an ICPI value of 1.86, which is characteristic for virulent NDV strains [26]. The strain from Benin (JX546245) has an ICPI value of 1.65[27] while that of China, ZJ1 (AF431744.3) has 1.89 ICPI value[28]. According to the World Organizationfor Animal Health, a virulent version of NDV is indicated by an ICPI value of 0.7 or above or by the presence of at least three basic amino acids at the site of fusion protein cleavage, whereas values above 1.5 are typical of velogenic viruses[29].Therefore,thisstudyhasverifiedthat the majority oftheNigerianNDV strains are evolutionarily related to the extremely virulent strains.

However, several NDV strains from Nigeria (MH99694, MH996947, MH996948, MH996949, and MH996950) have been reported to have low virulence while having a closer evolutionary link with the virulent Tanzanian strain (MK583011)[30]. It is likely that the homology being lower than the threshold interpopulation distance identified in this study was caused by evolutionary divergence with weak similarities as several of these strains from Nigeria have bootstrap values below 70%.

The referenced NDV vaccine strains employed in this study's sample analysis (NDVac_NVRI13, NDVac_LAPRO14, NDVac_RAN15, and NDVac_I216)were also phylogenetically distinct from

other NDV strains circulating in Nigeria (Figure 2). Due to its mesogenic pathotype, the NDVac_RAN15 from India appeared as anout-groupintheNeighborJoiningphylogenictree(Figure2). However, it demonstrates an evolutionary relationship across populations with the virulent strains. The vaccine strains NDVac_LAPRO14 from Hungary and NDVac_NVRI13fromNigeria share several characteristics. Interestingly, the lentogenicvaccinestrainfrom Nigeria and the avirulentNDVac_I216fromGhana exhibit 100% resemblance.

4. CONCLUSION AND RECOMENDATIONS

4.1 Conclusion

The findings of this investigation are consistent with the prevalence of NDV in Kano as reported by Adamu *et al* [10] and the prevalence of NDV in Zaria as reported by Wakawa*et al* [19]. Due to the fact that improved chickens are frequently immunized and the virus is typically concentrated at the tracheal site of the birds, improved chickens have a greater likelihood of shedding NDV. Samples from this study share an evolutionary link with severalavirulent, lentogenic and mesogenic vaccines.Some strains of the Nigeria NDV are extremely virulent. Since it is well known that ND vaccines work better at reducing virus shedding when the vaccine strains are genetically more closely related to the challenge strain [31], the evolutionary distance between the vaccine strains and the prevalent field strain represents an important factor in efficient disease control. Hence, the findings should raise questions about current approaches to controlling NDV.

4.2 Recommendations

Cutting-edge vaccinations are therefore urgently required to solve the shortcomings of the traditional live attenuated vaccines in light of the aforementioned restrictions.Hemagglutinin-neuraminidase (HN) also plays a role in the antigenic variation of NDV and since there is no published report on the characterization of HN in NDVgeneisolated from Nigeria, it is wise to examine the HN gene as well.

ETHICAL APPROVAL

The study was approved by the Ethics Committee of Laboratoire Central de L'Elevage (LABOCEL), Niamey,Niger Republic.

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