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https://dx.doi.org/10.4314/nvj.v44i2.4 **ORIGINAL ARTICLE**

MOLECULAR AND SEROLOGICAL DETECTION OF NEWCASTLE DISEASE VIRUS IN LIVE-BIRD MARKETS, JOS, PLATEAU STATE NIGERIA.

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ABSTRACT

Newcastle disease (ND) is a highly infectious viral disease of birds caused by the Newcastle disease virus (NDV) and it has been reported in domestic birds in Nigeria. Waterfowls and village poultry in live bird markets (LBM) acts as reservoirs, potentially reintroducing the virus to commercial poultry. This study aims at molecular and serological detection of NDV at live bird markets in Plateau State, Nigeria. A cross sectional analysis involved 309 pooled cloacal and tracheal swabs over three months were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR), RT-qPCR, virus isolation and haemagglutination and haemagglutination-inhibition test (HA &HI). Virus isolation was attempted in 9 to 11 days old specific antibody negative (SAN) embryonated chicken eggs and fifteen samples showed haemagglutination. Subsequent tests confimed nine of these as NDV positive through haemagglutination and haemagglutination inhibition (HI) tests. ConventionalRT-PCR and RT-qPCR further validated five of the nine NDV positive isolates. The phylogenetic analysis of partial F gene nucleotide sequences revealed that all three isolates belonged to class II genotype XIV.2_XIVb. This finding underscores the persistent threat of NDV to local poultry, necessitating comprehensive virological surveillance to understand, isolate and characterize the virus in Nigeria. Therefore, monitoring for emerging lineages and sub-lineages in Nigeria birds is crucial for safeguarding commercial poultry production.

Keywords: Local chicken, Live bird market, Newcastle disease virus, Phylogenetic tree, Nigeria.

INTRODUCTION

Newcastle disease (ND) is a highly contagious and lethal viral disease that affects all bird species. The virus responsible is Newcastle disease virus (NDV), an enveloped virus with a single stranded negative sense RNA genome that is linear and non-segmented. It belongs to the family *Paramyxoviridae* in the subfamily *Avulavirinae*, genus *Orthoavulavirus* (ICTV, 2018, Dimitrov *et al.,* 2019; Ferreira *et al.,* 2021). According to the "rule of six," the RNA genome of NDV is approximately 15 kb and codes for six major proteins: nucleoprotein (NP), matrix protein (M), fusion protein (F), haemagglutinin neuraminidase (HN), phosphoprotein (P), and large RNA polymerase (L), in the order 3′-NP-P-M-F-HN-L-5′. (Kolakofsky *et al.,* 2005). The disease affects a large number of hosts, about 241 species belonging to 27 orders, out of the 50 known orders of birds (Madadger *et al.,* 2013). Several wild birds of various ages as well as chickens, turkeys, ducks, pigeons, guinea fowl, and Japanese quail are among the more often impacted species (Nanthakumar *et al.,* 2000; Zhang *et al.,* 2011). Ducks are generally considered as natural NDV reservoirs, and even for NDV strains that are fatal to chickens, they exhibit little to no clinical symptoms following infection (Liu *et al.,* 2010; Stanislawek *et al.,* 2022). A virulent NDV infection can be fatal to chickens, leading to a large reduction in egg production and a high death rate. One of the main issues still plaguing established or emerging poultry industries in many nations, including Nigeria, is the disease.

Newcastle disease virus is classified into three major pathotypes based on the clinical signs induced in the infected chicken: Velogenic (highly virulent), mesogenic (intermediate virulent) and avirulent (lentogenic strain) (Lamb and Kolakofsky, 2001). Studies comparing the deduced amino-acid sequence at the cleavage site of the NDV, varying in virulence for chicken showed that virulent viruses usually have the motif ^{112}R/K-R-Q-K/R-R-F^{117} and avirulent viruses have ^{112} G/E-K/R-Q-G/E-R-L^{117} (Dortmans *et al.,* 2009). Clinical signs seen in affected birds by this disease may vary widely and are dependent on factors like the virus strain, host species, age, immune status, environmental stress and concurrent infection. In chickens, the disease may vary from sudden death with 100% mortality to subclinical disease. Due to the enormous financial losses linked to the disease, particularly in chickens and turkeys, it is a serious threat to the poultry industries and has a global distribution (Miller et al., 2010). NDVs can be divided into classes I and II according to the results of the phylogenetic analysis of the F gene, which has been done in a number of studies (Diel *et al.,* 2012; Samal *et al.,* 2012). Class II has at least 18 genotypes identified, compared to Class I which has nine (Snoeck *et al.,* 2013; Dimitrov *et al.,* 2016). Major factors influencing NDV pathogenicity include the cleavability of the fusion protein precursor (F0) and the presence of several basic residues in the fusion protein cleavage site (Martin-Garcia *et al.,* 2012). In Africa, velogenic NDVs have been reported to have been isolated from sick and seemingly healthy poultry (Damena *et al.,* 2016; Molini *et al.,* 2017). Because of the poultry industry's explosive growth, high stocking densities, and inadequate biosecurity protocols, which encouraged the disease's endemicity and spread, NDV has been reported to be endemic in Nigeria (Okwor and Eze, 2010). Even though Nigeria experiences annual outbreaks of the NDV, little is known about the virus's current spread in some of the nation's regions. In Nigeria, NDV was first recorded in

