Vol. 12(2), pp. 20-26, April-June 2020
DOI: 10.5897/JVMAH2019.0825
Article Number: 2DBAB7763449
ISSN: 2141-2529

http://www.academicjournals.org/JVMAH

Journal of Veterinary Medicine and Animal Health

Full Length Research Paper

Molecular characterization and phylogenetic analysis of Newcastle disease virus isolated from poultry in North Central States of Nigeria

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Received 22 December, 2019; Accepted 30 March, 2020

Newcastle disease (ND) is a highly contagious viral disease constituting a continuous threat to the poultry industry worldwide. This study evaluated the genetic characteristics of Newcastle disease viruses (NDVs) obtained from backyard commercial poultry farms and live bird markets during active and passive surveillance in different regions of Plateau and Nasarawa States of Nigeria between 2009 and 2017. The partial fusion (F) gene coding sequence and cleavage site of five NDV isolates was determined. This was aligned and compared with sequences of representative NDV from the GenBank. Deduced amino acid sequence of the protein revealed that four isolates had virulent motifs (^{112}RRQKRF^{117}) while one had an avirulent motif (^{112}GRQGRL^{117}). One virulent strain was recovered from an apparently healthy duck. Phylogenetic analysis based on comparison with different classes of NDVs revealed that two isolates clustered with genotype XIVb NDVs, another two isolates clustered with genotype XVIIa while one isolate clustered with genotype II. The phylogenetic analysis revealed that the velogenic isolates clustered with published class II genotype XIVb and XVIIa closely related to isolates from Benin and Niger republic. This highlights the need for ND control programmes to place more stringent measures on cross-border trade of live bird and poultry products to prevent the introduction of new strains of NDV that would be more difficult to control.

Key words: Chickens, duck, live bird market, Newcastle disease virus, genotypes, Nigeria.

INTRODUCTION

Newcastle disease (ND) is a highly contagious and fatal disease of poultry which is notifiable to the World Organization for Animal Health (OIE, 2018). The disease is present worldwide and affects many domesticated and wild bird species. The etiologic agent of the disease, Newcastle disease virus (NDV), is classified under the

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genus Avulavirus within the subfamily Avulavirinae and family Paramyxoviridae (Amarasinghe et al., 2019). The disease has a wide host range including approximately 241 species of 27 orders, out of known 50 orders of birds (Madadger et al., 2013).

More commonly affected species include chickens, turkeys, ducks, pigeons, guinea fowl, Japanese quail and many wild birds of all ages (Nanthakumar et al., 2000; Zhang et al., 2011). In general, ducks are considered natural reservoirs of NDV and show few or no clinical signs after infection even for NDV strains lethal to chickens (Stanislawek et al., 2002; Liu et al., 2010). Chicken infection with virulent NDVs can be devastating due to the resulting high mortality, significant drop in egg production and is characterized by very rapid spread. The disease remains one of the major problems affecting existing or developing poultry industries in many countries including Nigeria.

The NDV is an enveloped, single stranded negative-sense RNA virus whose genome is approximately15kb. Its genome has six open reading frames (ORFs) which encode for six major structural proteins, namely, nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and the RNA-dependent RNA polymerase (L) (Ganaret al., 2014). The phylogenetic analysis of the F gene was carried out in different studies and based on the F gene, NDVs can be categorized into Classes I and II (Diel et al., 2012; Samal et al., 2012).

So far, class I has nine genotypes, while Class II has at least 18 genotypes identified (Snoeck et al., 2013; Dimitrovet al., 2016). The cleavability of the fusion protein precursor (F0) and the presence of a number of basic residues in the fusion protein cleavage site are major determinants for NDV pathogenicity (Martin-Garcia et al., 2012). In Africa, studies have isolated velogenic NDVs from sick and seemingly healthy poultry (Damena et al., 2016; Molini et al., 2017). In Nigeria, NDV has been noted to be widespread due to rapid expansion of the poultry industry, high stocking densities and inadequate biosecurity measures which created conditions conducive for the spread and maintenance of the endemicity of the disease (Okwor and Eze, 2010). ND is endemic in Nigeria, with frequent outbreaks in commercial, backyard and village poultry farms (Nwanta et al., 2008; Sa'idu and Abdu, 2008). Despite rampant ND outbreaks that occur annually in Nigeria, the information about the NDV circulating in some regions of the country is still scanty.

