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(RESEARCH ARTICLE)

# Molecular characterization and phylogenetic studies of a virulent newcastle disease virus detected in indigenous chickens in plateau state, Nigeria

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## Abstract

A study of Newcastle disease virus (NDVs) using molecular characterization in indigenous chickens in Plateau State was carried out. The birds were obtained from three geographical locations in Plateau State. Three ribonucleic acids (RNAs) of Newcastle disease viruses (NDVs) obtained from normal indigenous chickens, which were never vaccinated against Newcastle disease (ND) were characterized. These RNAs were named Lc 3, Lc 4 and Lc5, respectively. These three samples were genotypically analyzed using reverse transcription polymerase chain reaction (RT-PCR) with primers specific to the viral fusion (F) protein gene. The deduced amino acid sequences at the F0 of these three RNA between positions 112 and 117 were all the same. The three NDV RNA had phenylalanine (F) residue at position 117, confirming their high virulence to indigenous chicken in Plateau State, Nigeria. The existence of virulent NDVs in circulation in indigenous chicken could be a serious threat to commercial chicken. The fragment of the deduced amino acid sequences were compared phylogenetically and were all found to belong to the genotype 5 g. To the best of our knowledge, this is the first reported genotype Vg strains that possess the sequences of 112R-R-Q-K-R-F117 within the F0 protein in indigenous chickens in Plateau State, Nigeria. They also grouped/clustered together with a Niger Republic strain (FJ772475 chicken-2602-605-Niger-2008, FJ772481chicken-2602-625-Niger-2008), and another strain from Cameroon (FJ772484chicken-3490-147-cameroon-2008). This study presents for the first time to the best of our knowledge report of not only the presence of the velogenic strain, but has gone ahead to establish the amino acid sequences, and the phylogenic ancestral history of these novel Nigerian NDV isolates, and their accession numbers as well as their relatedness to already established isolates. The information obtained in this study could be useful for improving the knowledge on the epidemiology, spread, efficiency of prevention/control strategies currently in place in Nigeria and indeed the West African sub-region.

**Keywords:** Molecular characterization; Phylogenetic studies; Newcastle Disease Virus; Indigenous chickens; Plateau State; Nigeria

## 1. Introduction

Newcastle disease (ND) - a List A infection considered as one of the two most important diseases of chickens along with highly pathogenic avian influenza [1] is an economically important disease causing heavy production loss to the farmers besides high mortality worldwide. The disease affects 27 of the 50 orders of birds [2]. The disease is caused by Avian Paramyxo virus type 1 (APMV 1) of the genus *Avula virus* under the family *Paramyxo viridae.* The virus has a negative sense, single stranded - RNA genome that consist of approximately 15 kbp (15,186 bp). The genome contains six major genes that encode the structural proteins in the order 3'-NP-P-M-F-HN-L-5.

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The disease is present in endemic form with frequent outbreaks in commercial poultry. Besides commercial poultry, the disease also affects the village chickens and it remains as a constant threat to the backyard poultry [3, 4]. While the commercial poultry are routinely vaccinated, the indigenous chickens in Nigeria are not usually vaccinated due to social and financial reasons [5, 1].

The first documented evidence of ND in Nigeria was in Ibadan in South-Western part of the country. However, the first laboratory confirmation was made in 1953, from outbreaks in the former Benue and Ibadan Provinces [6]. The pathogenicity of ND strains is determined by the amino acid sequence at the site of F-protein cleavage site [7, 8]. Chickens remain the most susceptible and important natural host [9]. However, Solomon *et al* [10] isolated virulent strains of NDV in apparently healthy domestic ducks and guinea fowls in Plateau State.

Motivated by recent technological achievements in molecular modeling studies, this necessitated the use of Reverse transcription polymerase chain reaction (RT-PCR [16] technique for detecting the presence of the viral RNA in wet tissues of indigenous chickens in Nigeria as well as their sequences.