the 1950's (Hill *et al.,* 1953) but the molecular characterization of the Nigerian isolate was just recently available. Seven NDV genotype I, II, IV, VI, XIV, XVII, and XVIII members have all been found in the country (Snoeck *et al.,* 2013; Shittu *et al.,* 2016; Bello *et al.,* 2018; Welch *et al.,* 2019).

The mainstays of NDV prevention and control are the stringent implementation of biosecurity protocols and extensive immunization campaigns, both of which have been effectively implemented for many years across the globe (Alexander, 2000). Even in vaccinated birds, the high frequency of NDV infection is not only associated with inadequate immunization or immunological suppression; it may also result from viral mutation that modifies the virus's genomic sequence, changing its biological characteristics and virulence (Kattenbelt *et al.,* 2006). According to the growing number of reports of ND outbreaks in Nigerian flocks vaccinated against the virus, birds may not be entirely protected by the conventional vaccinations or vaccine failure may have resulted in more virulent new NDV variants. Despite research on NDV in Nigeria, little is known about the genetic makeup of the viruses that cause the disease to be endemic. Thus, the purpose of our research is to assess the characteristics of ND viruses that were isolated from local poultry in the northern and central regions of Plateau State, Nigeria.

MATERIALS AND METHOD

Study Area

Plateau State can be found in between Lat. 08° 24′N and longitude 008° 32′ and 010 º 38′ E. It is situated in the tropical zone, with a temperature of 22ºC and mean minimum low temperature of 18ºC. The range of the mean yearly rainfall is 131.75 to 146 cm and the highest rainfall is recorded during the wet season months of July and August. Temperatures appear highest between the months of March and April (National Bureau of Statistics, 2009). It has a population of approximately 3.5 million people (NADIS, 2006a). Jos is divided into three LGAs namely Jos North, Jos South and Jos East (National Bureau of Statistics, 2009) and also into three senatorial districts or zones that comprises of Northern, Central and Southern zones. The people of Plateau State are predominantly farmers. The estimated number of poultry in Plateau State is eight million (Livestock population figures, FMARD, 2018, Animal husbandary Department, Federal Ministry of Agriculture). Because of the comparatively cool climate in Plateau State, poultry farming flourishes there. This research was conducted in major live-bird markets (LBMs) in the Northern and Central zones, specifically Yankaji old and Mangu LBMs. The State was chosen because of the fact that some farms in the state had experienced repeated outbreaks of Newcastle disease.

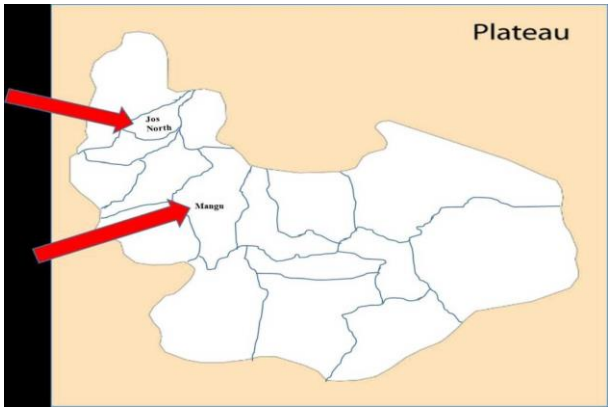


Figure. 1: Map of Plateau State displaying the research area (Chinyere C.N).

Sample Collection and Transportation

A multi-stage random sampling of local birds including chicken, ducks, turkey and Geese

brought to the LBM in Jos metropolis were sampled for this study. A total of 309 pooled cloaca and trachea swabs samples were collected. Each bird was sampled by inserting a polyester tipped swab stick on the cloaca and oropharyngeal cavities. Both samples were combined and placed in a 1.8ml cryovial containing 1 ml of viral transport medium (VTM) containing penicillin, streptomycin, gentamicin and amphotericin B. The samples collected were transported in a cool box with ice packs to the Influenza Laboratory of National Veterinary Research Institute, Vom, Plateau State, Nigeria. The swabs were kept at -80^{o}C until analyzed.