Prevention and control of ND primarily depend on the strict application of biosecurity measures and intensive vaccination programs that have been successfully used throughout the world for many years (Alexander, 2000). The frequent incidence of NDV infection, even in vaccinated birds, is not only related to improper vaccination or immune suppression but may also be due to viral mutation leading to changes in the genomic sequence of the virus, thus altering its biological properties and virulence (Kattenbelt et al., 2006). The increasing report of outbreaks of ND in vaccinated flocks in Nigeria suggests that new NDV variants may be more virulent and birds may not be completely protected by the present conventional vaccines. Although NDV has been studied in Nigeria, genetic information about the viruses involved in the endemicity of the disease is still largely incomplete. Our study is aimed at evaluating the genetic characteristics of ND viruses obtained from backyard commercial poultry farms and live bird markets in two north central (Plateau and Nasarawa) States of Nigeria.

MATERIALS AND METHODS

Study area and sample collection

Samples were collected from Plateau and Nasarawa States, north central Nigeria during surveillance studies between 2009 and 2017. Tracheal and cloacal swabs from live birds and pooled tissue such as trachea, lungs, spleens and intestines from dead birds were collected from both local and exotic chickens including ducks from live bird markets (LBMs).In addition, tissues (lungs, trachea, spleen and intestines) from dead birds recovered from outbreaks in backyard and commercial chickens were also included in this study. A total of 150 swab samples were collected in 1.5ml glycerol-based (50%) virus transport medium containing penicillin (10,000 units/ml), streptomycin (10,000 mg/ml), gentamicin (5000 mg/ml), and amphotericin B (50 mg/ml). Samples were immediately placed in a flask with ice packs and transported to the laboratory and stored at -80°C until required.

Newcastle disease viral isolation

Virus isolation, extraction and RT-PCR was performed at the Virology Department of the National Veterinary Research Institute (NVRI), Vom, Nigeria. For virus isolation, 200 µL of supernatant from swab samples were inoculated into three 9-11 day-old specific antibody negative (SAN) embryonated chicken eggs through the allantoic cavity as per standard procedures (OIE, 2008). Inoculated eggs were incubated at 37°C for 3- 4 days and dead embryos observed 24 h post inoculation (PI) were discarded. The allantoic fluid (ALF) was harvested from dead eggs after 24 h PI and at the expiration of the incubation period. The ALF were tested for haemagglutination (HA) activity and positive ALF were plated on blood agar plate to exclude bacteria contamination. NDV was identified by haemagglutination (HA) and haemaglutination inhibition (HI) tests (OIE, 2008). A total of 5 NDV strains were isolated and stored at -80°C for RT-PCR and sequencing analysis.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Viral RNA was extracted from ALFs using the QiagenQIAamp®Viral RNA Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. The partial fusion (F) gene was amplified using the following primer pairs: NDV-F4235F: 5'-ATACTCTGGAGYCAAACYGCG-3' and NDV-F5492R: 5'-GGGACAAGTGCHGAGGCAAA-3'. The F gene of the NDV isolates was amplified using the One-Step RT-PCR kit (Qiagen, Germany) in a 25µl reaction mixture containing 5.0 µl of 5× PCR buffer, 1.0 µldNTP mix (10 mM each), 1.0 µl of each primer (10µM), 0.5 µl RNase Inhibitor (40 U/µl, Promega), 0.5µl RT-PCR Enzyme Mix, 5.0 µl of RNA template, and nuclease-free water was added to make up to the final volume. The RT-PCR procedure was conducted on a GeneAmp PCR system 9700 thermocycler (Applied

Table 1. Newcastle disease virus isolates showing location, source, type and health status of chickens and a duck from Plateau and Nasarawa State, Nigeria.

S/N	Isolate ID	Location	Source/type of bird	Health status
1	PLJS-109/2017	Jos north, Plateau	Commercial farm layers	Dead
2	PLJN-T11/2009	Jos north, Plateau	LBM local chicken	Sick
3	NSKR-DK56-59/2009	Karu, Nassarawa	LBM duck	Apparently healthy
4	PLJN -TSI/2009	Jos north, Plateau	LBM local chicken	Sick
5	PLJS-137B /2017	Jos north, Plateau	Commercial farm layers	Dead

LBM:-Live bird market.

Biosystem, CA, USA) which amplified a 1274bp fragment of NDV genome based on the following cycling condition: 50°C at 30 min, 94°C for 15 min; 40 cycles of 94°C for 30 s, 55°C for 1 min and 68°C for 2 min and a final extension at 68°C for 10 min. The PCR products were separated on gel electrophoresis using 1.5% agarose stained with ethidium bromide (SIGMA, Germany) and visualized using Gel Documentation system (Biostep, Germany).