## 2. Material and methods

### 2.1. Study area

This study was carried out in Plateau State of Nigeria. The State is located in the Central geopolitical zone of the country, bounded by Bauchi State to the east, Taraba State to the south east, Nasarawa State to the south and Kaduna State to the North. It has an average ambient temperature of 24 $^{o}$C and relative humidity of 50%. The State has an approximate human population of 3.5 million with over 30 ethnic groups [12, 13]. Plateau State has an estimated commercial poultry population of 808,298. The highest concentration of this figure are found in the two local government areas (LGAs) of Jos North and South, accounting for 620, 540, while the remaining 15 LGAs account for 187, 758 [14].

Plateau State has an altitude ranging from 1, 200 metres to a peak of 1, 829 metres above sea level in Shere hills. The State has a land area of 30, 913 km$^2$. Its mean annual rainfall varies between 1, 317 mm in the Southern part of the State and 1460 mm on the high Plateau [15]. These samples (poultry) were collected (purposive) from the three geographical zones, i.e., Southern, Central and Northern Zones of Plateau State, Nigeria.

### 2.2. Category of birds sampled

(1) Carcasses or live birds captured for gaming purposes (2) Birds caught during hunting (3) Diseased or hunted birds(4) Apparently healthy, sick or moribund indigenous poultry in selected areas of study and (5) Wild birds or local chickens found dead or are dying and submitted to the Central Diagnostic Laboratory, National Veterinary Research Institute, Vom, Plateau State, Nigeria. The indigenous chickens that were sampled were not vaccinated against ND based on the information obtained from the owners of the birds.

### 2.3. Molecular characterization of detected NDVs

Aliquots of the various organ/tissues dispensed in 1 ml cryogenic vials were prepared at the National Veterinary Research Institute (NVRI), Vom and were later shipped to Southeast Poultry Research Laboratory (SEPRL) Athens the United States of America, for molecular characterization.

The technique used in the Southeast Poultry Research Laboratory (SEPRL) Athens is an established procedure for detection of the Avian Paramyxovirus-1 (APMV-1) Matrix Gene and the Virulent Newcastle Disease Virus (vNDV) Fusion gene RNA by Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR). This protocol uses the Roche LightCycler® 2.0 as well as the National Veterinary Services Laboratory (NVSL) Testing. The matrix APMV-1 assay is designed to detect APMV-1 Matrix RNA from vaccine/lentogenic strains as well as vNDV (mesogenic/velogenic strains). The primers used for RT-PCR were generated by alignment of published NDV nucleotide sequences, representing part of the open reading frame for the matrix gene.

### 2.4. Extraction of viral RNA and RT-PCR

RNA was extracted from tissue homogenates using commercial high pure RNA isolation kit (Roche, Germany) as recommended by the manufacturer.

### 2.5. Polymerase chain reaction (PCR)

The thermocycler conditions were as follows:

40 min at 42 °C, 7 min at 95 °C, 30 s at 94°C followed by 55 cycles of 30 s at 53 °C, 30 s at 72 °C. This has already been tested by one-step RT- PCR method. The assays were performed on PTC200 M J research system.

Detection of PCR products: PCR products were separated in 2% agarose gel 1x TBE buffer stained with ethidium bromide and visualized by ultraviolet (UV) trans-illumination. The samples were also tested by Real time RT-PCR method using the primers and probe (FAM and BHQ) for both Matrix gene (M gene) and Fusion gene (F gene).

One-step real-time RT-PCR kits (QuantiTect Probe RT-PCR kit) (Qiagen GmbH, Hilden, Germany) was used. The reaction mixture for both assays contained 5 µl of RNA preparation, 12.5 µl of 2× Buffer Mix, 900nM of each primer and 0.25 µl of Taq Mix. In the M-gene and F gene assay, 250nM of M-probe was added. The RT-PCR thermal profile consisted of an initial RT step of 50 °C for 30 min, followed by denaturation at 95°C for 15 min and 45 cycles of 20 s at 95°C and60 s at 58°C in the F-gene assay and 52 °C in the M gene assay. In order to test the RT-PCR efficiency, the LaSota strain RNA extracted from the antigen solutions provided in house (CVL) were diluted serially 10-fold in sterile, nuclease-free water.

The Jones-Taylor-Thornton (JTT) model and the maximum composite likelihood model were used to calculate pair wise distances to determine the relatedness of strains (homology) and their distances from their epidemiological ancestries. This was also used to construct neighbour-joining phylogenetic tree, base pair distances, which indicates the relatedness or otherwise to isolates from Nigeria exotic commercial chickens.