Virus isolation

Virus isolation was performed by inoculating 9-11 day old embryonated chicken eggs that are specific-antibody-negative (SAN) with a supernatant fluid of the swab specimens and incubate for five days at a duration of 24hours interval. Egg inoculation, incubation, candling and virus harvesting were conducted in accordance with the OIE Manual of Standards for Diagnostic Tests and Vaccines (OIE, 2012). At the end of the incubation period, allantoic fluid (ALF) was harvested from the eggs and screened by Haemagglutination (HA) test. The HA positive ALF were further subjected to heamagglutination-inhibition (HI) test and RT-PCR for confirmation. Virus stocks were stored at -70^{0}C until further used.

Extraction of viral RNA

The genomic viral RNA was extracted from the swabs using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol and was stored at -80°C until use.

RT-qPCR

The samples were screened for L gene using RT-qPCR for the detection of avian paramyxovirus type 1 using Qiagen One-step RT-qPCR kit according to the manufacturere's instructions. The Reverse and forward primers sequence for the NDV-L gene ND-F - 5′-GAG CTA ATG AAC ATT CTT TC -3′, ND-R- 5′-AAT AGG CGG ACC ACA TC TG - 3′, Probe L Pro MGB 6FAM 5′-CCA ATC AAC TTC CC -3′, Probe L Pro MGB2 VIC 5′-AAT AGT GTA TGA CAA CAC -3′ respectively was used. The L gene segment was reversed transcribed and amplified using applied biosystem (AB) thermocycler rotogen PCR. Reverse transcription was achieved by incubation with reverse transcriptase at 50^{0}C for 20min and initial PCR activation 95^{0}C for 15min. RT-qPCR was performed by denaturing cDNA at 94^{O}C for 45sec, annealing at 50^{0}C for 45sec. The above program was repeated for 40 cycles.

RT-PCR for FUSION (F) GENE

Those that were positive for NDV L gene were further amplified for F gene using a Qiagen One-step RT-PCR kit according to the manufacturer's instructions. The reverse and forward primers sequence for the NDV-F 5296F-5′ATTGGTAGCGGCTTGATCACTG-3′ and NDV-F 6295R 5′ CGTTCTACCCGTGTACTGCTCTTT-3′ respectively was used. The F gene segment was reversed transcribed and amplified using Applied Biosystem thermocycler PCR platform which amplified a 1000bp fragment of the NDV

F gene. Reverse transcription was achieved by incubation with reverse transcriptase at 50^0C for 30min and initial PCR activation 94^0C for 15min. RT-PCR was performed by denaturing cDNA at 94^OC for 30sec, annealing at 55^0C for 1min and extending the fragment at 68^0C for 90sec. The above program was repeated for 40 cycles with a final extension at 68^0C for 10 min and PCR products maintained at 4^0C.

Agarose gel electrophoresis

The PCR products were separated in 1.5% agarose gel in TBE buffer stained with ethidium bromide and compared with molecular marker (100bp) and the gel was visualized on UV trans-illuminator a Biostep dark hood DH-40/50 imaging system (Germany).

SEQUENCING AND PHYLOGENETIC ANALYSIS

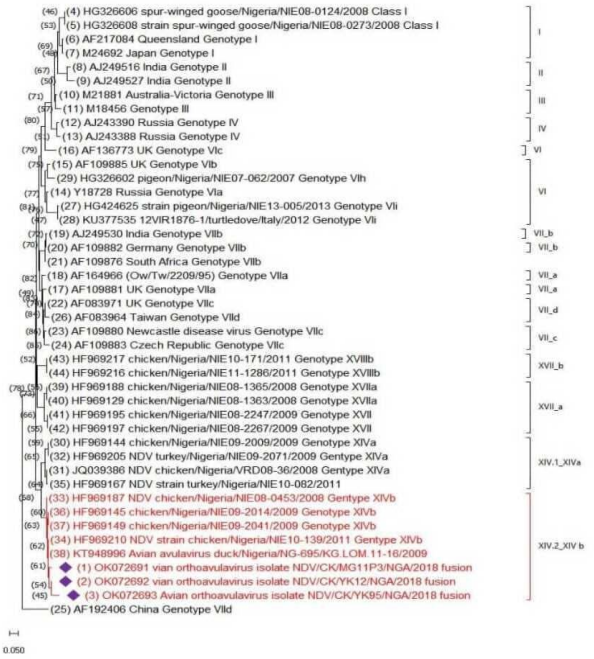
Positive PCR products were purified with QIAquick PCR purification kit (Qiagen, Germany) and sent to a commercial sequencing company Macrogen Inc, Netherland for sequencing using ABI3730XL sequencer (Applied Biosystems) in both forward and reverse directions. Thereafter, consensus sequences were generated through CAP3 alignment editor and phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis MEGA X. Genotype nomenclature according to Dimitrov et al., 2019 were used to identify the genotype XIV.2_XIVb.