Sequencing and phylogenetic analysis

The PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Germany). The purified PCR products were directly sequenced in both directions on an ABI3730XL sequencer (Applied Biosystems) by a commercial sequencing company (Macrogen Inc., Korea) using the corresponding forward and reverse primers used in the PCR amplification reaction of the F gene. The DNA sequences were assembled using an online fragment merger tool (http://hvdr.bioinf.wits.ac.za/fmt/). Translation of nucleotide sequences was done using Molecular Evolutionary Genetic Analysis (MEGA 6.0). Phylogenetic tree of the isolates was constructed using the Kimura two-parameter model with 1000 bootstraps in MEGA 6.0. Previously described genotype nomenclature was used for the identification of genotypes (Diel et al., 2012; Snoeck et al., 2013).

GenBank accession number

The obtained sequences of the isolated NDV were submitted to the GenBank database and available under the following accession numbers: PLJN-TS1/2009/MN046106; PLJN-T11/2009/MN046107; NSKR-DK56-59/2009/MNO46108; PLJS-137B/2017/MN046109 and PLJS-109/2017/MN046110.

RESULTS

Virus isolation

A total of five NDV isolates were successfully obtained, two from commercial poultry farms and three from Live bird markets(LBMs) (Table 1) including samples from both live and dead birds collected in this study.

Cleavage site analysis

Deduced amino acid sequences of the F gene cleavage site were used to determine the pathotypes involved. The fusion gene of virulent/mesogenic NDVs is characterized by the presence of multiple basic amino acids at the cleavage site while the fusion gene of lentogenic strains is characterized by the presence of monobasic amino acids. Deduced amino acid sequence of the cleavage site of fusion (F) protein revealed that four isolates out of the five used for this study had virulent motifs while one had an avirulent motif. All the four virulent isolates exhibited sequence motif of ^{112}RRQKRF^{117} at the cleavage site (Table 2).

Phylogenetic analysis

Phylogenetic analysis of partial F gene nucleotide sequences of the NDV isolates was done by comparing them with already published F gene sequences of both Class I and II NDVs. Phylogenetic analysis based on comparison with different classes of NDVs revealed that 2 isolates clustered with genotype XIVb NDVs, another two isolates clustered with genotype XVIIa while one isolate cluster with genotype II (Figure 1).

DISCUSSION

Recent reports from West and Central Africa described the presence of novel virulent NDV strains belonging to new genetic lineages closely related to genotype VII namely, genotypes XIV, XVII, and XVIII (Cattoli et al., 2010; Samuel et al., 2013; Snoeck et al., 2013). Many of these isolates were obtained from live bird markets and village poultry, and in several cases the absence of apparent clinical signs was reported. In this study, the results of the phylogenetic analysis indicated that two isolates clustered with genotype XIVb NDVs, another two isolates clustered with genotype XVIIa while one isolate clustered with genotype II. The genotype II isolates are a mixture of velogenic and lentogenic viruses such as LaSota and B1 strains used globally for vaccination and disease control (Seal et al., 2005; Kim et al., 2007). The isolates in this genotype have been majorly recovered from domestic chickens and wild birds found in North and

Table 2. Newcastle disease virus isolates showing cleavage motif site, pathotypes and genotype from chickens and a duck in Plateau and Nasarawa State, Nigeria.

Isolate ID	Cleavage site motif (112 – 117)	Pathotype	Genotype
PLJS-109/2017	RRRKRF	Virulent	Genotype XIVb
PLJN-T11/2009	RRRKRF	Virulent	Genotype XIVb
NSKR-DK56-59/2009	RRQKPF	Virulent	Genotype XVIIa
PLJN -TSI/2009	RRQKRF	Virulent	Genotype XVIIa
PLJS-137B /2017	GRQGRL	Avirulent	Genotype II