## 3. Results

Immunohistochemical technique was used to screen out those samples that were positive. Only three (3) were positive at the level of immunohistopathological analysis showing evidence of the presence of NDV antigen as indicated by red fluorescence.

**Table 1** Location within Plateau State where Immunohistochemical positive samples were obtained from indigenous chickens in Nigeria

| Sample ID | Location | Zone/Local Government Area |
|---|---|---|
| Lc1 | Miango | Northern -Bassa |
| Lc4 | Perka-Ampang west | Central -Mangu |
| Lc5 | Garkawa | Southern -Mikang |

Lc1-, Lc4 and Lc5: Indigenous chicken numbers 1-3

Three of these samples were positive by IHC technique, NDVs were detected following screening using real time PCR, they were then sequenced and phylogenetic analysis of the F gene nucleotide sequence including the $F_0$ cleavage site of the three (3) viral RNAs were done to assess genetic relatedness to other already reported strains in some other breeds of poultry, particularly those recovered from commercial chickens.

### 3.1. Molecular analysis

Genotypic and phylogenetic analysis of the partial *F* gene nucleotide sequences of the three Nigerian isolates from indigenous chickens including the $F_0$ cleavage site of isolates was carried out to determine their genetic relatedness. This was done by comparing this with corresponding sequences of reference strains using Mega 5.0 software [16]. Based on the topology of the tree, the sequences of the three isolates in this study, clustered together with sub-lineages reported by Cattoli *et al* [17] and Snoeck *et.al*. [18] as shown in figure 1. Based on the phylogenetic analysis, the predicted cleavage site of all the three isolate is $^{112}$R-R-Q-K-R-F$^{117}$. The isolates had multiple basic amino acids R (aginine) and K (lysine) as well as F (phenylalanine) at position 117 (Figure 2). And clustered within the sub-lineage 5 g (Figure 1).

An assessment of genetic relatedness of the 3 isolates in this study to other Nigerian strains, particularly those in commercial chickens and NDV strains from other parts of the world showed that the isolates in this study were similar

to isolates reported in exotic breeds of chickens in Nigeria as well as some close neighbouring countries in West Africa (Figures 1 and 2).

On Figures 1 and 2 genotype sub types are indicated at the right hand side. The ruler line represents the distance scale length of phylogenetic tree created by MEGA 5.0, which is 0.005. The phylogenetic tree shows viruses isolated from indigenous chickens in this study based on comparison of partial nucleotide sequences bolded in red colour bars and viruses of other genotypes as references. The NDV RNA from Nigeria indigenous chicken share 100% identity or homology with isolates from commercial chickens in Nigeria and those in chickens from other parts of West Africa [17].

Phylogenetic analyses based on the fusion protein amino acid cleavage sites and the nucleotide sequences in this study revealed that the NDV isolates in indigenous chickens designated as LC3 LC4 and LC5 had 100% homology. LC3 and LC4 were both detected in birds from the same location, i.e., Perka village in Ampang West District of Mangu LGA, in the Central geographical zone of Plateau State. However, LC5 even though homologous to LC3 and LC4 is from a completely different location (Garkawa), in Mikang LGA, in the Southern geographical zone of Plateau State (Table 2).

On the basis of the results of pair wise alignments of the fusion protein genes, the isolates also had 100% homology, indicating that the isolates are very closely related and share a common ancestry with the NDV isolated from exotic commercial chickens in Nigeria (Figure 2). Table 1 shows accession number, the year of sample collection, the country of origin and the genotype of the three NDVs isolates detected in indigenous chickens in this study as of 27/02/2013 by the GeneBank.