GenBank accession number

NDV Sequences obtained from this study were added to the GenBank database under accession numbers OK072691-OK072693.

Phylogenetic analysis

The partial F gene nucleotide sequences from the NDV isolates in this study were subjected to phylogenetic analysis and compared with those from classes I and II that were retrieved from the NCBI database. The results revealed that the three isolates all belong to class II genotype XIV.2_XIVb (**Figure 2**).



RESULT

The result of the study confirmed that 5 out of 15 (33%) from virus isolation in 309 pooled tracheal and cloacal swabs that were examined using both the conventional RT-PCR and RT-q PCR were positive for NDV. Nine out of the 15 (60%) harvested were positive by HI. Three positive isolates (two from the Yankaji live-bird market and one from Mangu live-birds market) were sequenced to confirm their clade. Fig. 2: Phylogenetic tree. The maximum likelihood phylogenetic tree of the NDV isolates isolated during this study, along with other published sequences obtained from GenBank, is displayed in Figure 2. The Tamura-Nei model and the Maximum Likelihood method were used to infer the evolutionary history (Tamura and Nei, 1993). The displayed tree has the highest log likelihood (-32194.90). There were 39 nucleotide sequences in this analysis. Codon positions included were 1st+2nd+3rd+Noncoding. The final dataset contained 15232 positions in total. In MEGA X, evolutionary analyses were carried out (Kumar *et al.,* 2018).

DISCUSION

The endemicity of Newcastle Disease Virus (NDV) in Nigeria is highlighted by the discovery of the virus in seemingly healthy local poultry in this study conducted at the live-bird market in Plateau State. It may also buttress the assertion that local poultry are relatively more resistant to NDV with a lower mortality rate than commercial poultry. The NDV isolates in this study belong to the class II genotype XIV2._XIVb, according to the results of the phylogenetic analysis. Members of this genotype are widely distributed in West and Central African countries which Nigeria is part of with high virulent and mainly isolated from domestic birds. Similar findings have been reported in the North-Central states of Benue and Kogi, as well as in the North-West states of Nigeria, including Sokoto, Kaduna, and Jigawa. Others are Taraba and Yobe (North- East), Lagos state (South-West) (Bello *et al.,* 2018), (Helen *et al.,* 2020). Because of the local diversity of the genotype XIV subgenotype XIV.1_XIVa and XIV.2_XIVb, they are also known as regional NDV genotype. Similar results was obtained by Catherine *et al.,* 2019 where she reported a wild distribution XIV.2_XIVb in domestic birds such as pigeon, chicken, duck, turkey etc from 2002 - 2015 from various parts of the country. Phylogenetically, the genotype XIV.2_XIVb from this study clusters around other Nigerian XIV.2 XIVb genotype isolated in 2008 and 2011 with GenBank accession number HF969187, HF969145, HF969149, HF969210 and KT948996 (Fig.2). Elsewhere, genotype XIV has been reported in Burkina Faso with Burkina Faso/2415-580/2008 (BF/08), South Africa with Chicken/South Africa/08100426/2008 (SA/08), and Niger with Niger/1377-7/2006 (Niger/06) (Susta *et al.,* 2015). According to the study's findings, strains from LBMs and outbreaks from commercial chicken farms shared sequence similarities (genotype XIVb) (Welch *et al.,* 2019). Previous reports have noted some observed sequence similarity between strains recovered from outbreaks in commercial and backyard poultry farms in Jos, Plateau state, and those from LBMs in Sokoto and Kano states

(Solomon *et al.*, 2012). This may be due to horizontal spread by live poultry vendors from rural settlements and live bird markets to commercial and backyard poultry farms. Because different species of birds from different places interact uncontrollably, live-bird markets have remained a major factor in the spread of NDV (Choi *et al.*, 2005; Abah *et al.*, 2017). However, given the mindset of farmers who replace their LBM stock with existing poultry on their farms, the possibility of LBM spillover to backyard and commercial farms cannot be completely ruled out.

CONCLUSION

As part of an early warning system, Nigeria must maintain active, flock-based serological, virological, and molecular surveillance for NDV in order to detect the virus quickly and establish long-term control. The combination of these different methods ensures prompt detection of the virus and needs a proactive measures for the prevention of major outbreaks.

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