South America, Africa, Asia, and Europe (Dimitrov et al., 2016). Two isolates recovered in this study clustered in Class II genotype XIVb, one was obtained from a commercial poultry farm and the other from an apparently local chicken from a live bird market. The isolates belonging to genotype XIV are the most predominantly obtained strains of NDV in Nigeria. Both subgenotypes XIVa and XIVb have been recovered from domestic birds found in the North-West (Sokoto, Kaduna, Jigawa), North Central (Benue, Kogi), North-East (Taraba and Yobe), and South-Western parts (Lagos) of this country (Mohammed et al., 2018). Recent reports from Nigeria (Catherine et al., 2019) showed widespread distribution of genotype XIVb isolated from domestic bird species between 2006 and 2015 from many states in the country. Phylogenetically, the genotype XIVa isolates form a clustered with some NDV strains from Niger Republic while the Nigerian genotype XIVb isolates tend to be more closely related to the 2009 isolates from Benin Republic. From the result of this study, there was sequence similarity between strains recovered from outbreaks in commercial poultry farms and those from LBMs (genotype XIVb). Some observed sequence similarity between strains recovered from outbreaks in backyard and commercial poultry farms in Jos, Plateau state, and those from LBMs in Sokoto and Kano states, Nigeria have previously been reported (Solomon et al., 2012). This may be due to horizontal spread by live poultry vendors from rural settlements and LBMs to commercial and backyard poultry farms. Live bird markets have continued to be a high risk area for transmission of NDV due to the high concentration and interactions of wide variety of birds coming together from different locations (Choi et al., 2005; Abah et al., 2017). Furthermore, spill over from the LBMs to backyard and commercial farms remains a possibility because of the attitude of farmers who buy replacement stock from LBMs and introduce them into existing poultry on their farms. A recent report of NDV in Nigeria demonstrates that most genotypes are widely distributed across the country, but genotype XVII has only been seen in the northern states (Bello et al., 2018). On the basis of phylogenetic analysis, genotype XVIIa isolates from Nigeria are closely related to NDVs isolates from Niger Republic, Cameroun, Burkina Faso, and Mali. The ecological distribution of genotype XVII isolates to date is restricted to the West and Central Africa (Shittu et al., 2016a; Snoeck et al., 2013) where they are believed to considerably militate against poultry production. Another report indicated that representatives of these isolates have recently been shown to cause atypical velogenic viscerotropic ND characterized by end stage morbidity and high mortality in chickens (Susta et al., 2015).

In the present study, one virulent velogenic NDV obtained from an apparently healthy domestic duck in Karu, Nasarawa State, Nigeria was genotypically characterized. This isolate clustered with genotype XVIIa of class II. In ducks, many NDVs have been isolated in recent years and most of them belong to class I NDVs and are low-virulence strains, occasionally a high virulence strain is isolated but little is known about their potential to cause disease in domestic ducks. Also, previous reports of isolation of virulent NDVs from apparently healthy birds have been documented (Byarugaba et al., 2014; Echeonwu et al., 1993; Snoeck et al., 2013). In all of these previously reported works, free-range local chickens and ducks were involved. The perpetuation of velogenic strains in apparently healthy birds poses a threat to commercial poultry in affected African countries. Molecular epidemiology research indicates that most NDVs isolated from ducks and pigeons belong to genotypes II, III, VI, and IX (Diel et al., 2012; Gaikwad et al., 2016).

Recently, reports of a velogenic strain of NDV isolated from an apparently healthy free-roaming domestic duck in Nigeria (duck/Nigeria/903/KUDU-113/1992) were documented (Shittu et al., 2016b). Phylogenetic analysis of the fusion protein gene and complete genome classified the isolate as a member of NDV Class II, genotype XVII (Shittu et al., 2016b). Ducks are identified as natural reservoirs of NDV, they play important role in the transmission of NDV to chickens and other poultry species (Kang et al., 2014).

Conclusion

The NDVs isolated from this study clustered with genotype XIVb and XVIIa and sequence similarity was observed between the NDV strains recovered from