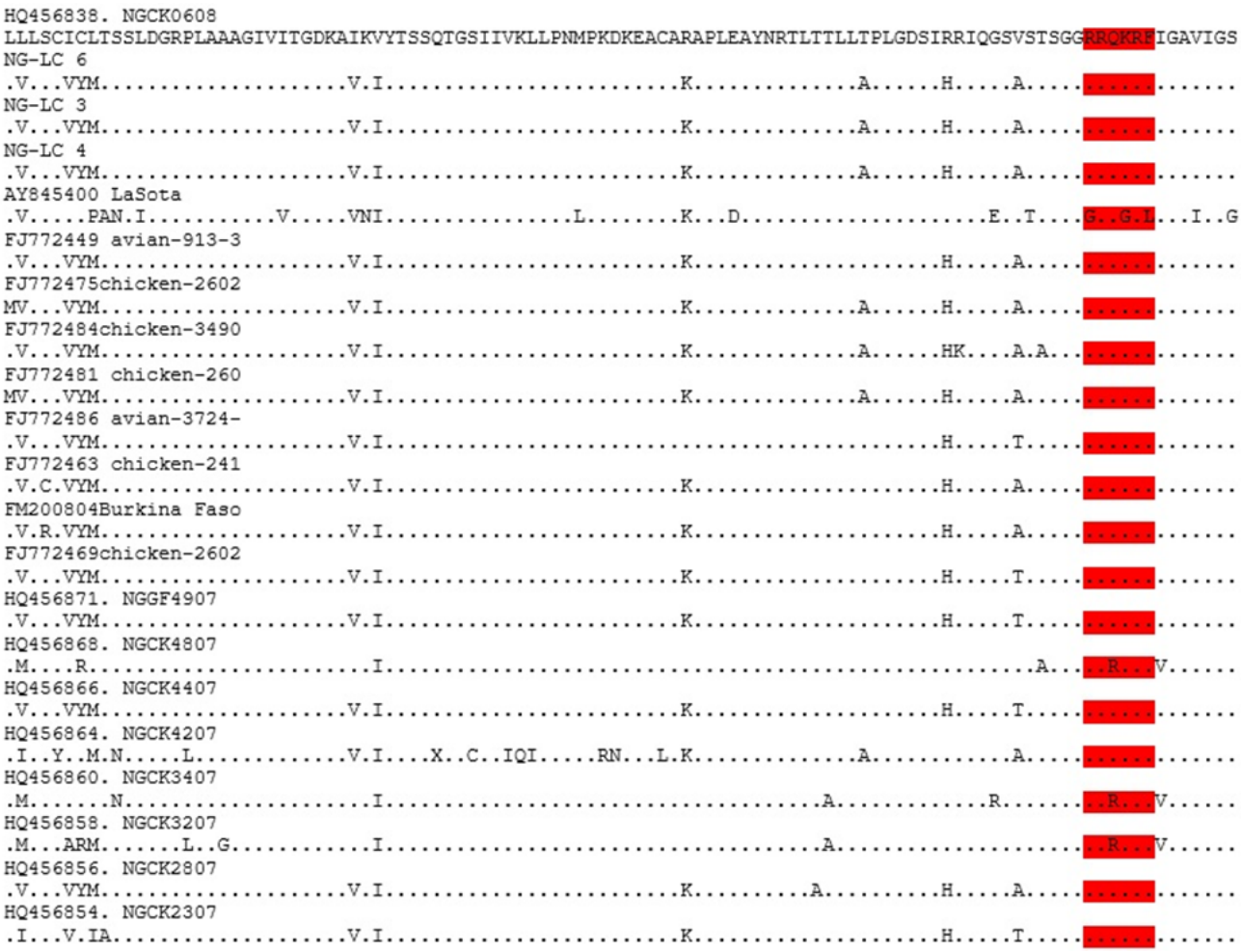


**Figure 1** Amino acid sequence alignment showing the cleavage site (shaded) of the fusion protein of the three Nigerian isolates in indigenous chickens, with the NDV sequence in this study in red bold-face

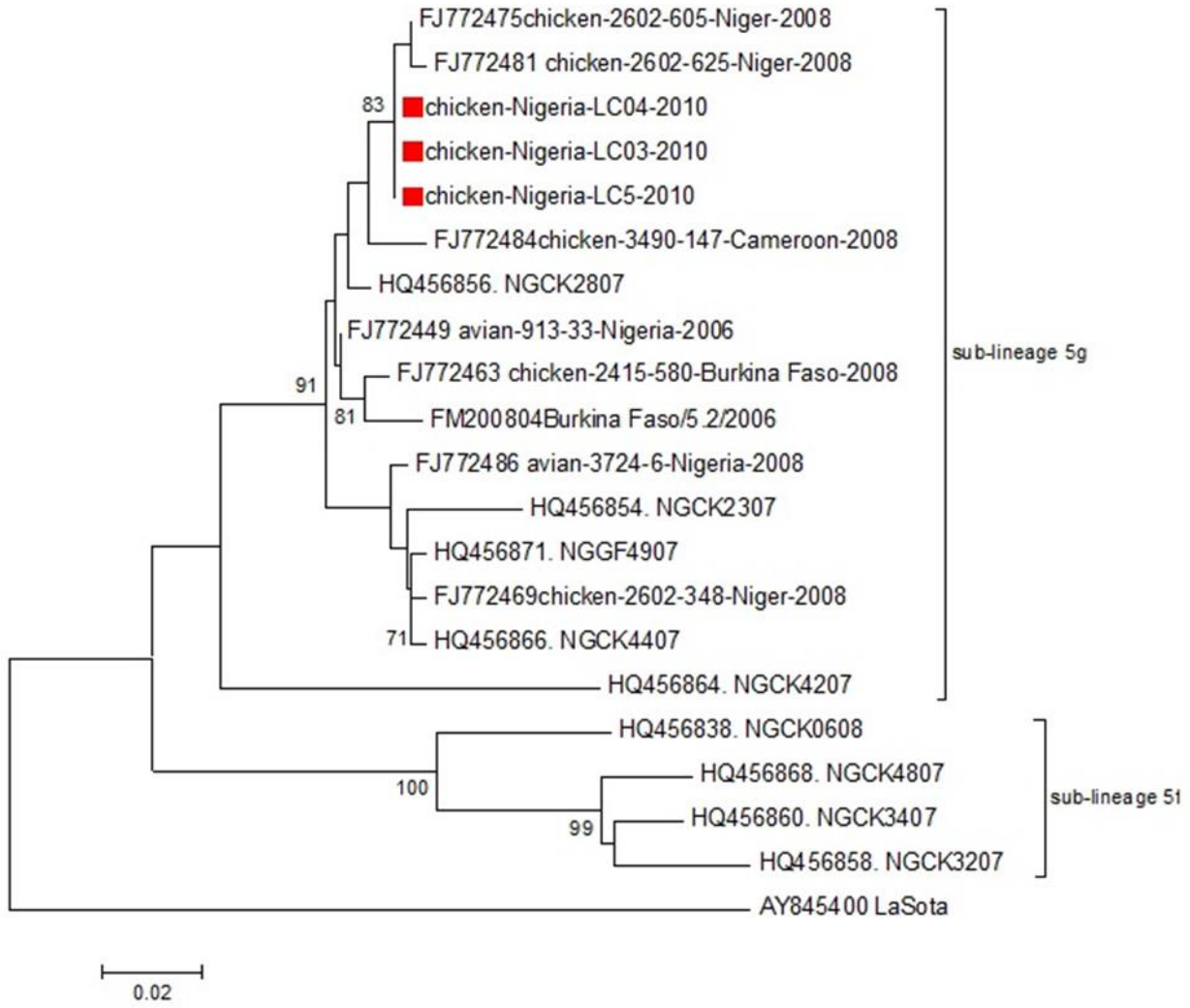


**Figure 2** A rooted Neighbour-joining phylogenic tree based on a 314-bp (base pairs) of representatives of sub-lineage 5 with the strains in this study designated as Chicken Nigeria-LC04,Chicken Nigeria-LC5 and Chicken Nigeria-LC03 in red squares

**Table 2** Lineage cleavage motif, virus strain and accession numbers of the three RNA in this study

| Virus strain | Isolate | Cleavage site | Lineage | Accession |
|---|---|---|---|---|
| Kumbish NG-739/Lc3 | chicken-Nigeria-LC04-2010 | $^{112}$R-R-Q-K-R-F$^{117}$ | sub-lineage 5 g | KC 689333 |
| Kumbish NG-740/Lc4 | chicken-Nigeria-LC03-2010 | $^{112}$R-R-Q-K-R-F$^{117}$ | sub-lineage 5 g | KC689334 |
| Kumbish NG-741/Lc5 | chicken-Nigeria-LC5-2010 | $^{112}$R-R-Q-K-R-F$^{117}$ | sub-lineage 5 g | KC689335 |