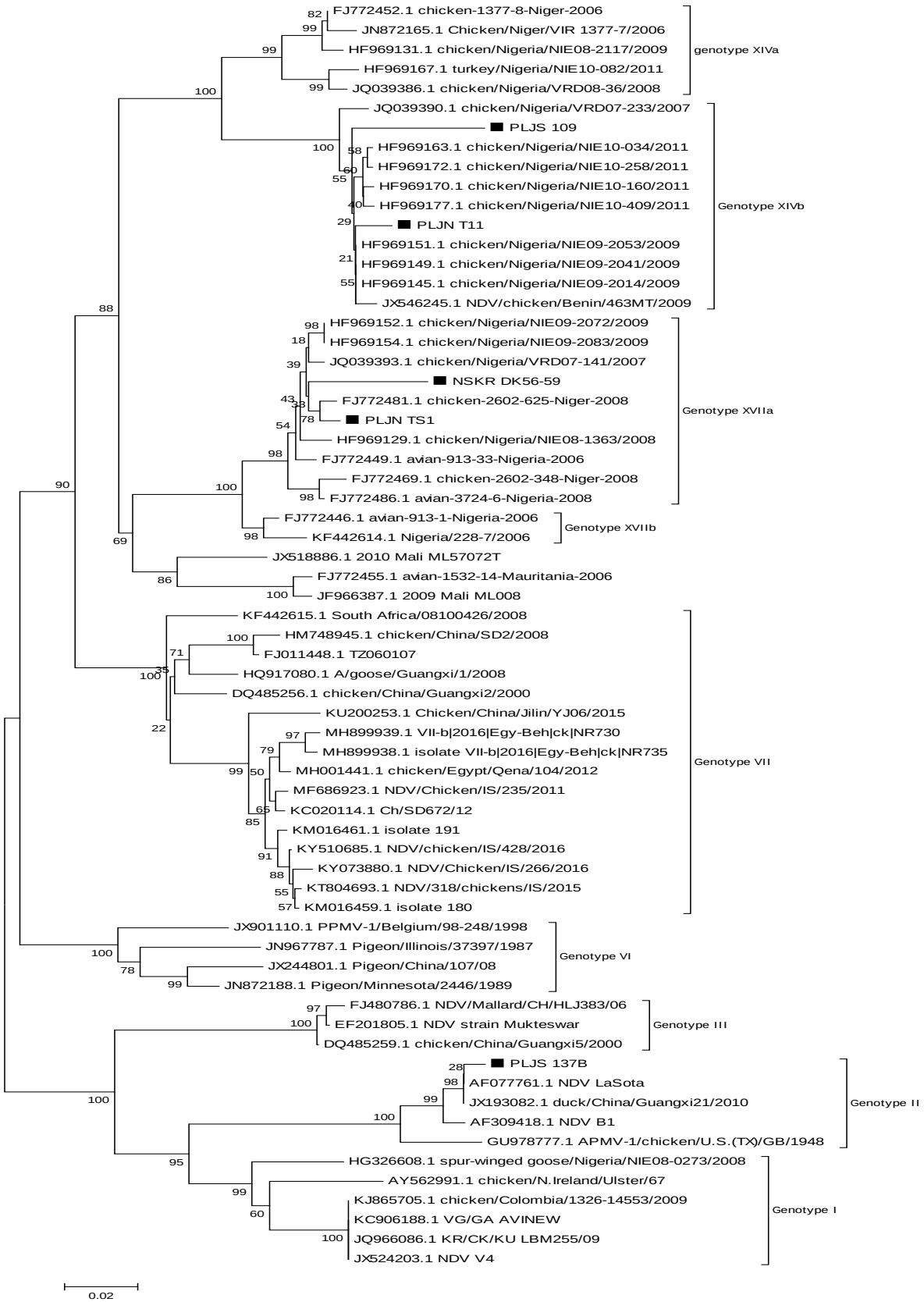

Figure 1. Maximum likelihood phylogenetic tree of the partial fusion gene of the five NDV isolates alongside other published sequences retrieved from the GenBank. The black square close to the node represent isolates from this study.

outbreaks in commercial poultry farms and those from LBM from Plateau and Nasarawa States, Nigeria. The identification of NDV of genotypes XIV and XVII in Nigeria and West Africa including Benin republic and Niger is indication that these NDV strains became established in the country and the region and larger nucleotide distances identified between the isolates provide enough evidence to suggest that these viruses continuously evolve locally as a result of active transmission (Catherine et al., 2019). However, their emergence in other parts of the continent within the next few years would not be unexpected given the poor transboundary biosecurity measures in most of the African countries including Nigeria. Thus, there is a need to intensify disease surveillance in live bird markets, households and commercial poultry farms so that disease epidemics due to these NDV strains can be quickly detected and contained in order to avert high economic losses.

Recommendations

There is need for continuous surveillance and epidemiological studies of NDV in all bird species. Further studies need to be taken to determine whether the emergence of new sub- lineages and genotypes could be responsible for ND outbreaks in vaccinated flocks. Challenge studies using the emerging NDV strains need to be carried out with a view to producing homologous vaccines that would further reduce virus shedding and control this disease.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful to all staff of the Regional Laboratory, Virology Division, National Veterinary Research Institute (NVRI) Vom, Nigeria for their technical assistance. The authors also thank all the poultry farmers who participated in this study for their cooperation during sample collection.

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