## 4. Discussion

Newcastle disease (ND) is endemic in Nigeria [19]. The devastating effect of ND in both indigenous and exotic chickens in Nigeria cannot be over emphasized. The main emphasis of this study lies in the molecular study of the virus in indigenous chicken with a view to improving on the current prevention and control measures being use in exotic chickens raised under intensive system. This will also provide information on whether to include indigenous chickens in the present prevention and control measures or design an entirely new programme for our indigenous stocks. In addition, there is dart of knowledge and or reports on the molecular characterization of NDV in indigenous/village chickens, despite the fact that up to 80% of the poultry population in Nigeria is kept by rural households as free-range chickens [20].

This study presents the report of not only the presence of the velogenic strain, but has gone ahead to establish the amino acid sequences, and the phylogenic ancestral history of these novel isolates, and their accession numbers as well as their relatedness to already established isolates. The F protein sequences ($^{112}$RRQKRF$^{117}$) at the $F_2$-$F_1$ cleavage site of all 3 NDV strains in this study suggest a high level of virulence. This is in line with the reports of Collins *et al*., [21]; Aldous and Alexander [1]; and Solomon *et al*. [18] in exotic chickens. The isolates in this study had multiple basic amino acids R (aginine) and K (lysine) as well as F (phenylalanine) at position 117 [21, 22, 10]. It should be noted too that this is the first time to the best of our knowledge we are reporting this amino acid sequence; and the amino acid arrangement of the new strains in this study have slight difference, indicating some degree of substitution in the arrangement. Comparison of the nucleotide sequences of the 3 isolates in this study with that of genotype 5f as reported by Solomon, *et al*. [10] in commercial chickens showed the highest similarities in homology, topology and pair wise distances.

All the three detected NDV RNAs in this study clustered together with a Niger Republic strain (FJ772475chicken-2602-605-Niger-2008, FJ772481chicken-2602-625-Niger-2008), they also clustered together with another strain form Cameroon (FJ772484chicken-3490-147-cameroon-2008); these also grouped together with the Nigerian strain (HQ456856.NGCK2807 and FJ772449avian-913-33Nigeria-2006) which were all isolated from commercial chickens (Figure 1). This is not surprising, especially for Niger republic and Cameroon which have very close trade and trans-human ties with Nigeria.

The homology between the sequences from this study and the previous strains isolated in commercial poultry and even the ones isolated from apparently health Muscovy ducks and guinea fowl in Nigeria and commercial chicken [10] which might have been responsible for a duck/guinea fowl to commercial chicken transmission or indigenous chicken/duck/guinea fowl to commercial chicken transmission or vice versa.

## 5. Conclusion

In conclusion, the information obtained in this study could be useful for improving the knowledge on the epidemiology, spread, efficiency of prevention/control strategies currently in place in Nigeria and indeed the West African sub-region. We strongly suggest that continuous research of the ND virus is required to help early detection of virulent strain or mutated strain for the quick and rapid intervention measures for eventual eradication of this disease; which is indeed a serious threat to the poultry industry in Plateau State and Nigeria, as it has also showed that there is probably only one virulent major phylogenetic group of NDV circulating in both commercial and indigenous chickens in Plateau State and possibly in the entire country as this will also rapidly curtail the likely evolution of new lineages and spread of velogenic NDV strains in Nigeria and the sub-region may continue unabated for a long time to come. This study presents for the first time to the best of our knowledge report of not only the presence of the velogenic strain, but has gone ahead to establish the amino acid sequences, and the phylogenic ancestral history of these novel Nigerian NDV isolates, and their accession numbers as well as their relatedness to already established isolates.

## Compliance with ethical standards

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### *Disclosure of conflict of interest*

The authors declare that there is no conflict of interest.

### *Statement of ethical approval*

Ethical approval for the work was granted by National Veterinary Research Institute Vom, Nigeria.

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